

# Micro Biotec'13

PORTUGUESE CONGRESS OF  
MICROBIOLOGY AND BIOTECHNOLOGY

6<sup>th</sup> - 8<sup>th</sup> December | Aveiro Portugal

## Abstracts Book





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## WELCOME ADDRESS

It is a great pleasure and privilege to welcome you to Aveiro and to the Portuguese Congress of Microbiology and Biotechnology (MicroBiotec'13).

This congress is an initiative of the Portuguese Society for Microbiology and the Portuguese Society for Biotechnology and is being hosted by the University of Aveiro for the first time.

The programme provides a unique opportunity for debate, discussion and the exchange of fresh ideas and information in the fields covered.

We are pleased to offer you an excellent scientific programme, with stimulating lectures with the involvement of international and national experts.

In addition, your contributions, that are highly welcomed, are presented in oral sessions or poster exhibitions that undoubtedly increase the knowledge of the latest scientific developments in the scientific domains covered by the congress.

We hope that it will be an enjoyable event that will provide an opportunity for delegates to discuss and share knowledge, ideas and expertise with colleagues and peers.

We wish you a fruitful meeting and a pleasant stay in Aveiro!

On behalf of the organizing committee,

António Correia & Manuel António Coimbra

## MENSAGEM DE BOAS VINDAS

É um grande prazer e privilégio dar-lhes as boas vindas a Aveiro e ao Congresso Português de Microbiologia e Biotecnologia (MicroBiotec'13).

Este congresso é uma iniciativa da Sociedade Portuguesa de Microbiologia e da Sociedade Portuguesa de Biotecnologia e é organizado pela primeira vez pela Universidade de Aveiro.

O programa oferece uma oportunidade única para o debate, a discussão e a troca de ideias e informações inovadoras nos domínios abrangidos.

O excelente programa científico conta com a participação e com palestras proferidas por especialistas nacionais e estrangeiros.

Para além disso, as contribuições de todos os participantes foram muito bem-vindas sendo apresentadas em sessões orais ou na exposição de painéis que, sem dúvida, aumentam o conhecimento sobre os últimos desenvolvimentos científicos nos domínios do congresso.

Esperamos que seja um evento agradável e que proporcione uma oportunidade para discussão e partilha de conhecimentos, ideias e experiências com colegas e pares.

Desejamos-lhes uma reunião proveitosa e uma agradável estadia em Aveiro!

Em nome da comissão organizadora,

António Correia & Manuel António Coimbra

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## SCIENTIFIC PROGRAM

December 6<sup>th</sup>

- 09:00–10:30      **Registration**
- 10:30–10:45      Auditorium  
**Opening Ceremony**
- 10:45–11:35      **Plenary Lecture – Chair: Isabel Sá Correia**  
S1: Industrial and Food Microbiology and Biotechnology  
**Sylvie Dequin**  
**INRA, Montpellier, France**  
Comparative genomics as a tool for understanding evolution and improving wine yeasts traits
- 11:35–12:25      **Plenary Lecture – Chair: Manuel Mota**  
S2: Environmental Microbiology and Biotechnology  
**Alfons Stams**  
**Wageningen University, Netherlands**  
Key features of syntrophic methanogenic communities
- 12:25–12:55      The Horizon 2020 Program – Dr. Maria João Fernandes (FCT: FP7 National Contact Point)
- 12:55–14:00      **Lunch**
- 14:00–14:25      Auditorium – Chairs: Tim Hogg and Arlete Faia  
**KL01 António Vicente**  
**Universidade do Minho**  
Applications of nanotechnology in the agro–food sector
- Lecture Hall 1 – Chairs: Nelson Lima and António Correia  
**KL02 David Smith**  
**CABI, UK**  
Microbial resource research infrastructure (MIRRI) facilitating improved pathways to discovery
- Lecture Hall 2 – Chairs: Raquel Sá–Leão and Ilda Santos  
**KL03 Marta Aires–de–Sousa**  
**ESSCVP**  
Methicillin–resistant *staphylococcus aureus*: a problem inside and outside portuguese hospitals
- 14:25–14:40      Auditorium  
**OP01 Ana Oliveira**  
**ESB, CBQF, Universidade Católica Portuguesa**  
Effect of controlled atmosphere and storage temperature on the phenolics and carotenoids of pasteurized peach purées
- Lecture Hall 1  
**OP05 Christophe Espírito Santo**  
**IMAR, CMA, Universidade de Lisboa**  
Sequenced mobile elements from submarine hydrothermal vents include simultaneously genetic determinants for heavy metal and antibiotic resistance

Lecture Hall 2

**OP10 Elsa Fortes–Gabriel**

**IHMT, Universidade Nova de Lisboa**

Leptospirosis in Angola: first isolates of *Leptospira* spp. from rodents and serological survey among human suspected of malaria

14:40–14:55

Auditorium

**OP02 Anouk Duque**

**REQUIMTE/CQFB: IBET**

Population analysis of a pha producing bioreactor subjected to feedstock shifts

Lecture Hall 1

**OP06 Diogo Pinho**

**Genoinseq, Next Generation Sequencing Unit, Biocant**

Assessment of *Quercus suber* soil microbiome using DGGE fingerprints and barcoded pyrosequencing

Lecture Hall 2

**OP11 Nuno Faria**

**ITQB, Universidade Nova de Lisboa**

Massive dissemination of methicillin resistant *Staphylococcus aureus* in bloodstream infection in Portugal: emrsa–15 establishment and diversification

14:55–15:10

Auditorium

**OP03 Nuno Silva**

**CICECO, Universidade de Aveiro**

Production of bacterial cellulose membranes by *Gluconacetobacter sacchari* using industrial biomass residues and their potential application

Lecture Hall 1

**OP07 Isabel Henriques**

**CESAM, Universidade de Aveiro**

Enterobacteria carrying oxa–48–like genes isolated from non–human sources

Lecture Hall 2

**OP12 Pedro Farias**

**Instituto Piaget, CIIERT, Lisboa**

Hospital's environmental microbial communities: a survey in two hospitals with different dimensions

15:10–15:25

Auditorium

**OP04 Daniel Camarneiro Silva**

**INESC, Microsistemas e Nanotecnologias and IN–Institute of Nanoscience and Nanotechnology, Lisboa**

Aqueous two–phase system in a microfluidic platform to accelerate bioprocess design and optimization of monoclonal antibodies

Lecture Hall 1

**OP08 Olga Lage**

**FC, Universidade do Porto**

Bacteria associated with marine erylus sponges: their diversity and bioactivity

Lecture Hall 2

**OP13 Isabel Miranda**

**FM, Universidade do Porto**

*Candida albicans* CUG mistranslation is a mechanism to create cell surface variation



15:25–15:40

Auditorium

**Flash oral presentations**

**P315/F01 Maria da Conceição Fernandes**

**CEBAL, Instituto Politécnico de Beja**

Bioethanol production from extracted olive pomace

**P302/F02 Sara F. Carvalho**

**CICECO, Universidade de Aveiro**

Ionic-liquid-based aqueous biphasic systems: an alternative approach for the separation of extracellular polysaccharides

**P072/F03 Carlos Boto**

**CIEPQPF, University of Coimbra**

Immobilization of lipoxygenase into cellulose derivatives for mycotoxins detection in food

Lecture Hall 1

**OP09 André Costa**

**CMEB, Universidade do Minho**

Antibacterial and antifungal activity of a new solvent-cast film based on a functionalized elastin-like polymer

Lecture Hall 2

**Flash oral presentations**

**P338/F09 Vânia Ferreira**

**University of Aveiro**

Detection of premature stop codons leading to truncated internalin a among food and clinical strains of listeria monocytogenes

**P337/F10 Maria Cunha**

**Universidade Nova de Lisboa**

Identification of type III secretion substrates of chlamydia trachomatis using yersinia enterocolitica as a heterologous system

**P221/F11 M. Filomena Raposo**

**Universidade Católica Portuguesa/Porto**

Bioactive compounds from marine microalgae — Health applications

15:40–15:50

Auditorium

**Flash oral presentations**

**P073/F04 Manuela Pintado**

**CBQF, Universidade Católica Portuguesa/Porto**

Food safety evaluation for cassava and yam producers in Nigeria

**P096/F05 Margarida S. Afonso**

**CICS, Universidade da Beira Interior**

Resveratrol production in bioreactors: assessment of cell physiology states by flow cytometry and plasmid segregational instability by real-time QPCR

Lecture Hall 1

**Flash oral presentations**

**P163/F06 Célia M. Alcobia Gomes**

**Instituto Politécnico de Coimbra ESTeSC, DAS**

Microbiological contamination by users of the waiting rooms of the hospital emergency departments

**P171/F07 Ana Rita R. Lopes**

**LEPAE, Faculdade de Engenharia/Universidade do Porto**

Crop rotation and cropping phase in rice paddies and their relationship with variations of the soil microbial communities

Lecture Hall 2

**Flash oral presentations**

**P233/F12 Diana Machado**

**IHMT, Universidade Nova de Lisboa**

Efflux inhibitors against drug resistant *mycobacterium tuberculosis*: antimicrobial agents and enhancers of macrophage killing activity

**P200/F13 Inês Correia**

**Universidade do Porto**

Dialysis effluent protozoa colonization in portuguese patients undergoing peritoneal dialysis

15:50–15:55

Lecture Hall 1

**Flash oral presentation**

**P165/F08 Miguel A. Ramos**

**CBQF, Universidade Católica Portuguesa/Porto**

Combining ectomycorrhizal fungi and bacteria: a powerful tool to improve tree performance

15:55–17:30

**Coffee break and Poster Session S1 and S8**

17:30–18:20

Auditorium

**Plenary Lecture – Chair: Luisa Peixe**

**S3: Health Microbiology and Biotechnology**

**Craig Winstanley**

**University of Liverpool, UK**

*Pseudomonas aeruginosa* population dynamics during chronic lung infections

18:20–19:20

Lecture Hall 1

**General Assembly SPM**

18:50–19:20

Lecture Hall 2

**General Assembly SPBT**

21:30–23:00

Fábrica Centro Ciência Viva de Aveiro

**Manuel Mota**

Perspectives for the Future of Biotechnology and Microbiology

## December 7<sup>th</sup>

09:00–09:50

Auditorium

**Plenary Lecture – Chair: Isabel Sá Correia**

**S4: Molecular Microbiology and Microbial Physiology**

**Professor Nicolau van Uden Award – Cecília Leão**

**Universidade do Minho**

Yeast dealing with stress in fermentative environments: revisiting physiological models in light of molecular biology

10:00–11:00

**Coffee break and Poster Session S2 and S6**

11:00–11:25

Auditorium – Chairs: Pedro Morada-Ferreira and Manuela Côrte-Real

**KL04 Paula Ludovico**

**Universidade do Minho**

The yeast chronological aging model: proteotoxic stress and age-related dysfunctions

Lecture Hall 1 – Chair: João Queiroz

**KL05 Margarida Diogo**

IST, Universidade de Lisboa

Integrated platform for production and purification of human pluripotent stem cell-derived neural precursors

Lecture Hall 2 – Chair: José Teixeira

**Cost action session** – EP4Bio2Med, funded by COST Action TD1104

**KL06 Giovanna Ferrari**

University of Salerno, Italy

Pulsed electric field technology: Current applications in the agro-food sector and potential use in biotechnology

11:25–11:40

Auditorium

**OP14 Marta Mendes**

IBMC, FC, Universidade do Porto

Ros-mediated links between primary and secondary metabolism in streptomyces: the example of pimaricin production in *Streptomyces natalensis* ATCC 27448

Lecture Hall 1

**OP18 Ângela Sousa**

CICS, Universidade da Beira Interior

Purification of human papillomavirus16 e6/e7 plasmid DNA-based vaccine using an arginine modified monolithic support

Lecture Hall 2

**Cost action session** – EP4Bio2Med, funded by COST Action TD1104

**OP22 Javier Raso**

University of Zaragoza, Spain

Fundamentals of microbial inactivation by pulsed electric fields

11:40–11:55

Auditorium

**OP15 Carlos São-José**

FF, DMI, CPM-URIA, Universidade de Lisboa,

Phageduction: a new technology for cell-targeted DNA delivery

Lecture Hall 1

**OP19 Marta Silva**

Requimte-CQFB, FCT, Universidade Nova de Lisboa

Bacterial sensing of key environmental parameters: structural and functional characterization of chemotaxis sensor proteins from *G. sulfurreducens*

Lecture Hall 2

**Cost action session** – EP4Bio2Med, funded by COST Action TD1104

**OP23 Valentina Ganeva**

University of Sofia, Bulgaria

Liberation of water-soluble proteins from yeast by pulsed electric field treatment

11:55–12:10

Auditorium

**OP16 Michaela Simcikova**

IBB, IST, Universidade de Lisboa

Development of a minicircle production system based on para resolvase-mediated *in vivo* recombination

Lecture Hall 1

**OP20 Paula Morais**

FCT, IMAR-CMA, Universidade de Coimbra

Natural hotspots for multiple resistances gain: arsenic resistance in heterotrophic aerobic bacteria from marine hydrothermal vents

Lecture Hall 2

**Cost action session** – EP4Bio2Med, funded by COST Action TD1104

**OP24 S. Šatkauskas**

**Vytautas Magnus University, Lithuania**

Pulsed electric field assisted extraction of biologically active constituents from fresh blueberries

12:10–12:25

Auditorium

**OP17 Paulo Jorge Dias**

**IBB, IST, Universidade de Lisboa**

The major facilitator superfamily multidrug resistance transporters (MFS-MDR) in hemiascomycetous yeasts: phylogenetic and syntenic analyses

Lecture Hall 1

**OP21 Vitor Ramos**

**FC, Universidade do Porto**

Cyanobacterial diversity in microbial mats from one of the largest hypersaline coastal lagoons complex: an in-depth polyphasic approach

Lecture Hall 2

**Cost action session** – EP4Bio2Med, funded by COST Action TD1104

**OP25 Nadia Boussetta**

**UTC, France**

Electrically assisted extraction of polyphenols: application of pulsed electric fields and high voltage electrical discharges

12:25–12:40

Auditorium

**Flash oral presentations**

**P201/F14 Maria Correia**

**Universidade Católica Portuguesa**

Bacterial resistance in the context of oral health

**P256/F15 Joana Barbosa**

**CESAM, Universidade de Aveiro**

Directed evolution of the lantibiotic lichenicidin

**P276/F16 Christian Gomes Ramos**

**Instituto Superior Técnico**

The MtvR sRNA is involved in the regulation of *hfq*

Lecture Hall 1

**Flash oral presentations**

**P172/F19 Ângela Cunha**

**CESAM, Universidade de Aveiro**

Bacterioneuston ecology of the estuarine system Ria de Aveiro: an overview

**P162/F20 António L. P. Amaral**

**Instituto Politécnico de Coimbra, Instituto Superior de Engenharia de Coimbra**

Study of chemical oxygen demand and ammonia removal efficiencies by image analysis and multivariate statistics tools

**P164/F21 Francisca A. Silva**

**CICECO, Universidade de Aveiro**

New findings on the (eco)toxicity of cholinium-based ionic liquids

Lecture Hall 2

**Cost action session** – EP4Bio2Med, funded by COST Action TD1104

**OP26 Ricardo Pereira**

**Universidade de Minho**

Production of whey protein hydrogels through application of electric fields

- 12:40–12:50** Auditorium  
**Flash oral presentations**  
**P277/F17 Mónica Nunes**  
 IHMT, Universidade Nova de Lisboa  
 Development of real-time PCR assays targeting the flagellin gene for the identification of *borrelia burgdorferi* sensu lato genospecies  
**P269/F18 Adriano Gigante**  
 Faculdade de Farmácia, Universidade de Lisboa  
 The impact of LysB in mycobacteria phage-mediated lysis
- Lecture Hall 1  
**Flash oral presentations**  
**P301/F22 Gonçalo Silva**  
 CICS, University of Beira Interior  
 Enthalpy contributions to lysozyme adsorption onto a cation-exchange support: effect of pH under linear and overloaded conditions  
**P291/F23 Mafalda R. Almeida**  
 CICECO, University of Aveiro  
 Purification of immunoglobulin Y (IgY) using aqueous biphasic systems
- Lecture Hall 2  
**Cost action session** – EP4Bio2Med, funded by COST Action TD1104  
**Flash oral presentation**  
**P080/F24 Bojidar Galutzov**  
 University of Sofia, Bulgaria  
 Electroinduced release of recombinant laccase from *Saccharomyces cerevisiae* based on cell electropermeabilization  
 General Discussion
- 13:00–14:30** Lunch
- 14:30–15:20** Auditorium  
**Plenary Lecture – Chair: Raquel Aires Barros**  
 S5: Bioprocess engineering  
 Alois Jungbauer  
 Univ of Natural Resources and Life Sciences, Vienna, Austria  
 Continuous downstream processing, a challenge for the biochemical engineer
- 15:30–15:55** Auditorium – Chairs: Célia Manaia and Manuel A. Coimbra  
**KL07 Jorge Leitão**  
 IST, Universidade de Lisboa  
 Post-transcription regulation in bacterial pathogenesis: roles played by RNA chaperones and sRNAs
- Lecture Hall 1 – Chairs: Milton Costa and Francisco Gírio  
**KL08 Manuel Santos**  
 Universidade de Aveiro  
 Fungal statistical proteomes: can they shake current concepts of the gene?
- Lecture Hall 2 – Chairs: Ivonne Delgadillo and Sónia Mendo  
**KL09 Jorge Saraiva**  
 Universidade de Aveiro  
 High pressure technology: 20 years of increasingly food commercial successes and future food and biotechnological potential applications
- 15:55–16:10** Auditorium  
**OP27 Diana Costa**  
 HSRC, CICS, Universidade da Beira Interior  
 Plasmid DNA based carriers for improved cancer therapy

Lecture Hall 1

**OP31 Catarina Batista**

DMI, CPM-URIA, FF, Universidade de Lisboa

Functioning, regulation and genetics of an Esat-6-like secretion system in *Bacillus subtilis*

Lecture Hall 2

**OP35 Vitor Gaspar**

CICS, HSRC, Universidade da Beira Interior

Delivery of DNA biopharmaceuticals by non-viral nanoparticulated carriers

**16:10-16:25**

Auditorium

**OP28 Nuno Mira**

IBB, IST, Universidade de Lisboa

The genome sequence of the food spoilage yeast *zygosaccharomyces ballii* ISA1307

Lecture Hall 1

**OP32 Clara Pereira**

IBMC, Universidade do Porto

Phosphoproteome analysis to unravel the role of the ceramide-activated protein phosphatase Sit4p in mitochondrial function and lifespan in yeast

Lecture Hall 2

**OP36 Filipa Rosa**

Faculdade de Engenharia, Universidade Católica Portuguesa

Bypassing the need for reporter genes

**16:25-17:25**

**Coffee break and Poster Session S3 and S5**

**17:25-17:40**

Auditorium – Chairs: Célia Manaia and Manuel A. Coimbra

**OP29 Pedro Oliveira**

SFSN, University College Cork, Ireland

The impact of fungal infections on processing and quality of beer

Lecture Hall 1 – Chairs: Milton Costa and Francisco Gírio

**OP33 Ângela França**

Universidade do Minho

*Staphylococcus epidermidis* biofilm lifecycle and its virulence: from planktonic growth, to biofilm structure and systemic dissemination

Lecture Hall 2 – Chairs: Ivonne Delgadillo and Sónia Mendo

**OP37 Teresa Conceição**

LMG, ITQB, Universidade Nova de Lisboa

High prevalence of nosocomial MRSA carriage in Luanda, Angola

**17:40-17:55**

Auditorium

**OP40 Tony Collins**

CMEB, DB, Universidade do Minho

Optimising the production of a silk-elastin-like protein in *E. coli*: overcoming acetate accumulation and plasmid instability

Lecture Hall 1

**OP34 Margarida Casal**

CBMA, Universidade do Minho

SATP, a succinate-acetate transporter protein in *Escherichia coli*

Lecture Hall 2

**OP38 Miguel Cacho Teixeira**  
IBB, IST, Universidade de Lisboa

Unveiling 5-flucytosine resistance mechanisms in yeast, using chemogenomics and membrane proteomics approaches

17:55–18:05

Auditorium

**Flash oral presentations**

**P094/F25 Bruno Firmino Arez**  
LNEG, Unidade de Bioenergia

Optimization of a ssf process using invertase applied to fossil fuels biodesulfurization

**P095/F26 Felisbela Oliveira**

IBB, Centre of Biological Engineering, Universidade do Minho

Influence of moisture content, temperature and inoculum size on lipase production by filamentous fungi under solid-state fermentation of olive pomace

Lecture Hall 1

**Flash oral presentations**

**P332/F30 João Menino**

Life and Health Sciences Research Institute (ICVS/3Bs)

TLR9 activation dampens the early inflammatory response to *paracoccidioides brasiliensis*, impacting host survival

**P331/F31 Joana Cruz**

Instituto Nacional de Investigação Agrária; Center for Biodiversity, Functional and Integrative Genomics

Black rot disease in Portugal: a divergent pathosystem for *Xanthomonas campestris* pv. *campestris* and galician – portuguese cabbages

Lecture Hall 2

**OP39 Helena Passos**

DQ, CICECO, Universidade de Aveiro

A new process for the recovery and concentration of bisphenol A from human fluids

18:05–18:20

Auditorium

**Flash oral presentations**

**P097/F27 Viviana G. Correia**

CREM, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Characterization of the *gam* cluster of a *Bacillus subtilis* gut strain

**P074/F28 Zuzana Ciesarová**

VÚP Food Research Institute Bratislava, Slovak Republic

Trial production of diabetic biscuits with reduced acrylamide level

**P006/F29 Filipe D. R. Carvalho**

CBCE, DB, IST, Universidade de Lisboa

Towards a rational approach for enzyme immobilization in a microreactor framework

Lecture Hall 1

**Flash oral presentations**

**P330/F32 Carla Barradas**

CESAM, DB, Universidade de Aveiro

Diversity of *Botryosphaeriaceae* species from *Eucalyptus* spp. in Portugal

**P250/F33 Patrícia Branco**

Laboratório nacional de energia e geologia (LNEG)

Interactions between *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii*: cell-cell contact mechanism

**P278/F34** Susana R. Chaves

DB, CBMA, Universidade do Minho/IBMC, Universidade do Porto

Ceramide metabolism modulates mitochondrial outer membrane permeabilization and apoptosis

Lecture Hall 2

**Flash oral presentations**

**P202/F35** Nuno Cerca

CEB-IBB, Universidade do Minho

The role of *gardnerella vaginalis* in mixed species biofilms occurrence in bacterial vaginosis and its prevalence in Portugal

**P359/F36** Joana S. Boura

Wake Forest School of Medicine, NC, USA

Gene delivery strategies to efficiently over-express functional HLA-G in bone marrow-derived mesenchymal stromal cells

**P360/F37** Vânia Roberto

DCBM, University of Algarve

Proliferative and mineralogenic activities of seaweeds and marine plants extracts in a fish *in vitro* cell system

18:30–19:20

Auditorium

**Plenary Lecture – Chair: Jorge Pedrosa**

S6: Cellular Microbiology and Pathogenesis

**Maria Mota**

FM, Universidade de Lisboa

Studying malaria infections: understanding to intervene

20:30–23:00

Congress dinner

December 8<sup>th</sup>

09:00–09:50

Auditorium

**Plenary Lecture – Chair: Hermínia de Lencastre**

S7: Genomics and Systems Biology

**Edward Feil**

University of Bath, UK

An overview of transmission patterns and population structure of *Staphylococcus aureus* inferred from next-generation sequencing

10:00–11:00

Coffee break and Poster Session S4 and S7

11:00–11:25

Auditorium – Chair: Jorge Pedrosa

**KL10** Gustavo Goldman

USP, S. Paulo, Brazil

Signal transduction mechanisms for cellulase production and glucose sensing in filamentous fungi

Lecture Hall 1 – Chair: Manuel Santos

**KL11** Isabel Gordo

Instituto Gulbenkian de Ciência

Pathoadaptation of *Escherichia coli* to macrophages



Lecture Hall 2 – Chair: Adelaide Almeida

**KL12 Paula Teixeira**

**ESB, Universidade de Porto**

Listeriosis in Portugal: from retrospective studies to the detection of the first confirmed human outbreak

**11:25–11:40**

Auditorium

**P373/F38 Lisete Silva**

**QOPNA, Universidade de Aveiro**

Evaluation of the stress-induced growth of the portuguese clinical isolate *Helicobacter pylori* ci-5 strain – a structural characterization of cell-surface lipopolysaccharides

**P329/F39 Isabel Fernandes**

**Universidade de Aveiro**

When fungal proteomics meets cork oak decline

Lecture Hall 1

**OP42 Lia Godinho**

**CREM, FCT, Universidade Nova de Lisboa**

Toxicity of sugar phosphates in a *Bacillus subtilis* strain defective in *araR*

Lecture Hall 2

**OP44 Sónia Almeida**

**LMMHP, ITQB, Universidade Nova de Lisboa**

Low prevalence of *Streptococcus pneumoniae* carriage among the elderly in Portugal

**11:40–11:55**

Auditorium

**OP41 Patrícia M. R. Pereira**

**QOPNA, DQ Universidade de Aveiro, LPET, IBILI, FM, Universidade de Coimbra**

Carbohydrate-driven porphyrinoids to induce cell demise by photodynamic therapy

Lecture Hall 1

**OP43 Beatriz Oliveira**

**IBET**

Fungal activity in untreated surface water: mycotoxins production and estrogenic activity

Lecture Hall 2

**OP45 Luís Raiado-Pereira**

**IBB, IST, Universidade de Lisboa**

Purification of pharmaceutical grade plasmid DNA by hic membrane chromatography – impact of plasmid size and process throughput

**12:00–12:50**

Auditorium

**Plenary Lecture – Chair: José Teixeira**

**S8: Emergent technologies**

**Henk Haagsman**

**University of Utrecht, Netherlands**

Cultured Meat

**12:50–13:00**

Closing ceremony and prize awards

## Session 1

# Industrial and Food Microbiology and Biotechnology

# Plenary Lecture

## COMPARATIVE GENOMICS AS A TOOL FOR UNDERSTANDING EVOLUTION AND IMPROVING WINE YEASTS TRAITS

Sylvie Dequin

*INRA, UMR1083, Sciences Pour l'Enologie, F-34060 Montpellier, France*

Wine yeasts have been associated to winemaking since millennia and display unique properties among the *S. cerevisiae* species. The aim of my lab is to unravel the evolutionary mechanisms which shaped wine yeast genome and metabolic networks. We are particularly interested in deciphering the genetic bases which are behind different traits/phenotypes. Our goal is also to develop new yeast strains adapted to current market demands.

I will discuss our recent findings regarding the contribution of horizontal gene transfer to the evolution of wine yeast genomes. Using comparative genomics, we found large introgressions from distant yeasts that enlarge the physiological abilities of wine yeasts and may favour the colonization of new ecological niches. Among the new genes acquired, a high-affinity fructose carrier and an oligopeptide transporter with a wide spectrum have been identified, which can provide nutritional advantages during the fermentation of grape must. Population genomic approaches have also the potential to point out key genes involved in the adaptation to specific niches. From the comparison of the genome sequence of wine and flor yeasts, we have identified divergent regions specific to flor strains. These regions contain genes involved in key functions associated with velum formation.

Advances in next generation sequencing have also the power to boost strain development programs. The elucidation of genetic variation between yeast strains using comparative genomics or genetic-genomic approaches to map quantitative trait loci (QTL) led to the identification of allelic variants for improved fermentation rate, aroma properties or decreased sulfite production. On the other hand, non-targeted approaches such as experimental evolution under selective cultivation conditions have been increasingly used for the improvement of wine yeasts. Successful engineered strains producing high amount of esters that contribute to fruity and floral aroma in wine have been obtained. Reverse engineering of such mutants is a major challenge in modern yeast biotechnology. I will present a successful example of the use of genome-wide approaches to identify relevant genetic changes of evolutionary engineered wine yeast.

# Keynote Lectures

## KL01

### APPLICATIONS OF NANOTECHNOLOGY IN THE AGRO-FOOD SECTOR

**António A. Vicente**

*IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering,  
University of Minho, Campus de Gualtar. 4710-057, Braga, Portugal*

Nanotechnology has a great potential to generate innovative solutions, providing food engineers and food technologists with a set of valuable instruments to deal with the growing demands of consumers regarding the foods they eat: safety, quality, health effects and innovation. Nanotechnology is being applied e.g. in the improvement of food safety and in the encapsulation of functional ingredients.

Layer-by-layer technology, which may consist in alternately submerging substrates in aqueous solutions of polyelectrolytes with opposed charges, may be applied to produce multi-layer coatings of nanometric thickness in various surfaces. These coatings may be prepared to incorporate bioactive compounds and promote their controlled release. They may be used to coat food systems, such as fruits and fresh-cut vegetables.

One other nano-system with great potential for application is nanoparticles based in food-grade materials. In this case, it is necessary to study/optimize the formulations and the processing conditions to obtain nanoparticles with the targeted properties. Once again, these systems may be used as carriers of bioactive compounds or as vehicles of such compounds already inside the human body.

It is possible that most of these applications will be difficult to adopt commercially due to their presumably excessive price or by difficulties in scaling-up their production. This just means that there is an enormous room for the development of scientific and technical work around this subject.

## KL10

## SIGNAL TRANSDUCTION MECHANISMS FOR CELLULASE PRODUCTION AND GLUCOSE SENSING IN FILAMENTOUS FUNGI

Gustavo Goldman

*Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE) and FCFRP, Universidade de São Paulo*

Despite recent advances in the understanding of lignocellulolytic enzyme regulation, less is known about how different carbon sources are sensed and the signaling cascades that result in the adaptation of cellular metabolism and hydrolase secretion. Therefore, the role played by non-essential protein kinases (NPK) and phosphatases (NPP) in the sensing of carbon and/or energetic status was investigated in the model filamentous fungus *Aspergillus nidulans*.

Eleven NPKs and seven NPPs were identified as being involved in cellulase, and in some cases also hemicellulase, production in *A. nidulans*. The regulation of CreA-mediated carbon catabolite repression (CCR) in the parental strain was determined by fluorescence microscopy, utilising a CreA::GFP fusion protein. The sensing of phosphorylated glucose, via the RAS signalling pathway induced CreA repression, while carbon starvation resulted in derepression. Growth on cellulose represented carbon starvation and derepressing conditions. The involvement of the identified NPKs in the regulation of cellulose-induced responses and CreA derepression was assessed by genome-wide transcriptomics (GEO accession 47810). CreA::GFP localisation and the restoration of endocellulase activity via the introduction of the  $\Delta creA$  mutation, was assessed in the NPK-deficient backgrounds. The absence of either the *schA* or *snfA* kinase dramatically reduced cellulose-induced transcriptional responses, including the expression of hydrolytic enzymes and transporters. The mechanism by which these two NPKs controlled gene transcription was identified, as the NPK-deficient mutants were not able to unlock CreA-mediated carbon catabolite repression under derepressing conditions, such as carbon starvation or growth on cellulose.

Collectively, this study identified multiple kinases and phosphatases involved in the sensing of carbon and/or energetic status, while demonstrating the overlapping, synergistic roles of *schA* and *snfA* in the regulation of CreA derepression and hydrolytic enzyme production in *A. nidulans*. The importance of a carbon starvation-induced signal for CreA derepression, permitting transcriptional activator binding, appeared paramount for hydrolase secretion.

Financial support: FAPESP and CNPq, Brazil

# Oral Presentations



## OP01

## EFFECT OF CONTROLLED ATMOSPHERE AND STORAGE TEMPERATURE ON THE PHENOLICS AND CAROTENOIDS OF PASTEURIZED PEACH PURÉES

Ana Oliveira<sup>1</sup>; Elisabete Alexandre<sup>1</sup>; Marta Coelho<sup>1</sup>; Cláudia Lopes<sup>3</sup>; Domingos P. F. Almeida<sup>2</sup>; Manuela Pintado<sup>1\*</sup>

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Modified atmosphere is a possible way to promote extension of the storage period and quality allowing the regular supply of industrial fruit matrices year-round and across the globe.

The aim of this study was to evaluate phytochemicals profile (focused on carotenoids and phenolic compounds) of pasteurized peach puree stored under reduced oxygen atmospheres throughout 90 days.

Peach (*Prunus persica* (L.) Batsch ‘Catherine’) purées were pasteurized at 90 °C during 5 minutes and then flushed with three different gas mixtures: 10% oxygen+90% nitrogen, 100% nitrogen and air during 3 minutes at 0.4 bar. Purées were stored in the dark under sterile conditions during 90 days at 4 and 23 °C.

Total antioxidant activity was affected by atmosphere with losses of 14 and 43% in air, 21 and 46% in 10% O<sub>2</sub>+90%N<sub>2</sub>, and 44 and 5% for 100%N<sub>2</sub>, stored at 4 and 23 °C, respectively. Total phenolic content was not significantly affected by storage atmosphere conditions at both storage temperatures, decreasing less than 15% during the 90-day storage period.

Among individual phenolic compounds identified in peach stored at 4 °C, the levels of (+)-catechin, neochlorogenic acid and chlorogenic acid increased under 10%O<sub>2</sub>+90%N<sub>2</sub>, by 67, 13, and 14%, respectively. The different atmosphere conditions at 23 °C presented no effect in concentration of (+)-catechin and neochlorogenic acid but chlorogenic acid concentration at 10%O<sub>2</sub>+90%N<sub>2</sub> showed a significant increase of 22%.

Total carotenoid content was affected by atmosphere with reductions of 53 and 75% in air, 69 and 79% in 10%O<sub>2</sub>+90%N<sub>2</sub>, and 41 and 67% for N<sub>2</sub>, stored at 4 and 23 °C, respectively. In the individual carotenoids, at 4 °C, the levels of lutein+zeaxanthin, β-cryptoxanthin and β-carotene under 100% N<sub>2</sub> increased by 27, 18 and 59%, respectively, while at 23 °C their concentrations decreased by 44, 17 and 2%, respectively. Refrigerated storage conditions under 100% N<sub>2</sub> preserved better carotenoids than room temperature where atmospheric conditions make no differences.

These results suggest that phenolic compounds are relatively well preserved during storage of peach purée while carotenoid levels can undergo significant changes. Storage temperature is more relevant than atmosphere composition, but anoxic conditions (100% N<sub>2</sub>) combined with refrigerated storage assure the best storage conditions for phytochemical preservation on peach purées to be used in food industry.

## OP02

### POPULATION ANALYSIS OF A PHA PRODUCING BIOREACTOR SUBJECTED TO FEEDSTOCK SHIFTS

Gilda Carvalho<sup>1</sup>; Anouk F. Duque<sup>2</sup>; Catarina S.S. Oliveira<sup>2</sup>; Ines Pedras<sup>2</sup>; Soren M. Karst<sup>3</sup>; Per H. Nielsen<sup>3</sup>; Maria Ascensão Reis<sup>2</sup>

<sup>1</sup>REQUIMTE/CQFB; IBET; <sup>2</sup>REQUIMTE/CQFB; <sup>3</sup>Univ. Aalborg

The production of polyhydroxyalkanoates (PHA) from industrial wastewater or by-products as feedstock using mixed microbial cultures is an attractive alternative to pure cultures. Additional economical advantages arise from having the flexibility to use different substrates, depending on their seasonality, price and composition. This study investigated the microbial ecology dynamics and corresponding performance of a mixed culture PHA producing system where a cheese whey substrate was followed by sugar cane molasses (SCM) and back to the cheese whey (CW). The system consisted of an anaerobic acidogenic fermenter (AnMBR) to convert the feedstock into organic acids, which were then used as PHA precursors in an aerobic feast and famine system sequencing batch reactor (SBR).

The acidogenic culture was responsive to the substrate shift, with an increase of the biomass and organic acids to substrate yields in the period fed with cheese whey. Furthermore, there was an increase in the amount of HV precursors (propionate and valerate) when cheese whey was replaced by molasses, resulting in lower HB:HV ratios of the polymer. Thus, it is possible to manipulate the polymer composition by using different feedstocks, which has advantages in terms of obtaining polymer with specific mechanical and thermal properties.

High throughput sequencing was performed to follow the changes in microbial populations in the AnMBR and the selection SBR as a consequence of the shift in feedstock. In the acidogenic reactor, a clear change in ecology accompanied the substrate shift. During the SCM phase, the Actinomycetaceae family constituted 75–93% of the total amplicon count, whereas a more dynamic population was detected during the CW phase. The Streptococcaceae family dominated during the first 18 days of this phase, followed by *Lactobacillus delbrueckii* 42 days after the introduction of CW.

In the selection SBR, quantitative FISH showed a decrease in the *Azoarcus* and *Thauera* populations with the introduction of fermented CW. The sum of *Thauera*, *Azoarcus* and *Paracoccus* biovolume was less than 50% throughout this phase, whereas these three groups covered most of the population when fed with fermented SCM. However, both systems had high storage capacity (56% for SCM and up to 65% for CW), suggesting that the remaining population also contained high abundance of other organisms with PHA-storing capacity.

In fact, when the feedstock shifted from SCM to CW, the resulting organic acids composition increased in propionate and valerate in detriment of acetate which resulted in a dramatic change in the population composition. High throughput sequencing results showed a very dynamic population, indicating temporary presence of other organisms with PHA-storing potential, including some members of the Bacillales order such as *Clostridium* and *Paenibacillus*. These results indicate that the feedstock has a high impact on the microbial population composition, and that certain feedstocks may be more prone to develop more diverse microbial populations, contributing to higher level of functional redundancy.

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## OP03

# PRODUCTION OF BACTERIAL CELLULOSE MEMBRANES BY *GLUCONACETOBACTER SACCHARI* USING INDUSTRIAL BIOMASS RESIDUES AND THEIR POTENTIAL APPLICATION

Nuno Hélder Silva<sup>1</sup>; Eliane Trovatti<sup>1</sup>; Luísa Serafim<sup>1</sup>; Carmen S. R. Freire<sup>1</sup>; Armando J. D. Silvestre<sup>1</sup>; Carlos P. Neto<sup>1</sup>

<sup>1</sup>CICECO, University of Aveiro

Bacterial Cellulose (BC), a highly pure form of cellulose, is gaining considerable importance due to its unique physical and mechanical properties. In static media, BC is produced in the form of a swollen membrane (~99% water) by several bacteria from the *Gluconacetobacter* genus such as *G. sacchari* which has demonstrated to be a high potential BC producing strain [1]. In order to decrease the high cost of culture media, several residues from agro-forestry industries, including grape skins and olive oil mill residues, were successfully evaluated as economic carbon and nutrient sources for the production of BC [2,3]. Finally, the potential of the produced BC membranes as drug delivery systems was also investigated. In vitro diffusion studies with Franz cells were conducted for lidocaine and ibuprofen, using human epidermal membranes [4,5]. The results obtained indicate that these BC membranes can be successfully applied for the dermal drug delivery.

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## OP28

## THE GENOME SEQUENCE OF THE FOOD SPOILAGE YEAST ZYGOSACCHAROMYCES BAILII ISA1307

Nuno P Mira<sup>1</sup>; Martin Münsterkötter<sup>2</sup>; Filipa Dias-Valada<sup>1</sup>; Júlia Santos<sup>3</sup>; Margarida Palma<sup>1</sup>; Filipa C. Roque<sup>1</sup>; Joana F Guerreiro<sup>1</sup>; Fernando Rodrigues<sup>3</sup>; Maria João Sousa<sup>4</sup>; Cecília Leão<sup>3</sup>; Ulrich Güldener<sup>2</sup>; Isabel Sá-Correia<sup>1</sup>

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The yeast species *Zygosaccharomyces bailii* exhibits a remarkable but poorly understood intrinsic resistance to weak acids used as food preservatives, being the most significant spoilage yeast especially in acidic food products. The nucleotide sequencing and annotation of the genome of *Z. bailii* ISA1307 are reported. This strain was isolated from a continuous sparkling wine production plant. The genome sequence obtained is distributed through 154 scaffolds with a total size of approximately 21.2 Mb, corresponding to 96% of the genome size estimated by flow cytometry. Results show that *Z. bailii* ISA1307 is a strain whose genome includes 4,385 duplicated genes (approximately 90% of the total number of predicted genes) and around 1155 single-copy genes. The functional categories that include a higher number of *Z. bailii* ISA1307 genes are “Metabolism and generation of energy”, “Protein folding, modification and targeting” and “Biogenesis of cellular components”. The molecular mechanisms underlying relevant physiological traits of the ISA1307 strain and of other strains of *Z. bailii* species, such as poor sensitivity to the Crabtree effect, fructophilicity and resistance to acid stress, will be discussed, based on the functional annotation of predicted genes.

The knowledge of *Z. bailii* ISA1307 genome sequence is instrumental to accelerate systems-level understanding of weak acid resistance mechanisms envisaging the rational control of this highly threatening spoilage yeast species. It is also essential for inspiring and guiding novel biotechnological applications by exploring *Z. bailii* as an attractive cell factory, in particular, to allow fermentation processes to be performed under otherwise restrictive conditions, in particular related with its high resilience to acidic stress.

## OP29

## THE IMPACT OF FUNGAL INFECTIONS ON PROCESSING AND QUALITY OF BEER

Pedro M. Oliveira<sup>1</sup>; Alex Mauch<sup>1</sup>; Fritz Jacob<sup>2</sup>; Elke Arendt<sup>1</sup>

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Infected malt with filamentous fungi entering into the brewing supply chain can have a major impact on the processability and quality of malt, wort, and beer produced. *Fusarium culmorum* is one of the main fungal species responsible for one of the most devastating diseases affecting small grain cereals: *Fusarium* Head Blight. The main objective of this study was to investigate the impact that an initial *in vitro* 20% *F. culmorum* infection in barley grains has on malting, brewing and in the final beer quality. Due to fungal infection, germinative energy of infected barley grains decreased by 45% and its water sensitivity increased dramatically. Infected malt grains were characterized by extreme structural proteolytic, (hemi)-cellulolytic and starch deterioration with increased friability and fragmentation. Furthermore, malt loss was over 27% higher in comparison to control, with *Fusarium* mycotoxin deoxynivalenol (DON) accumulated in grains. *F. culmorum* caused major changes in enzymatic activity and thus leading to a different protein profile, with especial incidence in storage protein fractions. The infected malt grist also had a major impact on wort and final beer organoleptic qualities with a 67% darker colour and transferred 78% of the mycotoxin accumulated in grains to beer. Wort containing infected malt had a lower pH, more amino nitrogen compounds, higher  $\beta$ -glucan and a 46% increase in the purging rate than the control. It caused premature yeast flocculation, and resulted in a considerably different amino acid and flavor profiles, contributing to an increased beer staling character. This study shows clearly that 20% initial infected mature barley grains do not meet the standards required for use in malting or brewing.

## OP30

# INVESTIGATING CANDIDATE FACTORS INFLUENCING BOVINE SPONGIFORM ENCEPHALOPATHY

Thomas Kennedy<sup>1</sup>

<sup>1</sup>*Department of Agriculture, Food and the Marine*

Obtaining the appropriate sample is critical to ensure BSE test result integrity. For BSE, this is the brain stem at the level of the obex – the area where abnormal Prion Protein (PrP<sup>Sc</sup>) is most consistently deposited and fortuitously first detectable. Occasionally suboptimal samples (SO) occur where the obex is absent or unidentifiable, in which case negative results are questionable. The relationship between SO occurrence and factors such as animal age, breed category [dairy, beef breeds native to the British Isles and Continental beef breeds], gender, dehiding method (upward or downward) and sampler identity (n=13) was investigated.

A stepwise logistic regression model was applied to a dataset containing records of 23,646 animals sampled at the abattoir over a 2-year-period. Details relating to SO occurrence were obtained from rapid test laboratory reports. Details relating to the animal's age and date of slaughter, gender and breed were obtained from the Animal Identification and Movement database. Dehiding method changed mid study.

SO incidence was 0.26%. Results indicate that samplers Sahlmk (OR = 5.9; 95% CI = 1.9 – 18.4), Sdlrkl (OR = 3.5 ; 95% CI = 1.2 – 10.5), Semada (OR = 5.3; 95% CI = 2.0 – 13.7), bulls (OR = 2.7; 95% CI = 1.4 – 5.3), native beef breeds (OR = 2.3; 95%CI = 1.2 – 4.5) and continental beef breeds (OR = 2.4; 95% CI = 1.3 – 4.3) had a significant positive effect on SO occurrence. Age and hide removal method were found not to have any significant effect. The results inform a basis for risk ranking animals prior to sampling. The importance of sampler training and motivation is also indicated. Samplers are encouraged to perfect their technique by sampling animals younger than the statutory prescribed age (currently 48 months for unhealthy animals in all EU member states and 72 months in for healthy animals in some member states) prior to taking official samples.

## OP40

OPTIMISING THE PRODUCTION OF A SILK-ELASTIN-LIKE PROTEIN IN *E. COLI*:  
OVERCOMING ACETATE ACCUMULATION AND PLASMID INSTABILITY.

Tony Collins<sup>1</sup>; Mário Barroca<sup>1</sup>; Fernando Branca<sup>1</sup>; João Azevedo-Silva<sup>1</sup>; André da Costa<sup>1</sup>;  
Raul Machado<sup>1</sup>; Margarida Casal<sup>1</sup>

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Silk-elastin-like proteins (SELPs) combining the physicochemical and biological properties of silk and elastin have a high potential for use in the pharmaceutical, regenerative medicine and materials fields. Their development for use is however restrained by their production levels. Here we describe the production optimisation for a novel recently described SELP in the pET-*E. coli* BL21(DE3) expression system. Both batch production in shake flasks and fed-batch production approaches were investigated and optimised. In both cases a comprehensive empirical approach examining all process variables (media, medium composition, inducer, induction time and period, temperature, pH, aeration, agitation, pre- and post-induction growth rates) and a detailed characterisation of the bioprocesses were used in an attempt to maximise production and identify the factors limiting higher production levels. The major factors limiting SELP yields have been identified as acetate accumulation, plasmid instability on induction and a heightened host cell metabolic burden during SELP production. To circumvent these limitations we have optimised the fed-batch production approach and engineered the production plasmid for an improved stability. Using the optimised conditions, approximately 0.5 g/l of purified SELP was obtained in shake flasks and as much as 5 g/L was obtained when using the fed-batch approach. These are the highest reported SELP productivities to date and represent, respectively, approximately 10- and 100-fold increases on that previously reported.

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# Poster Presentations



## Biocatalysis & Biotransformation

P002

### ENHANCEMENT OF CASTOR OIL BIOTRANSFORMATION INTO AROMA BY *YARROWIA LIPOLYTICA* MUTANTS

Adelaide Braga<sup>1</sup>; Isabel Belo<sup>1</sup>

<sup>1</sup>IBB–Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho

The food industry has a great interest in biotechnological production of  $\gamma$ -decalactone by *Yarrowia lipolytica*, due to its increasing consumers acceptability in comparison with similar products obtained by chemical synthesis. This yeast is able to produce  $\gamma$ -decalactone by transformation of a hydroxylated C<sub>18</sub> fatty acid. However, lower yields of  $\gamma$ -decalactone were obtained (up to 4–5 g L<sup>-1</sup>), mainly due the degradation of newly synthesized lactone and the partial use of ricinoleic acid or intermediate at the C<sub>10</sub> level, which is simultaneously the precursor for other  $\gamma$ -lactones.

Thus, the purpose of this work is to enhance the biotransformation of castor oil, source of ricinoleic acid, into  $\gamma$ -decalactone exploring different operation mode strategies in bioreactor (batch and fed-batch) and compare the yields obtained with wild type strain with those achieved by mutant strains.

Different experiments were conducted in a 3.7-L bioreactor using an aeration rate of 5.1 L min<sup>-1</sup>, agitation 650 rpm and pH 6.0 (previously optimized conditions [1]). The influence of castor oil concentration and cell density on  $\gamma$ -decalactone production was investigated. Two different cell and castor oil concentrations (30 g L<sup>-1</sup> and 60 g L<sup>-1</sup>) were used for the biotransformation. In the expectation of achieving higher  $\gamma$ -decalactone concentrations, a step-wise fed-batch strategy was also attempted.

In a first approach, this study was conducted with *Yarrowia lipolytica* W29 (ATCC20460) and the highest  $\gamma$ -decalactone productivity of 215.4 mg L<sup>-1</sup> h<sup>-1</sup> was obtained in a batch mode of operation with 60 g L<sup>-1</sup> of cells and 60 g L<sup>-1</sup> of castor oil. After that,  $\gamma$ -decalactone production with two *Yarrowia lipolytica* mutants was studied. Experiments performed with *Y. Lipolytica* MTLY40–2P, with a deletion of all the POX 3–5 genes and a multicopy insertion of *POX2* [2], resulted in an increased accumulation and an inhibition of  $\gamma$ -decalactone degradation. Since this yeast is also known to be a lipase producer and these enzymes catalyze the hydrolysis of triacylglycerides into glycerol and free fatty acids, a *Y. lipolytica* JMY3010 mutant, that overexpress extracellular lipase by the LIP2 gene (encoded the main extracellular lipase activity) cloned under the control of the TEF promoter [3], as also used.

With these different approaches is possible to increase aroma productivity and a greater enhance in  $\gamma$ -decalactone production was achieved (up to 7–9 g L<sup>-1</sup>) through conjugation of a bioprocess optimization and genetic engineering approach.

1.  $\gamma$ -decalactone production from castor oil by *Yarrowia lipolytica* in a bioreactor by operational factors selection. 9<sup>th</sup> European Congress of Chemical Engineering and 2<sup>nd</sup> European Congress of Applied Biotechnology. The Hague. The Netherlands, April 21–25, 2013.
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## Biocatalysis & Biotransformation

### P003

#### OPTIMIZATION OF SALTS ADDITION FOR XYLITOL PRODUCTION BY *DEBARYOMYCESS HANSENI* AND FURTHER SCALE-UP TO A 50-L BIOREACTOR

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Xylitol is a pentahydroxy sugar alcohol. Since 1991, xylitol is considered a food additive (European Union code, E-967) according to the Codex Alimentarius. Xylitol has a sweetening power equivalent to sucrose (edulcorant power=1) but almost half its calories (2.4 kcal/g for xylitol versus 4 kcal/g for sucrose). Consequently, xylitol can replace sucrose on a weight for-weight basis, particularly considering that xylitol has a pleasant cool and fresh sensation due to its high negative heat of solution. Additionally, xylitol is not fermentable by cariogenic bacteria of the human oral cavity decreasing dental plaque and inhibiting demineralization of tooth enamel. Xylitol also has been used as diabetic sweetener and for parenteral nutrition. Moreover, it has negative heat of dissolution producing a fresh sensation, low viscosity in solution, absence of the Maillard reaction and a high chemical stability. In spite of these properties, the low production of xylitol worldwide over other traditional sweeteners (the production of traditional sweeteners is estimated in 51,000 tonnes per year) or alcohol type sweeteners (75,000 tonnes per year) is based on its high cost of production by chemical means (10 times higher than the price of other traditional sweeteners). Xylitol produced by chemical means (the only way to be obtained at industrial level) has a market price of approximately 6.39 €/kg. However the market of xylitol is increasing and at present is estimated to be \$340 million/year. Its high price in comparison with sucrose or sorbitol drives to look for lower cost technologies. In the present work, a 2<sup>\*\*</sup>(8-2) central composite design with 85 experiments was carried out to optimize the concentration of salts to carry out the xylose to xylitol bioconversion by *Debaryomyces hansenii*. The best results were used to scale-up the process to a 50-L bioreactor using synthetic xylitol or vine trimming hemicellulosic hydrolyzates. Under these conditions, up to 16.4 g/L xylitol were produced after 27h fermentation with a product yield of 0.68 g/g and a global volumetric productivity of 0.61 g/L·h.

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## Biocatalysis & Biotransformation

P004

### POTENTIAL OF VINE-TRIMMING WASTES AS IMMOBILIZATION CARRIER FOR SOLID-STATE FERMENTATION TO INCREASE THE BIOTRANSFORMATION OF FERULIC ACID TO VANILLIN

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The biocatalytic production of fine flavors and fragrances have been expanding rapidly during the past several years due to the legislations in the US and Europe that stated that natural flavor substances could be prepared by enzymatic or microbial processes. Ferulic acid, a phenolic substrate widely distributed in the plant kingdom, can be used to produce vanillin, the most characteristic component of vanilla flavor. The price of "natural" vanillin is very high (3,750 €/Kg) due to its limited availability. The difference of price and the growing demand from consumers for natural products have triggered the interest of producing vanillin from natural sources by biotransformations. However, it is necessary to provide a process for producing industrially interesting vanillin concentrations, so it is necessary to study alternative processes such as solid-state fermentation (SSF) using microorganisms immobilized on inert supports in order to scale up the biotechnological processes to reach a wide market. In this work, raw vine-trimming wastes or the solid residue obtained after different fractionation treatments were evaluated for their suitability as *Amycolatopsis* sp. immobilization carrier. The results point out that in spite of showing the lowest water absorption capacity, raw material were the most appropriated substrate to conduct the fermentation, achieving up to 262.9 mg/L phenolic compounds after 24h fermentation, corresponding to 42.9 mg/L vanillin, 115.6 mg/L vanillyl alcohol and 104.4 mg/L vanillic acid.

## Biocatalysis & Biotransformation

P005

### USE OF BACTERIAL CELLULOSE AS A NOVEL CARRIER FOR IMMOBILIZATION OF COMMERCIAL LACCASE

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Immobilized enzymes have several advantages over free enzymes, such as enzyme stability improvement, biocatalyst reuse and easier product separation. In order to increase the industrial use of laccases, there is a high interest in discovering the ideal carrier to immobilize such enzymes. Bacterial cellulose (BC) is a singular form of cellulose produced by many bacteria. Owing to its unique properties (e.g. high mechanical strength and excellent water absorption capacity), BC is attracting a lot of interest as a new functional material. Additionally, BC contains a lot of functional groups, which makes it a potential carrier for enzyme immobilization.

Hence, the aim of this work was to investigate the immobilization of laccase onto lyophilized BC, as well as the influence of immobilization conditions on the performance of the immobilized enzyme. To understand the effects of lyophilized BC on the biocatalyst, the properties of the immobilized enzyme were studied.

To perform the immobilization of commercial laccase (Novozym® 51003), lyophilized BC pieces were incubated in laccase solution. Response surface methodology (RSM) and a 3<sup>3</sup> Box–Behnken full factorial design were employed to study and optimize the effects of immobilization conditions (enzyme concentration, contact time and pH) on the activity recovery of immobilized laccase. Then, the properties of free and immobilized laccase were investigated.

Beyond indicating that all the selected variables were significant ( $P < 0.05$ ), the results clearly suggested that enzyme concentration was the one with the highest impact on the activity recovery of immobilized laccase. The optimal conditions of enzyme immobilization were as follows: enzyme concentration, 0.15 µL laccase per L buffer solution; contact time, 4.8 h; and pH, 5.4. Under these conditions, the predicted and experimental activity recoveries of immobilized enzyme were 47.88 and 47.78 %, respectively. Thermal stability of the immobilized enzyme was found to increase notably. The immobilized laccase also showed high operational stability, since it retained 86 % of its initial activity after 7 successive cycles of biocatalysis. Kinetic studies showed that the values of the Michaelis–Menten constant and maximal rate of the reaction decreased upon immobilization (9.9 and 1.6 times, respectively).

## Biocatalysis & Biotransformation

P006/F29

### TOWARDS A RATIONAL APPROACH FOR ENZYME IMMOBILIZATION IN A MICROREACTOR FRAMEWORK

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Biocatalysis is a well established modern technology for the chemical modification of molecules. Several strategies have been applied to enhance the development pace of biocatalytic processes, including the use of microreactors and operation in microfluidic environments. These require low volumes of consumables, mass and heat transfer limitations are overcome and continuous flow operation is privileged.

Immobilization is another approach to improve enzyme performance. Despite the extensive research over many decades in this field, empiricism still prevails over rational design. In order to offset this pattern, and have more cost-effective systems, a trend towards a more comprehensive characterization of the bioconversion system is emerging.

The present work is within such scope, using as model system the immobilization of invertase in glass microchannels, aiming at the production of invert sugar syrup through sucrose hydrolysis. Invertase immobilization was achieved by coating the inner surface of the reactor with successive layers. The process starts with the activation of the glass surface with 3-aminopropyltriethoxysilane (APTES) followed by the introduction of a spacer, glutaraldehyde. Lastly, the enzyme solution was introduced in the presence of sodium cyanoborohydride. The self-assembly monolayers applied in the immobilization protocol were monitored and characterized through X-ray Photoelectron Spectroscopy (XPS) and Water Contact Angle (WCA) analysis; Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) analysis. *In situ* real-time enzyme immobilization kinetics was monitored by Quartz Crystal Microbalance (QCM) with dissipation.

The immobilization approach proved to be effective, resulting on successful continuous use of the microreactor for a period of over 30 days with roughly constant full conversion of a sucrose solution of 50g.L<sup>-1</sup> ( $\tau_{max} = 1$  h). AFM and WCA data confirmed that the applied coatings changed the surface properties of the microchannels. Still, XPS assays suggest that the chemical interactions between the several components of the coatings are more complex than what is often described in the literature and that the enzyme is unevenly distributed on the silica substrate. QCM studies revealed a significant increase in the enzyme surface loading when coatings are applied. The use of key analytical techniques allowed a deeper understanding of the immobilization phenomenon.

## Food Microbiology & Biotechnology

P007

### A MICROBIOLOGICAL ASSESSMENT SCHEME TO EVALUATE THE FEED SAFETY MANAGEMENT SYSTEM IN A CATEGORY 3 FAT MELTING ESTABLISHMENT

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Animal tissues used in the manufacture of petfoods pose identical risks to those used in human food production. Petfoods are often stored and prepared in the domestic kitchen providing an opportunity for cross contamination. Petfoods must therefore be produced under a strict feed safety management systems (FeSMS). The performance of a FeSMS in a Category 3 fat melting establishment integrated to a beef abattoir that produces greaves for pet food production was measured using a microbiological assessment scheme (MAS). The establishment is approved by the competent, is subject to official controls and maintains a FeSMS based on HACCP. Over a 10-month-period 685 samples taken at 7 critical sampling locations (raw materials, greaves post heat treatment, post centrifugation, post packaging, the environment, personnel and water) were analysed for 8 microbial parameters (Total Viable Counts, *Enterobacteriaceae*, *Salmonella*, *Listeria* spp, faecal enterococci, *Escherichia coli*, *Clostridium perfringens*, coliforms) . Findings were benchmarked against legal, industry and best practice norms. Results indicate that 100% of raw fat samples achieved the same acceptable criteria as the beef carcasses from which the fat was derived from as determined by Regulation (EC) No. 2073/2005. *Salmonella* was not isolated from greaves samples taken within process or post packaging, however, *Enterobacteriaceae* though compliant were detected at levels of 15cfu/g and 165cfu/g in two final product samples. 100% of water samples were compliant. Four environmental samples had TVC greater than 10cfu/cm<sup>2</sup> with *Listeria innocua* isolated from one sample . Two personnel swabs had TVC above the recommended 100cfu/cm<sup>2</sup>. The findings indicate that the MAS is an effective tool to assess FeSMS performance. Results indicate that safe petfood is being produced, however, deficiencies in personnel and environmental sanitation have been identified. Post cooking contamination from these sources remains a risk as is evidenced by the presence of *Enterobacteriaceae* in final product. The MAS also provides the establishment's management with key information as to where to prioritise resources and channel investment to improve the safety of their product.

## Food Microbiology & Biotechnology

P008

### AEROMONAS BIOFILM-FORMATION ON STAINLESS STEEL COUPONS

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*Aeromonas* spp. are waterborne bacteria that have been increasingly implicated in serious human infections; the main risk of acquiring aeromonads-associated infections being attributed to the consumption of water or contaminated food products. These bacteria are able to persist in water distribution systems and food processing environments, mainly due to their capacity to form biofilms. Obviously, the attachment of pathogenic bacteria onto food-contact surfaces and the subsequent biofilm formation is undesirable, since the detachment of cells from the biofilm structure can lead to the cross-contamination of food products and cause foodborne diseases. Moreover, bacteria in biofilm-state are indeed highly resistant to killing by antimicrobials, compared to planktonic cells, and therefore exhibit tolerance to such compounds; creating a constant source of cross-contamination within food environments.

In the present investigation five putative pathogenic strains were selected from a collection of *Aeromonas* spp. isolated from water, food, food-processing surfaces and clinical samples. Subsequently, the representative aeromonads were evaluated regarding their ability to form biofilm on stainless steel coupons. After coupon inoculation, followed by 48h of incubation at both refrigeration (4°C) and room (20°C) temperature, biofilm formation was measured by means of CFU counts. Additional assays evaluated the efficacy of three commercial disinfectants in removing pre-formed biofilm, as well as in preventing biofilm formation.

All aeromonads under analysis were able to form biofilms at both temperatures, despite their origin (environmental *versus* clinical). The disinfectants investigated demonstrated to be efficient in removing *Aeromonas*-biofilms from stainless steel coupons, but were unable to prevent biofilm formation.

In real food processing environments the presence of many other microbial species will add complexity to the behavior of multi-species biofilms, since all incorporated microorganisms will compete, cooperate and communicate with each other; highlighting the need for further investigations concerning this subject.

## Food Microbiology & Biotechnology

P009

### ALTERNATIVE METHODS FOR DETECTION OF *SALMONELLA* SPP. AND *LISTERIA MONOCYTOGENES*

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The microbiological quality of food has become a very important factor for food industries as well as for the regulation agencies.

Early detection of pathogenic microorganisms becomes crucial in order to prevent foodborne diseases. *Salmonella* spp. and *Listeria monocytogenes* are important foodborne pathogenic microorganisms. The detection methods of both microorganisms (ISO 6579:2002 and ISO 11290-1:1996, respectively), are quite lengthy, taking about 8 days to obtain results [1,2] Consequently, it is extremely important to validate faster methods with lower costs for laboratories.

Vibrational spectroscopic techniques, infrared spectroscopy (IR) and Raman spectroscopy, have been used since the 1980s as complementary methods for bacteria differentiation owing to their rapid “fingerprinting” capabilities and the molecular information that they can provide. These techniques present several advantages in the microbiological classification and identification fields: they are fast (requiring virtually no sample processing), non-destructive, multi-purpose (e.g., detection, enumeration, classification, identification), discriminating at different taxonomic levels (serotype, strain, species or genus) [3]. In this study it was evaluated the potential of mid infrared spectroscopy (MIR), to confirm the presence of *Salmonella* spp. and *Listeria monocytogenes* in food products. For this, *Salmonella* and *Listeria* (identified by API system) from food products (cheeses, sausages and prepared dishes) were isolated. The colonies that grew in OCLA medium (*Listeria*) and XLD medium (*Salmonella*) were directly analyzed by HATR-MID-IR. The multivariate analysis of the spectra easily allowed the discrimination of the different *Listeria* and *Salmonella* species. Moreover, it was possible to discriminate other bacteria that grew on XLD *Salmonella* selective medium (*Shigella* spp. and *Citrobacter* spp.) without the necessity of biochemical confirmation.

We conclude that MIR allows the rapid confirmation of two important food pathogens, *Salmonella* and *Listeria*, and even permits to differentiate different species of *Listeria* and *Salmonella*.

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## Food Microbiology & Biotechnology

### P010

#### ANTIBACTERIAL ACTIVITY OF DIFFERENT WATER BASED SEAWEEDS EXTRACTS AGAINST HUMAN PATHOGENIC BACTERIA

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Nowadays, it is becoming more and more urgent to find alternatives to antibiotics since it is recognized that the increasing number of microorganisms resistant to the available antibiotics is an emergent problem throughout the world and subject of much research. Bacterial infection causes high rate of mortality in human population. New compounds with new mechanisms of antibacterial action are urgently needed. Marine macroalgae are considered an excellent source of bioactive compounds so they have been studied as potential biocidal and pharmaceutical agents, with a broad range of biological activities including antibacterial activity. The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and side effects. However it is not only the resistance but also the cost of synthetic chemicals that impose the search for alternative drugs such as antimicrobial compounds from natural sources. These limitations demand for improved pharmacokinetic properties, which imply continued research for new antimicrobial compounds.

Aqueous extracts of *Sargassum muticum*, *Osmundea pinnatifida* and *Codium tomentosum* from the North Portuguese Coast by hot water extraction (HWE), enzyme-assisted extraction (EAE) and ultrasound-assisted extraction (UAE) were obtained and assessed for antibacterial activity by agar well diffusion method at two concentrations (2 and 8 mg/mL).

At 2mg/mL antibacterial activity was observed only against two gram positive species, namely *B. subtilis* (NCTC 3610) and two strains of *S. aureus* (ATCC 6538 and NCTC 8532). The activity index, calculated as the ratio of IZ of extract per IZ of positive control was higher than 0.50 for Viscozyme® L and cellulase EAE in *O. pinnatifida* and Flavourzyme EAE in *S. muticum* in comparison to lactic acid (30%) and chloramphenicol (1mg/mL), respectively. A four-fold increase in the concentration of lyophilized extract increased the evidence of eventual susceptibility of more pathogenic bacteria to these extracts although in some of them, their inhibition zone is minimal. The extracts showed higher antibacterial activity against Gram positive bacteria.

## Food Microbiology & Biotechnology

P011

### ANTIBIOFILM PROPERTIES OF *QUERCUS ILEX* ACORN EXTRACTS AGAINST MRSA AND MSSA

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Biofilms are complex structures that grant microorganisms some survival advantages namely nutrient availability and antimicrobial resistance. As such, the need to find new antimicrobial compounds that are active against these structures is in great demand. Phenolic compounds are described as capable of interfering with the cellular membrane and interact with both intracellular and extracellular proteins, thus they may present an alternative to traditional biofilm control.

Acorns have been reported as a rich source of phenolic compounds, particularly tannins, and thus are likely to exhibit some of the properties that are associated with these compounds, namely antimicrobial properties. As such, the present work aimed to assess the antibiofilm properties of hydroethanolic, phenolic rich, acorn extracts against two *Staphylococcus aureus* strains, one sensitive (MSSA) and one resistant (MRSA) to methicillin. To do so, both microorganisms were exposed to two different sub-MIC concentrations of extracts produced using acorn shells (fresh and roasted) and cotyledons (fresh or roasted). The amount of biofilm produced in the presence of the extracts, after 48 h, was then determined using the microtiter – crystal violet based assay. The results obtained showed that MRSA was the most sensitive strain, with biomass inhibition percentages ranging from 60 to 70%, while for MSSA all inhibition percentages ranged between 29 to 57%. No significant differences between shell and cotyledon were found when considering the results obtained for MRSA. The same was not observed for MSSA where biomass production was significantly higher for roasted cotyledon (2.5 to 20.6%). For this microorganism, it was interesting to note that lower concentrations of the extracts appeared to possess a higher antibiofilm activity (0.7 times more active for fresh or roasted shell extract and 0.6 times more active for fresh cotyledon extracts).

Overall, these results indicate that *Quercus ilex* acorns possess the potential to be an effective means of biofilm control though further studies are still required to fully ascertain their true potential.

## Food Microbiology &amp; Biotechnology

P012

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS FROM *MELALEUCA ARMILLARIS*Patrícia Pires<sup>1</sup>; Fernanda Delgado<sup>1,2</sup>; Paulo Antunes<sup>3</sup>; M. Helena Martins<sup>1</sup>; M. Manuela Goulão<sup>1</sup>; Cristina M. B. S. Pintado<sup>1,2</sup>

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The genus *Melaleuca* (family *Myrtaceae*) consists of about 230 species rich in volatile oils [1], which in general represent an interesting alternative to control spoilage and pathogenic foodborne microorganisms. Several studies evaluated the antimicrobial activity of *Melaleuca* essential oils against different microorganisms, including bacteria and fungi [2], showing that the results are very dependent of the plant origin.

The aim of this study was to assess the antimicrobial potential of the essential oils of *Melaleuca armillaris* against *Listeria monocytogenes* and *Pseudomonas aeruginosa* bacteria and *Yarrowia lipolytica* yeast. Leaves of *M. armillaris* were collected from the garden of Escola Superior Agrária (Castelo Branco, Portugal) between March and May 2013. Green and dried leaves were used for the essential oil extractions by hydrodistillation (different times of extraction essayed) according to the standard procedure described in the *European Pharmacopoeia*. The chemical composition of the ten essential oils of *M. armillaris* was studied by means of GC-MS analysis. The minimum inhibitory concentration (MIC) values were determined by the broth microdilution method for bacteria and by the broth macrodilution method for yeast, while the minimum microbicidal concentration (MMC) values were determined by the drop plate method onto solid medium. Chloramphenicol was used as reference antibiotic. The essential oils were tested in different concentrations for each microorganism. There was assayed at the doses of 83.20, 43.45, 22.48, 11.63, 6.02, 3.11, 1.61, 0.83 and 0.43 µl/mL to evaluate the antimicrobial activity of *L. monocytogenes*. For antimicrobial evaluation against *P. aeruginosa* was used the doses of 173.79, 89.89, 46.50, 24.05, 12.44, 6.43, 3.33, 1.72 and 0.89 µl/mL. For *Yarrowia lipolytica* the essential oils was tested at the doses of 76.15, 26.22, 8.76, 2.92, 0.97 and 0.32 µl/mL.

The antimicrobial activity results (intermediate value of three experiments) showed that *Listeria monocytogenes* and *Yarrowia lipolytica* were inhibited by the essential oils of *Melaleuca armillaris*, while growth of Gram-negative bacteria *Pseudomonas aeruginosa* was not affected even at the highest dose tested. The most frequent MIC and MMC values of essential oils tested against *Listeria monocytogenes* were, respectively, 11.63 µl mL<sup>-1</sup>/22.48 µl mL<sup>-1</sup> and 83.20 µl mL<sup>-1</sup>. Concerning to the yeast *Yarrowia lipolytica*, we observed MIC and MMC values of 8.756 µl/mL for the majority of the essential oils. In general, the results of these selected essential oils showed low antimicrobial activity when compared with the MIC values in the bibliographic references [3]. The relationship between inhibitory activity, plant origin and chemical composition of the essential oils from different provenances are also discussed.

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## Food Microbiology & Biotechnology

P013

### ANTIMICROBIAL ACTIVITY OF PROPOLIS NANOPARTICLES AGAINST SOME COMMON MEAT CONTAMINATION BACTERIA

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Raw meat is a highly perishable product that requires a great amount of care, from its handling to the conservation conditions at low temperatures. The reduction of microbial proliferation in meat is necessary to achieve an increase of shelf life, food safety, while maintaining product features.

For this reason, a technology based on natural antimicrobial agent may offer a potential alternative to protect and control the proliferation of microorganisms on food products.

Propolis is a natural resinous substance collected from the leaf buds of different tree species by honeybees and known for its biological properties (antibacterial, antifungal, antioxidant) (Koo et. al, 2000).

The aim of this work was to evaluate the antimicrobial activity of propolis nanoparticles in comparison with ethanol-propolis extract against some common meat contamination bacteria.

The ethanol-propolis extract was obtained from green propolis resin, in absolute ethanol under agitation during 15 days. To obtain the propolis nanoparticles, ethanol-propolis extract at 13.75% (w/v) was mixed with polyvinyl-alcohol solution at 0.1% (w/v).

Antimicrobial activity of propolis nanoparticles and ethanol-propolis extract was tested against 8 microorganisms typically present in meat. Minimum inhibitory concentrations (MIC) of both solutions were evaluated by agar-well diffusion method; all strains were susceptible and MIC values ranged from 0.57 to 2.29% (w/v) for propolis nanoparticles and from 0.68 to 6.88% (w/v) for ethanol-propolis extract. The MIC of propolis nanoparticles for *Escherichia coli*, *Staphylococcus aureus*, *Salmonella thompson*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Enterobacter helveticus*, *Lactobacillus buchneri* and *Leuconostoc mesenteroides* was 1.15%, 0.57%, 2.29%, 1.72%, 1.72%, 2.29%, 2.29%, 1.72%, respectively, and the MIC for ethanol-propolis extract to the same species was 3.44%, 0.68%, 3.44%, 3.44%, 3.44%, 6.88%, 6.88%, 3.44%, respectively.

The shown antimicrobial activity of propolis nanoparticles is of potential interest for food applications (e.g. in edible coatings formulation). Therefore, results obtained in this study, set the bases for future studies, using films as support for propolis nanoparticles, for application in meat products.

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## Food Microbiology & Biotechnology

### P014

#### ANTIMICROBIAL EFFECT OF SELECTED ESSENTIAL OILS ON *SALMOMELLA* SPP. ISOLATED FROM TRADITIONAL MEAT PRODUCTS ESTABLISHMENTS

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Food borne outbreaks caused by foods contaminated with *Salmonella* spp. have wide economical and public health significance. Alternative preservation of foods based on the utilization of essential oils (EOs) has been increased to achieve the consumers demand regarding the awareness of synthetic chemical additives.

The antimicrobial effect of 13 EOs: basil, tarragon, oregano, cinnamon, parsley, nutmeg, lemon, orange, black pepper, paprika, rosemary, laurel and cumin (kindly provided by Ventós Chemicals, Barcelona) were tested against 11 strains of salmonella (*Salmonella* sp. CECT 4155 and 10 strains previously isolated from meat products establishments). Strains were overnight cultured in XLD; colonies were suspended in NaCl 0,9% (turbidity McFarland 0.5). The antimicrobial screening was performed by the agar well diffusion (AWD) test and the disk diffusion assay (DDA). The indicator strains (100 ul) were inoculated onto Mueller Hinton agar. 20 u l were added to 6.5 mm wells (AWD) or poured onto a 6 mm paper disk (DDA). EOs were screened to determine the minimum inhibitory concentration (MIC) against the same salmonella strains using the concentration of 25%, 20%, 15%, 10% for the EOs with moderate inhibitory activity observed in the screening tests and 2.5%, 1%, 0.5% and 0.25% for the EOs with strong inhibitory activity in a 96-well microplate containing Mueller Hinton broth inoculated with salmonella to a final concentration of 5.7 Log CFU in each well. The experiment was made in triplicate. EOs of basil, tarragon, cinnamon and nutmeg presented a moderate activity (halo  $\leq$  20 mm.); EOs of oregano, rosemary, laurel and cumin showed a strong activity (halo  $>$ 20mm.). The rest of EOs not presented inhibitory effect. The differences among the inhibitory effect by EOs were statistically significant ( $p < 0.01$ ). The inhibitory effect was higher when tested with the DDA in comparison with AWD ( $p < 0.01$ ). The MIC values ranged from 10% to 25% and to 0.25% to 5% in EOs with moderate inhibitory action and EOs with strong inhibitory action respectively. The use of EOs as a natural preservative are an alternative to the chemical additives to improve the safety of the meat products, however, our results showed that it is necessary a proper evaluation of the antimicrobial effect of the EOs due to the wide range of its antimicrobial effect.

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P015

### APPLICATION OF A LAB ANTIFUNGAL HURDLE BIOTECHNOLOGY IN THE MALTING PROCESS

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The application of lactic acid bacteria (LAB) as a hurdle technology in malting industry can contribute towards product microbial safety and stability. The main objectives of this project were: firstly, to apply a LAB biological antifungal hurdle step in the malting process, in order to inhibit the *Fusarium* proliferation and mycotoxin deoxynivalenol (DON) accumulation in malt grains; secondly, to analyse changes in the malt quality attributes; and finally, to characterize the antifungal LAB cell-free-supernatant (cfs) and identify its antifungal compounds. *In vitro* *Fusarium* infected barley grains were used for malting. *Lactobacillus amylovorus* and *reuteri* cfs, produced in barley malt substrate, were applied in the early stages of malting. *Fusarium* proliferation was quantified by measuring the fungal DNA, using a PCR-photometry assay, and the mycotoxin deoxynivalenol (DON), using HPLC, in the final malt. Standard EBC methods and ANOVA statistical analysis were used to evaluate and compare the malt quality parameters between batches. LAB cfs acid-based antifungal compounds were quantified using LC-MS/MS. Our results show that *L. reuteri* R29 cfs hurdle application in malting successfully inhibits *Fusarium* growth by 40%, and reduces the mycotoxin DON by 80%. Malt quality attributes resulted in extra modified grains, lower pH, higher coloration, and higher extract (carbohydrates) yield. Furthermore, *L. reuteri* R29 cfs antifungal capacity is resistant to 100 °C and highly dependent on acids production with the presence of proteinaceous compounds. *L. reuteri* R29 showed particularly high levels of caffeic (63.89 µg.mL<sup>-1</sup>) and hydrocinnamic (71.24 µg.mL<sup>-1</sup>) acids in comparison to other strains.

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P016

### BACTERIAL DIVERSITY AND PROBIOTIC ACTIVITY IN SOLE AQUACULTURE

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*Solea senegalensis* aquaculture may be affected by bacterial infections and, therefore, negative impacts on the production yields. Because the use of antibiotics should be minimized, supplementation of fish feeds with probiotic cultures is a good practice, increasingly well-accepted. The aim of this study was to characterize the culturable gut microbiota of juvenile sole, subjected to dietary supplementation with commercial probiotics.

Two commercial probiotics were used: a multi-strain probiotic (PA) including *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp. and *Lactobacillus* sp. and a mono-strain culture (PB) corresponding to *Pediococcus acidilactici*. Both were tested at the recommended dose (PA1 and PB1) and at two-fold lower dosage (PA2 and PB2). Total heterotrophs, and presumable faecal coliforms, pseudomonads and aeromonads, and lactic acid bacteria were enumerated on different culture media.

Culturable proteobacteria, especially enterobacteria and aeromonads, were significantly more abundant in sole gut than lactic acid bacteria (3.71–7.84 and 1.2 – 5.8 log CFU g<sup>-1</sup>, respectively). The fish that fed with the mono-strain probiotic (PB), mainly at PB2 dose, presented significant increase on the counts of culturable bacteria of all groups tested. In fish fed with the probiotic PA (dose PA1) was observed a decrease in bacterial counts mainly of lactic acid bacteria. In the control diet was observed a reduction in the bacterial counts for all the culture media along the three sampling campaigns diet.

A group of 286 isolates recovered over the three sampling dates was tested for their antimicrobial activity, against *Aeromonas salmonicida* subsp. *salmonicida* LMG3780, *Photobacterium damsela* subsp. *piscicida* LG41/01, *Staphylococcus aureus* DSM 1104 and *Vibrio anguillarum* VCO1.06.05, aiming their possible use as probiotic agents. Antimicrobial activity was detected in 13 strains identified as *Vagococcus fluvialis*, *Enterococcus raffinosus*, *Vibrio furnissi*, *Bacillus methylotrophicus*, *Acinetobacter venetianus*, *Shewanella upenei*, *Shewanella hafniensis*, *Shewanella haliotis* and *Vibrio alfacensis*. All strains presented activity against *A. salmonicida* subsp. *salmonicida* and only two, identified as *V. furnissi* and *A. venetianus*, were active against *P. damsela* subsp. *piscicida*. The fact that none of these species is listed as fish pathogen and the absence of presumed acquired antibiotic resistance phenotypes make these isolates good candidates as probiotics.

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P017

### BIOAVAILABILITY OF PHENOLIC COMPOUNDS FROM BLUEBERRY LEAF INFUSIONS (*VACCINIUM CORYMBOSUM* L.) IN CACO-2 CELLS

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Phenolic compounds are widespread in plant-derived foodstuffs and therefore abundant in our diet. There are evidences regarding the positive association of their intake with several diseases prevention. Blueberry leaves are of particular interest as they have been described as being rich in phenolic compounds such as chlorogenic acid and quercetin glycosides. Bioavailability is a major issue regarding the biological impact of these compounds *in vivo* and remains unclear, with few data available on this matter. Studies in CaCo-2 cells (enterocyte cell line) tested the transport of blueberry leaf infusion (optimized to yield the highest amount total phenolics) across this cell line for different incubation times. From the different compounds originally identified only rutin (9%), quercetin-3-glucoside (Q3glu), chlorogenic (7%) and neochlorogenic acids (23%) were transported across the epithelia cells, after 60, 90 and 120 min. From these compounds, neochlorogenic acid and Q3glu exhibit the highest transport rates (23 and 22%, respectively of the original concentration) while for rutin and chlorogenic acid the transport was lower than 10% of the original amount of compound. It was interesting to note that two metabolites, that were not originally present, caffeic and p-coumaric acids were detected after 30 and 60 min, respectively.

From this work it was possible to conclude that neochlorogenic acid and Q3glu are more efficiently transported through the CaCo-2 membrane and that this process resulted in the detection of some metabolites that were not originally present.



P018

## CHITOOLOGOSACCHARIDES MODIFIED BY MAILLARD REACTION WITH GLUCOSE INHIBIT ADHESION OF ENTEROPATHOGENIC BACTERIA TO MUCIN

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Antiadhesive capacity is a relevant property attributed to some prebiotic oligosaccharides that may confer health benefits. Specifically, these oligosaccharides may directly inhibit infections by enteric pathogens due to their ability to structurally mimic the pathogen binding sites that coat the surface of gastrointestinal epithelial cells.

Chitooligosaccharides (COS) are oligomers composed of glucosamine and N-acetyl glucosamine units, linked by  $\beta(1\rightarrow4)$  bonds and it has been shown that they possess better biological activities than chitosan. Due to this structure, similar to prebiotic oligosaccharides, they appear to be a promising substrate to obtain new prebiotic ingredients with antiadhesive properties. However, previous research works indicate that COS cannot be used directly as prebiotic since the presence of the amino groups in its molecule confers it with an important antimicrobial activity, which could cause a decrease in the bacterial host population with negative health outcomes. Chemical modification of chitosan by substitution of their amino groups eliminate this antimicrobial effect and convert this new COS in a new interesting prebiotic ingredient.

In the present study, modified COS with glucose through the Maillard reaction were obtained. Chitosan was the starting material and two different mechanisms were used in this conversion. In the first one, COS were obtained by hydrolysis of chitosan, followed by addition of glucose through a Maillard reaction. The second mechanism consisted in a first step of chitosan modification with glucose through Maillard reaction and a second step of hydrolysis of this modified chitosan in order to obtain the final modified COS. The use of both mechanisms allowed the obtention of modified COS with a similar molecular weight and different degrees of substitution (DS).

Once, the modified COS were obtained, their ability, to inhibit the adhesion of several food pathogens (*Escherichia coli*, *Salmonella enteritidis* and *Bacillus cereus*) to mucin was evaluated “*in vitro*”. A classical mucin adhesion test was carried out using a fluorescence-based method for the detection of adhesive properties of pathogenic strains.

Results showed that both products were capable of inhibiting the adhesion of all tested pathogens. These substrates showed a strain-dependent effect, suggesting the involvement of different carbohydrate-recognition sites. The DS also had a clear effect on the anti-adhesive properties of the derivatives.

Although more studies are necessary to further evidence of their biological effects, this work is a basis for future work showing the ability of modified COS to competitively exclude intestinal pathogens and amplify COS uses as a potential prebiotic ingredient.

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## Food Microbiology & Biotechnology

P019

### CHITOSAN BASED FILMS ENRICHED IN GRAPE EXTRACTS WITH ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES

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The use of edible films and coatings in food protection and preservation from renewable biopolymers has recently increased since they offer several advantages such as being biodegradable and environmentally friendly. Chitosan is a polysaccharide that has the ability of forming transparent films with biocompatible, antioxidant, and antimicrobial properties. Further, chitosan films can be an efficient vehicle for incorporating functional compounds. Grape pomace is an abundant by-product, which is a rich source of phenolic compounds, lipids, polysaccharides, and proteins. Then, there is a high interest to use this by-product extracts in order to improve the chitosan films properties.

The purpose of this work consisted in the preparation of chitosan-based films for food applications. The films were prepared with incorporation of three different grape pomace extracts: 1) hot water extract, comprising 72 % of polysaccharides; 2) oil with 70 % of linoleic acid; and 3) waxes, in order to improve the properties of the chitosan films. The films properties were investigated, such as solubility in weak acid solutions, and the antioxidant, mechanical and antimicrobial properties.

The chitosan-based films with oil and waxes incorporated revealed lower solubility in water (pH=6.5) and in a wine-model solution (10% ethanol; pH=3.5) compared with a chitosan film and the antioxidant activity was higher, up to 100 % depending on the method used to evaluate (ABTS, DPPH, FRAP, or reducing power). The incorporation of all the extracts did not affect significantly their mechanical properties (tensile strength, percentage of elongation, and Young's modulus). All films demonstrated antimicrobial activity which was evaluated by measuring the optical density (640 nm) in apple juice, which remained stable after 4 days in juice with chitosan-based films incorporated whereas the control juice (without film) has an optical density 7 times higher. The chitosan based films also inhibited the growing of some fungi in bread.

In conclusion, the chitosan-based films prepared by incorporation of extracts obtained from grape pomace seem to be promising for several food preservation applications.

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## Food Microbiology & Biotechnology

P020

### CHITOSAN BASED FILMS STABLE IN ACIDIC MEDIA WITH ANTIOXIDANT AND ANTI-YEAST ACTIVITIES

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Chitosan-based films have been studied for use in food preservation due to their antioxidant and antimicrobial activities. Yet, chitosan films are soluble in aqueous acidic media.

The aim of this work was to produce chitosan-based films with higher antioxidant activity and low solubility in acidic media, maintaining its inherent antimicrobial activity. In order to increase the antioxidant activity of chitosan films, caffeic acid was grafted to chitosan. Genipin, a natural compound with cross-linking capacity for chitosan, was also used to produce chitosan films stable in acidic medium.

The antioxidant activity of the films was determined by the percentage of inhibition, using the ABTS method. The solubility test was performed by placing a film square in water (pH 3.5) at room temperature with agitation for 7 days. The solubility was determined by the difference of the film square weight before and after the test. The antimicrobial activity was performed against *Saccharomyces cerevisiae* to evaluate if the inherent chitosan property was maintained in the film after the grafting and the cross-linking reactions. The activity of the films against *S. cerevisiae* was performed by placing the film squares in Yeast Extract Peptone Dextrose (YEPD) medium at pH 3.5, with an initial concentration of approximately  $10^2$  CFU/mL and incubated for 14 days at 26°C. Cell viability was determined by CFU counts on YEPDA plates.

The grafting of caffeic acid to chitosan and cross-linking with genipin originated films with an antioxidant activity 75% higher than that observed for the films prepared only with chitosan. Moreover, the use of genipin allowed to obtain films that only lost 10% of their mass in acidic media, which represents a decrease of 70% in films solubility comparing with the films of chitosan and caffeic acid [1].

The films obtained by grafting and cross-linking reactions kept the antimicrobial activity of the chitosan, since no yeast cell growth was observed, whereas in the control (cell suspension without film) an increase from  $10^2$  to  $10^8$  CFU/mL was observed.

This study shows that a chitosan-based film grafted with caffeic acid and cross-linked with genipin can be prepared, imparting a good antioxidant activity and low solubility in acid pH, and maintained the antimicrobial activity. Therefore, this film can be a promising material to be used as an active polymer for application in acidic foodstuffs.

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## Food Microbiology & Biotechnology

### P021

#### COMBINED EFFECT OF PROBIOTIC LACTOBACILLUS SAKEI AND GARLIC ESSENTIAL OIL ON THE BEHAVIOR OF *SALMONELLA* SPP. DURING CHOURIÇO MANUFACTURING

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Meat fermentation is a technology that allows the production of safe products, sensorially interesting and with a potential advantage to the consumer health. Once these products are eaten raw, its viable microflora is ingested by the consumer. Thus, it is possible to obtain a probiotic advantage. The aim of the present work was to evaluate the technological and probiotic ability of *Lactobacillus sakei* isolated from *chouriço* produced in the region of Trás-os-Montes and to evaluate the effect of the selected starter culture and of garlic essential oil on growth and survival of *Salmonella* spp. in *chouriço*. It was observed that within the group of *L. sakei* most of the strains has a high ability to grow in adverse conditions simulating the main hurdles found in *chouriço*. Antagonistic activity was found using the spot on agar and well diffusion tests against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli* O157: H7. This trait showed great variability between strains. In the evaluation of potential probiotic strains, some were very sensitive to pH of 2.5. The majority showed no sensitivity to bile salts. The ability of adhesion to pig intestine was expressed by all strains, although some have shown greater ability. The strains tested showed an antibiotic resistance profile typical of *Lactobacillus* genus. From weighting of the various factors the strain 1284 was selected to inoculate *chouriço* due to the high antagonistic activity and tolerance to pH 2.5 and capacity for growth and acidification. The use of a starter culture with this strain of *L. sakei* (level of addition 6–7 log CFU/g) in combination with garlic essential oil (0.5%) on the growth and survival of *Salmonella* spp. during *chouriço* manufacturing process sausage resulted in products that are safer even before aw reaches low values. After 7 days of drying, which is the time used by some manufacturers to market *chouriço*, the combined use of the two factors resulted in a reduction of 1.5 log CFU/g, which is important from the standpoint of the safety.

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## Food Microbiology & Biotechnology

### P022

#### COMPARATIVE ANALYSES OF CULTURE-DEPENDENT AND METAGENOMIC APPROACHES FOR THE CHARACTERIZATION OF THE YEAST MICROBIOME FROM FRUIT AND VEGETABLE WASTES

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Although it is known that fungal communities are important players in agricultural ecosystems, little information is available regarding their composition and diversity. Conventional microbiological approaches fail to reveal about 99.99% of all microorganisms present. However, metagenomic approaches overcome this limitation and provide a holistic genetic “biodiversity fingerprint”. In this reasoning, the objective of this study was to evaluate the fungal (in particular yeast) microbiome associated with biowastes from fruit and vegetable transforming industries. We further aimed to compare the yeast communities that can be identified by culture-dependent methods and metagenomic analysis in quantitative and qualitative terms.

From twelve fruit and vegetable biowaste samples (apple, asparagus, cardoon, strawberry, green beans, mix of salad, peach, pear, pepper, chard fresh and processed chard), 608 yeast isolates were obtained and identified by ITS1–5.8S–ITS2 RFLP and sequencing. For metagenomic analysis, a DNA extraction protocol was optimized to maximize the recovery of microbial DNA in the presence of high concentrations of plant biomass. Partial ITS2 sequence was analysed by pyrosequencing (454 Life Sciences) and the data were examined using the SILVA database.

The composition of the yeast flora significantly varied among different biowastes, as revealed by both approaches. The number of genera/species identified by metagenomic and conventional approaches ranged between 9–47 and 3–10, respectively. The genera most frequently encountered by culture-dependent methods were *Candida*, *Cryptococcus*, *Galactomyces*, *Hanseniaspora*, *Pichia*, *Rhodotorula* and *Torulaspora*. The metagenomic approaches also revealed the predominance of *Candida*, *Hanseniaspora*, *Pichia*, and *Torulaspora* and, additionally, *Kazachstania*, *Saccharomyces*, *Wickerhamomyces* and *Rhodotorula* in more than 50% of the samples. Although the metagenomic approach exhibited a significant higher yeast biodiversity the genus *Cryptococcus* was only identified by the culture-dependent method. This is the first study describing the highly diverse yeast microbiome in agrofood biowastes.

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## Food Microbiology & Biotechnology

P023

### EFFECT OF DRYING AND FREEZING PRE-TREATMENT UPON SOUR CHERRY (*PRUNUS CERASUS*) BIOACTIVE COMPOUNDS

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Sour cherry (*Prunus cerasus* L.) is a phenolic rich fruit containing substantial quantities of anthocyanins. The Iberian Peninsula (the eighth European producer) has 1397 ha dedicated to sour and duke cherry production and harvests 2610 metric tons of fruit per year. In Portugal, the main sour cherry-producing area is the Óbidos region. In this region, all sour cherries are harvested by hand, at the firm-mature stage to reduce bruising, between late June and early July. However, and despite all the forethought put into preserving fruit quality, sour cherries quality rapidly decays with the softening of its texture and loss of organoleptic properties. In order to bypass this problem producers have taken to freezing, which only slows fruit decay, or to immediately process sour cherries into foodstuffs (e.g. Ginjinha – a traditional Portuguese liquor). In this work we evaluate the potential of drying sour cherry as a means to develop new foodstuffs and to storage the fruit. This assessment was performed through analysis of the evolution of its phenolic composition over drying time. Simultaneously the application of a pre-treatment (passive and fluidized bed freezing – treatments that would slow decay in the time lag between harvest and processing) was studied to address the impact on fruit composition over drying. The results obtained showed that freezing before drying allows for a better preservation of the total phenolics (with a degradation of 25% of total phenolics for non-processed fruit opposing to the 2–10%, for both freezing methods used). A similar trend was observed for individual compounds analysis, *i.e.* freezing before drying seems to be the best option when considering the preservation of phenolic compounds. However, an exception was observed for chlorogenic, since fluidized bed freezing allowed for a better preservation of the compound, while the passive freezing other led to its reduction to bellow the detection limit). On the other hand, while neochlorogenic acid did not show significant differences after 6 days of drying; it is interesting to note that, despite this result, at 2 days of drying the pre-processing appeared to possess some advantages.

Overall, freezing before drying appears to be an advantageous alternative as it allows for, not only the preservation of this highly perishable fruit but also for a better preservation of its bioactive constituents.

## Food Microbiology & Biotechnology

### P024

#### EFFECT OF EDIBLE COATINGS ON COLOUR AND POLYPHENOL OXIDASE ACTIVITY OF APPLE CUBES DURING COLD STORAGE

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When cutting fresh apple, a browning reaction occurs. This is due to the exposition of the fruit tissues to oxygen and to the presence of polyphenols. The colour of apple cut surfaces will turn from a nice, attractive colour to a brownish colour. The presence of the enzyme polyphenol oxidase (PPO) accelerates this reaction.

The purpose of this study was to investigate the effect of specific edible coatings applied to fresh-cut 'Royal Gala' apple (cubes) on colour and PPO activity during cold storage (4°C). The main constituents (polysaccharides) of the different types of edible coatings that were used were carrageenan, alginate, pectin, carboxymethyl cellulose and the sulphated exopolysaccharide (EPS) from the microalgae *Porphyridium cruentum* (Raposo et al. 2013 a,b).

Even though the results concerning the colour assessment and PPO activity did not follow a regular tendency during storage, in general a correlation could be observed: when the apple cubes became browner ( $L^*$  values decreased and  $a^*$  values increased), the PPO activity increased. In addition, EPS-coated apple cubes in particular did not present the best results.

This piece of research work shows that EPS is not the best coating to protect fresh-cut 'Royal Gala' apple. The coating based on pectin presented better results.

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## Food Microbiology & Biotechnology

### P025

#### EFFECT OF HIGH PRESSURE ON YOGURT PRODUCTION AND THE POSSIBILITY OF USING PRESSURE AS AN ON/OFF SWITCH TO STOP/RESTART FERMENTATION

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Due to the high nutritional value and health benefits associated to yogurt consumption, this product has gained increasingly popularity over the last years. High pressure (HP) is a novel technology with several applications in different fields, being a recent approach the change of performance of microbial fermentation under sub-lethal pressures. This new application can bring novel characteristics and features to the fermentative process and/or to the final product [1].

Therefore, the aim of this work was to evaluate the possibility of applying HP technology on yogurt production process at the usual process temperature. Fermentation process was performed under different pressure conditions (0.1–100 MPa) and samples were collected and analyzed over time. For that purpose, physicochemical (pH, titratable acidity and reducing sugars concentration) and microbiological (*Streptococcus thermophilus* and *Lactobacillus bulgaricus* counts) parameters were measured.

Results showed that with the increasing pressure, pH, titratable acidity and reducing sugars concentration had lower variation over time, which indicates that fermentation rate was decreased by HP. In order to mathematically model the process, activation volumes ( $V_a$ , cm<sup>3</sup>/mol) were calculated and positives values were obtained.

Furthermore, at pressures around 100 MPa, fermentation was not observed at all for several hours, but the respective microbial loads decreased only slightly over fermentation time. These results indicate that there was not considerable microbial inactivation, but rather microbial growth inhibition. When these samples came back to atmospheric pressure, fermentation restarted with a typical rate, leading to yogurt, apparently, with normal characteristics. This opens the possibility of using pressure as an on/off switch to stop/restart fermentation, with no need of refrigeration and so with much less energy costs. Experiments are under way to verify if this can be a general feature of microbial behavior under pressure and so with broad biotechnological applications.

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## Food Microbiology & Biotechnology

P026

### EFFECT OF PRESSURIZATION RATE, TEMPERATURE AND PH ON THE EFFICIENCY OF INACTIVATION OF LISTERIA INNOCUA BY HIGH PRESSURE

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High hydrostatic pressure processing (HPP) is a technology in food industry that overcomes some limitations of the traditional food preservation processes. HPP effectively inactivates microorganisms and enzymes at room temperature, improving product stability and extending their shelf time while, better preservation of nutritional values, flavors and texture is achieved. Optimized HPP protocols may combine pressure with physical and/or chemical agents in order to improve inactivation efficiency.

In this study, the effect of compression and decompression rates and the combination of pressure with different temperature and pH values was tested for *Listeria innocua* as a non-pathogenic surrogate of *Listeria monocytogenes*, the causative agent of food-borne listeriosis. Experimental conditions tested corresponded to pressure values between 100 and 600 MPa, compression rates between 1.5 and 11.9 MPa s<sup>-1</sup>, decompression rates between 3.2 and 23.8 MPa s<sup>-1</sup>, holding times of 1 and 5 minutes, temperature values of 4, 10 and 20 °C and pH values of 4, 7 and 10.

Results showed that complete inactivation occurred with exposure to pressure values of 400, 500 and 600 MPa, during 5 minutes. When sub-lethal pressurization conditions were imposed, the inactivation was significantly (p<0.05) more efficient with the lowest compression and decompression rates (1.5 and 3.2 MPa s<sup>-1</sup> respectively). Low temperature (4 and 10 °C) and acidic or neutral pH also enhanced the inactivation efficiency in relation to room temperature and alkaline medium.

The results confirm that compression and decompression rates are relevant parameters of the efficiency of HPP treatments on *L. innocua*. The inactivation efficiency can be significantly affected by temperature and by the pH of the medium. These results demonstrate that HPP protocols should be carefully designed taking into consideration the chemical properties and thermal stability of each particular food matrix.

## Food Microbiology & Biotechnology

P027

### EFFECT OF PROCESSING AND STORAGE ON NUTRITIONAL AND FUNCTIONAL QUALITY OF STRAWBERRY

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Being very perishable, strawberries require preservation, both as fresh or processed foods. This study aims at the identification of health-important biomarkers and evaluation of the effects of processing and storage on nutritional and functional quality of fresh-cut strawberry. The effect of cutting and package type on the antioxidant activity, total phenolics, total anthocyanins and relevant individual compounds content was studied and the related changes determined. Strawberries were cut vertically into two or four wedges, depending on fruit size, and packaged in 150 g clamshells or 50 g filmed packages, stored at 5 °C, and assessed for and nutritional and functional composition on days 0, 1, 2, 5 and 7 of storage. Total antioxidant activity was assessed by the ABTS method, total phenolics by Folin Ciocalteu's method and phenolic compounds and anthocyanins were analyzed by high performance liquid chromatography (HPLC-DAD). There was a significant effect of processing and package type on total phenolic compounds with processed strawberries stored in clamshells showing the highest values from day 2 to day 5 of storage. Total anthocyanins content increased in the first 2 days of storage and remained relatively constant throughout day 2 to 7 of storage. There was a significant effect of package type on processed strawberries anthocyanins content with whole strawberries stored in clamshells showing the highest values. Cyanidine-3-glucoside content was significantly affected by processing with processed strawberries showing lower cyanidine-3-glucoside content throughout storage in comparison with whole strawberries. Catechin concentration increased in the first day of storage of whole strawberries while decreasing in processed ones. Whole strawberries in both package types showed higher values of epicatechin content than processed strawberries. Rutin concentration was affected by processing and package type with whole strawberries stored in filmed packages showing the highest values during storage. Processing also affected the concentration of ellagic acid.

## Food Microbiology & Biotechnology

P028

### EFFECT OF REGULATED DEFICIT IRRIGATION ON THE PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ACTIVITY OF JAPANESE PLUMS

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Plums are known to contain large amounts of different phytochemicals, being considered a good dietary source of natural antioxidants, with potential beneficial-health effects. Fruits polyphenolic composition and antioxidant activity is determined by several factors, as harvest year and water availability.

Strategies of Regulated Deficit Irrigation (RDI), which consists in application of water restriction when fruits growth is less sensitive to water deficit, are increasingly used to ensure an efficient and rational water use. However, until now little information is available about the effects of RDI on fruits phytochemical and antioxidant contents. Therefore, the aim of the present study was to evaluate the effect of RDI strategies upon total phenols, *o*-diphenols and anthocyanins contents, and antioxidant activity of plums. The effect of harvest year on plums phytochemical composition and antioxidant potential was also evaluated. Three irrigation strategies, control and two RDI (RDI1 and RDI2), were applied to plum trees (cv. Angeleno) during two consecutive growing seasons (2011 and 2012). The control treatment was irrigated at 100% of evapotranspiration (ET<sub>c</sub>), while in RDI1 and RDI2, the water restrictions were applied during phase of slow fruit growth (phase II) (20 and 0% ET<sub>c</sub> in RDI1 and RD2, respectively) and post-harvest (60 and 30% ET<sub>c</sub> in RDI1 and RD2, respectively), maintaining a 100% ET<sub>c</sub> during phases of rapid fruit growth (phases I and III). The phytochemical composition and antioxidant activity was determined separately for peels and plums flesh.

Irrigation strategies and harvest year affected the phytochemical profile and antioxidant activity of peels and flesh. However, independently the harvest year and irrigation strategy, peels when compared to flesh, showed higher content of phytochemical compounds and antioxidant activity. In the flesh, which represents *ca.* 86% of plum edible fraction, were observed higher levels of phenols and *o*-diphenols, and higher antioxidant activity in RDI plums than in plums from control treatment (1.68 *vs* 1.48 mg phenols/g flesh; 0.29 *vs* 0.25 mg *o*-diphenols/g flesh; 0.37 *vs* 0.32 IC<sub>50</sub> in RDI strategies and control, respectively). Considering the whole plum, the RDI strategies increased the antioxidant activity of fruits comparatively with control treatment. These results showed that RDI strategies evaluated in this study may be applied to reduce the water supply, without compromising the plums nutritional value.

## Food Microbiology & Biotechnology

P029

### EFFECT OF STORAGE CONDITIONS IN THE QUALITY OF THE BEE POLLEN

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Bee pollen (BP), is the result of the agglutination of flower pollens, made by worker honey bees, with nectar and salivary substances and collected at the hive entrance. The collection of AP is a relatively recent development, dependent primarily on the basic concept of scraping pollen of the bees' legs as they enter the hive. The objective of this work was to evaluate the effect of storage conditions in the physical-chemical and microbiological parameters of organic bee pollen

In our study were identified eight (8) botanical families; *Rosaceae* was the dominant following by *Fagaceae*. Regarding to the chemical physical parameters analyzed was verified that, all of them were in accordance with the stipulated by law, except the humidity. Significant differences were observed in the among in humidity, pH, water activity, reducer's sugars, lipids, carbohydrates and energy value among the dehydrated and refrigerated BP samples.

They were found seventeen (17) fat acids; ten (10) belonging to the family of the saturated fatty acids (SFA), four (4) monounsaturated (MUFA) and three (3) polyunsaturated (PUFAs). PUFAs were the most abundant. Significant differences were observed in SFA, MUFA and PUFAs among the several analyzed samples.

Regarding to microbiological analyses, the aerobics mesophylics, moulds and yeasts were present in all of the analyzed samples. The first ones (aerobics mesophylics) exceeded the legislated in some dehydrated samples, while the values obtained for the moulds and yeasts were higher in the refrigerated samples. The indicators of sanitary quality and the toxigenic species were absent in all of the samples in study. The studied pollens are nutritionally well balanced, and contain high levels of moisture, proteins, fat, energy, ash, carbohydrates, reducing sugars, essential n-3 fatty acids and good ratios of polyunsaturated fatty acids (PUFA) / saturated fatty acids. In fact, the PUFA represent 66% of the total fatty acids.

## Food Microbiology & Biotechnology

### P030

#### EFFECT OF TEMPERATURE ON THE BIOACTIVE PROPERTIES OF BEE POLLEN

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Bee pollen is flower pollen collected by the honey bee, *Apis mellifera*, for the purpose of feeding its larvae in the early stages of development. It is recognized to be a valuable apitherapeutic product with potential for medical, health and nutritional applications. The objective of this work was to compare the effect of different storage conditions in the bioactive compounds and biological properties of bee pollen.

The amount in phenolic compounds determined by the method of *Folin–Ciocalteu* varied from 32.64 to 48.40 mg GAE/g, for the dehydrated and refrigerated samples, respectively. Significant differences were verified among the two conservation processes. The amount in total flavonoids determined by the method *chloride of aluminum* were not verified among significant differences in the samples (6.58 mg CAE/g) and (6.99 mg CAE/g). Concerning the amount of total flavonoids determined by the method of aluminum chloride were not significant differences between the dehydrated (6.58 mg of DEA / g) and refrigerated samples (CAE 6.99 mg / g).

The antioxidant capacity of BP extracts was assessed through the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) and reducing power. The concentration-dependent antioxidative capacity was verified in DPPH and reducing power assays. Low values of EC<sub>50</sub> on DPPH scavenging assay were obtained for fresh and dehydrated samples 0.74 and 1.16 mg/mL, respectively. For reducing power the values obtained for the refrigerated samples and dehydrated were similar. The high activity of refrigerated samples could be related with the conservation process. Regarding to phenolic compounds determined by HPLC method, were identified four (4) families, flavones being the most abundant one in the refrigerated and dehydrated samples.

We also verified that the presence of pollen differentially affected the growth of bacterium Gram-positive (*Staphylococcus xylosus*, *Staphylococcus epidermidis*), Gram-negative (*Shigella spp*, *Klebsiella pneumoniae*) and yeasts (*Candida parapsilosis*, *Candida membranifaciens*, *Candida glabrata*) under study, depending this on the microorganism and the method of BP conservation.

## Food Microbiology & Biotechnology

P031

### EFFECT OF THE TEMPERATURE IN THE HONEY QUALITY

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Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants, secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature." (Decreto-Lei nº214/2003). This product can suffer alterations if the conditions of conservation and storage are not appropriate. The objective of this work was to evaluate the effect of the temperature in the physical-chemical and microbiological parameters of monofloral honeys stored for 4 months.

The evaluated physical-chemical parameters were: moisture, pH, free acidity, electrical conductivity, ashes, reducer's sugars, apparent sucrose, hydroxymethylfurfural (HMF), diastase activity and color. Relatively to the moisture none of the samples exceeded the legislated limits. The maximum value obtained for the pH were 4.92 for the heather honey maintained to room temperature and the minimum were 3.77 for the rosemary honey in the same storage conditions. Free acidity decreased in all samples, and all storage conditions. Relatively to electrical conductivity and ash, it was found that, for rosemary and cherry honeys, independently of storage conditions, the values obtained were within legal limits. Regarding to heather and chestnut honeys, the values obtained for the mentioned above parameters exceeded the stipulated by law. In all of the analyzed samples an increase of HMF was verified along the time, being this more accentuated to 45°C. In this case, the obtained values exceeded broadly allowed it by law. Relatively to the reducer's sugars and the sucrose it was verified, in most of the samples a progressive decrease during storage, however, in all cases, the obtained values located inside of the legislated. In most of the samples, the diastase activity decreased over the time. The storage caused a darkening of the honey, especially for the chestnut honey, stored to 45°C.

Relatively to the microbiological analysis, the aerobics mesophylics were found in some of the analyzed samples; however they never exceeded the limits stipulated by law. The moulds and yeasts just were present in the chestnut tree honey stored in the freezer. The indicators of sanitary quality and the toxigenic species were absent in all of the samples in study.

## Food Microbiology & Biotechnology

### P032

#### EFFECTS OF HYDROGEN PEROXIDE ON PERSISTENT AND NON-PERSISTENT STRAINS OF *LISTERIA MONOCYTOGENES* RECOVERED FROM CHEESE PROCESSING PLANTS

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Listeriosis, a food-borne disease caused by consumption of contaminated food by *Listeria monocytogenes*, can produce infections in susceptible human populations, such as immuno-compromised persons, the elderly, pregnant women their fetuses and new-borns. A wide variety of foods have been found to be contaminated with *L. monocytogenes*, namely: soft cheeses, dairy products, pâtés, sausages, smoked fish, salads, infant cereals, cakes, cream, butter and, in general, refrigerated ready-to-eat commercially produced products, consumed without further cooking or reheating.

*Listeria monocytogenes* is particularly difficult to control, since it is widespread in nature, and because it possesses physiological characteristics that allow it to grow under conditions that are usually adverse for most human pathogenic bacteria. Due to its ubiquity, *L. monocytogenes* can produce several problems in food-processing plants. Despite research showing the lethality of sanitizers to *L. monocytogenes*, the pathogen is occasionally isolated from food-processing environments, even following cleaning and sanitizing of equipment surfaces. The frequency of food product recalls due to contamination with *L. monocytogenes* attests to the ability of the organism to persist in food-processing environments despite sanitation measures.

Several studies have been reported on susceptibility of *L. monocytogenes* to different industrial disinfectants such as quaternary ammonium compounds, alcohols, chlorinated compounds, and other oxidizing agents such as peracetic acid, ozone and peroxide derivatives.

The use of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) has been proposed as an alternative for decontaminating fruit and vegetables due to its low toxicity and safe decomposition products. It is effective against a wide spectrum of bacteria, yeast, molds, viruses and spore-forming bacteria. H<sub>2</sub>O<sub>2</sub> rapidly degrades into oxygen and water (nontoxic products) upon contacting organic material, thus having no long-term residual activity. H<sub>2</sub>O<sub>2</sub> is classified as Generally Regarded as Safe (GRAS) for use in food products as a bleaching agent, oxidizing and reducing agent and antimicrobial agent.

This study was performed to evaluate the ability of 31 persistent and 18 non-persistent strains of *L. monocytogenes*, isolated from cheese processing environments, to survive different concentrations of H<sub>2</sub>O<sub>2</sub> (i.e. 1.5 and 0.75 % v/v at different exposure times (5 and 20 minutes) at 22 °C. A 1.6 and 5.2 log reduction was observed for 1.5% v/v of H<sub>2</sub>O<sub>2</sub>, and 0.8 and 3.4 for 0.75 % v/v of H<sub>2</sub>O<sub>2</sub>, after 5 and 20 minutes of exposition, respectively. No significant differences in the resistance between persistent and non-persistent strains was observed for the conditions tested.

## Food Microbiology & Biotechnology

### P033

#### ENUMERATION OF COAGULASE-POSITIVE *STAPHYLOCOCCI* DURING RIPENING OF CHEESE MADE WITH RAW MILK FROM SHEEP

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The production of cheese from raw milk is a major economic activity for the producing regions, representing a source of income for many families. With growing consumer concern regarding the acquisition of safe and quality products, there is a need to conduct studies that guide the producers in compliance with good manufacturing practices and good hygiene practices. In accordance with Regulation (CE) N°2075/2005 modified by the Regulation (CE) N°1441/2007, on the microbiological criteria applicable to foodstuffs, coagulase-positive staphylococci (*S. aureus* and other species) should be enumerated at the cheese-making state where their number is expected to be the highest. It is consensual accepted that the production of staphylococcal enterotoxins constitutes a risk when coagulase-positive staphylococci exceeded the value of 10<sup>5</sup> CFU per gram of cheese during production. The survival and growth of coagulase-positive staphylococci depends largely on the manufacturing technology used, which varies for each type of cheese. Thus, the aim of this study was to determine the peak level of coagulase-positive staphylococci during ripening of raw ewe's milk cheese in Beira Baixa cheese production region, Portugal.

Two batches of cheese made with raw ewe's milk were produced in two dairies, one with heated curing chamber and the other with natural maturation. The count of staphylococci, according to the standard EN/ISO 6888-2 (1999), was performed to raw milk used in cheese and the cheese over time (0, 1, 3, 5, 7, 12, 18, 45) days, a total of 5 cheeses per batch and maturation period. It was also conducted an experimental trial production of cheese by using raw milk from sheep artificially infected with *Staphylococcus aureus* at a final concentration of 10 CFU / ml of milk. In this case, the count of *Staphylococcus aureus* was made to the milk and cheese (with 0, 1, 2, 3, 4, 5, 6, 7, 11 and 14 days of aging) with TEMPO STA automated system (bioMerieux) a total of 3 cheeses for ripening period. With this work we can conclude that the peak in the number of coagulase-positive staphylococci was found on day 3, with the value of 1.1x10<sup>5</sup> CFU / g of cheese.

#### References

EN/ISO 6888-2 (1999). Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium.



## Food Microbiology &amp; Biotechnology

P034

EVALUATION OF THE SAFETY PROPERTIES OF BACTERIOCIINOGENIC *LACTOBACILLUS SAKEI* SUBSP. *SAKEI* 2ARafael Chacon Ruiz Martinez<sup>1</sup>; Bernadette Dora Gombossy de Melo Franco<sup>1</sup>; Susana C. Fonseca<sup>2</sup>; Manuela Vaz-Velho<sup>2</sup>; Svetoslav Dimitrov Todorov<sup>1</sup><sup>1</sup>Universidade de São Paulo;<sup>2</sup>Instituto Politécnico de Viana do Castelo

The bacteriocin produced by *Lb. sakei* subsp. *sakei* 2a is active against *L. monocytogenes* ScottA, *L. innocua* and different serological types of *L. monocytogenes*. Addition of bacteriocin produced by *Lb. sakei* 2a to a 3 h-old culture of *L. monocytogenes* repressed cell growth in the following 8h. Treatment of stationary phase cells of *L. monocytogenes* ( $10^7 - 10^8$  CFU/ml) by the bacteriocin resulted in growth inhibition. Good growth of *Lb. sakei* 2a was recorded in MRS broth supplemented with 0.2% or 0.4% ox-bile or in MRS broth adjusted to pH 5.0–9.0. Auto-aggregation of *Lb. sakei* 2a was 62.59%. Different levels of co-aggregation were recorded between *Lb. sakei* 2a and *E. faecalis* ATCC19443, *Lb. sakei* ATCC15521 and *L. monocytogenes* ScottA. Growth of *Lb. sakei* 2a was not inhibited by commercial drugs from different generic groups. The inhibitory effect on growth of *Lb. sakei* 2a was recorded only in presence of Arotin [selective serotonin reuptake inhibitor antidepressant] Minimal Inhibition Concentration (MIC) 1.0 mg/ml, Atlansil [Antiarrhythmic] MIC 0.625 mg/ml, Diclofenac potassium [Non-steroidal anti-inflammatory drug (NSAID)] MIC 2.5 mg/ml and Spidufen [NSAID] MIC 15.0 mg/ml. Only two antibiotics tested in this study (Amoxil and Urotrobel) inhibited growth of *Lb. sakei* 2a with a MIC of < 0.5 mg/ml and 5.0 mg/ml respectively. Based on this, *Lb. sakei* 2a presented a good potential to be considered as a probiotic candidate based on the genetic and physiological tests.

However, *Lb. sakei* 2a generated positive PCR results on the DNA level for *vanA* (vancomycin resistance), *hyl* (hyaluronidase), *esp* (enterococcal surface protein), *ace* (adhesion of collagen) and *ciIA* (cytolysin), a high virulence profile when been examined for presence of virulence factors. It is important to underline that cytolysin have been described as a virulence and antibacterial factor.

Besides all beneficial properties studied for various LAB, a special attention need to be pay on the possible presence of virulence factors, production of biogenic amines and antibiotic resistance. This virulence determinants have been well detected and studied in Enterococci and Streptococci, however, in last few years report on presence of virulence factors in otherwise GRAS Lactobacilli have been showing the potential upcoming problems. Horizontal gene transfer of virulence factors between pathogenic and LAB, including probiotics is a highly possible scenario in case of uncontrolled application of probiotics.

## Food Microbiology & Biotechnology

P035

### EVALUATION OF THE SENSORY QUALITY OF MONOFLORAL HONEY

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Honey is an alimentary product much appreciated due to his aroma, flavor and nutritional characteristics. The sensorial analysis is important in the evaluation of the quality and authenticity of monofloral honeys. It considers attributes as color, aroma, consistence and flavor, which are interlinked and depend on the volatile compounds related with the fragrance of the flowers where the nectar was collected. The aroma and the flavor are also related directly with honeys' color.

The objective of this work was to evaluate the pollinic profile and the sensorial characteristics of four honeys from biological mode of production.

The pollen analysis indicated that all of the analyzed honeys were monofloral, presenting as dominant pollen *Lavandula sp.* (45.83%), *Erica sp.* (49.69%), *Prunus sp.* (61.91%) and *Castanea sp.* (69.01%).

The results obtained in the sensorial analysis performed by the consumer's panel were treated by the Generalized Procrustes method. It was verified that the attributes that the consumers managed to evaluate more easily and whose contribution for the global appreciation was more accentuated were the color, the flavor and the consistence.

The cherry tree honey presented higher values in the scale of preferences in terms of global appreciation, color, flavor, consistence and intermediate values regarding the aroma. Concerning the heather honey, aroma was the most valued attribute. Honeys from chestnut and rosemary were the ones that received lowest punctuation.

## Food Microbiology & Biotechnology

### P036

#### EXTENDED SHELF LIFE UNDER REFRIGERATION FROM 7 TO 21 DAYS OF A READY-TO-EAT MEAL BY HIGH PRESSURE PASTEURIZATION

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High Pressure Processing (HPP) is being increasingly applied in the food industry, improving food safety and extending shelf life, at the same time that nutritional and organoleptic properties are maintained with a better sensorial quality of the products [1, 2].

The aim of this work was to evaluate the HPP effect (400 MPa/ 1.5min, 550 MPa/5 min and 600 MPa/10 min) on microbiological and physicochemical parameters and on shelf life of a ready-to-eat fish meal ("Bacalhau com natas") during 21 days of storage at 4 °C.

After HPP treatment (day 0) the results showed a decrease of the initial microbial counts, since Aerobic Colony Count (ACC) value was reduced (from 1.97 log CFU/g) to a value of  $\leq 1.00$  log CFU/g for all HPP-samples. For *Enterobacteriaceae* (ENT), Total Coliforms (TC) and Yeasts and Moulds (YM), the counts were  $\leq 1.00$  log CFU/g in all samples. At day 14, the ACC was  $\geq 5.00$  log CFU/g for control samples, while in HPP-samples the values ranged from  $\leq 1.00$  to 3.46 log CFU/g. In the 7<sup>th</sup> and 14<sup>th</sup> days, control and HPP-samples showed  $\leq 1.00$  log CFU/g for ENT, TC and YM. In the 21<sup>st</sup> day, control samples presented unacceptable values for ACC/ENT/TC ( $\geq 5.00/\geq 2.00/\geq 2.00$  log CFU/g, respectively). The same behaviour was not verified for HPP-samples, particularly for the 600 MPa/10 min treatment that showed 3.67/1.58/1.22 log CFU/g for ACC/ENT/TC, respectively.

For physicochemical evaluation pH, titratable acidity (TA) and lipid oxidation (peroxide index (PI) and TBARS) were analysed. The pH and TA did not shown significant differences. However, there was an increase in PI (~20 to ~60 mEq peroxide/Kg lipid) in all samples. There was also a slight increase in TBARS in day 14 (~0.4 to ~0.6 mg MDA/Kg sample) followed by a decrease at the day 21 (~0.6 to ~0.3 mg MDA/kg sample).

The results allow the confirmation that the HPP treatment at 600 MPa/10 min, provide a product that is stable during at least 21 days of storage at 4 °C, which corresponds to an extended shelf life when compared to the actual 7 days of the ready-to-eat meal in consideration.

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## Food Microbiology & Biotechnology

P037

### EXTRACT FROM POMEGRANATE BY-PRODUCTS FOR MAINTAIN THE QUALITY OF READY-TO-EAT POMEGRANATE ARILS – PRELIMINARY STUDY

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Recent searches for new food additives and preservatives are focused on natural molecules. By-products of agro-industry are rich in polyphenolic compounds, which are known to have several biological activities, such as antioxidant and antimicrobial properties. So, these by-products are appointed as good sources of natural molecules for application on food industry.

Some studies reported that pomegranate peel extract have antioxidant and antimicrobial activities. So, the aim of the present study was explore the utilization of extract from pomegranate by-products for maintain the quality of ready-to-eat pomegranate arils during refrigerated storage. Pomegranate husk were carefully cut and arils manually extracted. Wastes from pomegranate peel were collected and used for extract preparation. The ground wastes were extracted with water at room temperature during 4 h with gentle agitation. The supernatant were collected and lyophilized. The powder extract was dissolved in distilled water, with final concentration of 0.5% of phenolic compounds. Arils were dipped for 2 min in distilled water (control) or in distilled water with extract. After dipping, arils were drained, randomly distributed into rigid polypropylene boxes (*ca.* 100g) and stored at 4°C. At days 0, 7 and 14 were evaluated the total soluble solids (TSS), titratable acidity (TA), pH, colour, total phenolic content and antioxidant activity of arils. Two packs were analysed for each treatment on each sampling day.

The content of TSS, TA and pH were not affected by storage time and treatments, averaging 14.1 °Brix, 0.13% citric acid and 4.12, respectively. The colour coordinates ( $L^*$ ,  $a^*$  and  $b^*$ ) of arils were not affected by treatments, and little changes was observed in colour over time. However, a Hue angle (H) increased during storage from 45.5 at day 0 to 51.6 at day 14, independently the treatment. The total phenolic content decreased over time in both treatments from 0.87 to 0.78 g gallic acid equivalents/ml between day 0 and 14 of storage. Antioxidant activity of arils also decreased in both treatments during storage (from 40 to 29% DPPH inhibition at days 0 and 14, respectively). Present results show that dipping of pomegranate arils in aqueous solution with 0.5% of phenolic compounds from pomegranate by-products did not influence the quality of fruits during storage time. Other phenol concentrations or incorporation in edible coatings should be explored.

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### FOOD STORAGE UNDER PRESSURE AT VARIABLE ROOM TEMPERATURE – CAN THIS BE A NOVEL FOOD PRESERVATION METHODOLOGY?

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Due to a stroke of luck, some foods were found in good conditions after 10 months at a depth of 1540 m (~15 MPa) at 3–4 °C after the sinking of the research submarine Alvin [1]. This opened the possibility to preserve foods under pressure – Hyperbaric Storage (HS) – at cold or even room temperatures [2, 3].

The aim of this work was to evaluate the feasibility of HS at and above room temperature of highly perishable foods, using watermelon and melon juices as case-studies. The HS was tested from atmospheric pressure (0.1 MPa) to 150 MPa, at different temperatures (~20, 25, 30 and 37 °C) and periods of time (8 to 60h), evaluated through microbiological and physicochemical analyses.

In all cases, the results for watermelon juice showed that after 24h at 0.1 MPa/20 °C the microbial loads were already above unacceptable limits (6 log CFU/mL). On the contrary, for the juice stored under pressure, microbial loads decreased in the beginning and remained unchanged up to 60h, being even lower than under refrigeration.

Concerning pressure level, in all cases, the microbial loads were lower under pressure (except for 25 MPa) comparatively to storage at 0.1 MPa, thus showing a clear inhibitory effect on microbial growth. In addition, for higher pressures ( $\geq 75$  MPa) a microbial inactivation effect was observed, resulting in microbial load reductions. This resulted in HS yielding similar (in some cases better) compared with refrigerated storage at 0.1 MPa. Moreover, under pressure, the temperature had no effect from 20 to 37 °C on microbial survival and growth.

Physicochemical parameters were generally not meaningfully affected by HS comparatively to refrigeration storage, with the juices showing a fresh like odour. On the contrary, at 0.1 MPa, the juices showed an increasingly bad smell along storage time, characteristic of clear spoilage.

This work showed the feasibility of HS at room temperature, opening the possibility of food preservation with nearly no energy costs, since no refrigeration is necessary. If industrially feasible this can be a breakthrough in food preservation.

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## Food Microbiology & Biotechnology

P039

### FUNGAL AGARICUS BISPORUS DISEASES: TAXONOMY AND REALITY

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This presentation intends to present some of the scientific results obtained concerning one of the most economically damaging diseases of the commercial mushroom *Agaricus bisporus*: cobweb disease, caused by species of *Cladobotryum* sp. New findings at the taxonomic level will be presented.

Identification and classification of *Cladobotryum dendroides*, one of the pathogenic agents reported as causing cobweb disease, has changed throughout time (*Polyporus circinatus*, *Dactylium dendroides*, and as belonging to *Fusarium* sp.), being the controversy still ongoing. This has a severe impact, for instance, in the selection of biomarkers to identify cobweb disease and in the study of its metabolic pathways. Galactose oxidase has been reported as being produced by *Dactylium dendroides*<sup>1,2,3</sup>. Nonetheless, it seems like the only strain that effectively produces this enzyme is *C. dendroides* NRRL 2903. Once again, no consensus exists concerning the identification and classification of this strain, appearing as *Fusarium graminearum* and *Fusarium austroamericanum* in CBS (Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, The Netherlands) and ATCC (American Type Culture Collection) banks, respectively. There is thus a severe confusion about the classification of the strain and consequently whether galactose oxidase (GOase; and also the mycotoxins deoxynivalenol and acetyl-deoxynivalenol) is produced only by the NRRL 2903 strain or if it is produced by other *Cladobotryum* species and to what species belongs *C. dendroides* NRRL 2903 strain. Experimental results to be shown will unveil the current reality.

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## Food Microbiology & Biotechnology

### P040

#### HIGH PRESSURE PROCESSING EXTENDS THE SHELF LIFE OF READY-TO-EAT BOILED EGGS FROM 7 TO 34 DAYS UNDER REFRIGERATION

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High pressure processing (HPP) is a technology with increasingly growing interest for food preservation due to the ability of shelf life extension, retaining better nutritional and organoleptic properties comparatively to thermal processing [1, 2]. Ready-to-eat boiled eggs are available in the catering services market or individually packed with brine solution and acidulants to extend shelf life, which is still very short.

The aim of this work was to evaluate the HPP effect (550 MPa during 2.5 and 10 min) on microbiological and physicochemical parameters of boiled eggs during 34 days of storage at 4 °C.

HPP reduced the initial microbial counts (day 0), since aerobic colony counts (AAC) were reduced from  $2.41 \pm 0.45$  log UFC/g to undetectable levels ( $\leq 1.00$  log UFC/g) for all HPP samples. For *Enterobacteriaceae* (ENT), total coliforms (TC) and yeasts and moulds (YM), the counts were  $\leq 1.00$  log UFC/g in all samples. At day 14, the ACC was  $\geq 5.00$  log UFC/g for control sample, while the HPP samples presented undetectable levels. At the 22<sup>nd</sup> day, for 550 MPa/2.5 min condition, ACC increased to  $3.32 \pm 0.49$  log CFU/g, reaching the unacceptable limit ( $\geq 5.00$  log UFC/g) at the 34<sup>th</sup> day. However, 550 MPa/10 min maintained ACC below the unacceptable limit up to 34 days. ENT counts were always below undetectable levels and TC counts presented values  $\geq 3.00$  log UFC/g on the control sample, but no microbial growth was observed in HPP samples.

Concerning lipid oxidation, the peroxides index (PI) of all samples decreased during storage ( $\sim 9$  to  $\sim 4$  mEq peroxide/Kg lipid), being the lowest values obtained for 550 MPa/10 min condition. For TBARS, the obtained results showed an opposite behaviour, increasing along storage for all samples ( $\sim 0.11$  to  $\sim 0.23$  mg MDA/Kg sample) and mainly in HPP samples.

The optimum HPP condition was 550 MPa/10 min, to obtain an acceptable product with extended shelf life, with at least 34 days of storage at 4 °C.

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## Food Microbiology & Biotechnology

### P041

#### HIGH PRESSURE PROCESSING IN THE INACTIVATION OF ENTEROTOXIC *STAPHYLOCOCCUS AUREUS*.

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Foodborne diseases have significant health and economic consequences, representing one of the main global public health problems. They are responsible for high levels of morbidity and mortality in the general population, with diarrhoeal disease as the second leading cause of death in children under five years old. In industrialized countries, one third of the population may be affected by foodborne diseases each year and, in developing countries, this number increases dramatically. Therefore, the development of effective and alternative food preservation methods is imperative. One food processing technology that meets these demands is high pressure processing (HPP). This is a non-thermal food preservation method that causes microbial inactivation with minor damage in food properties, avoiding food spoilage and gastroenteritis. Although it has been shown that HPP effectiveness depends on the microorganism, the influence of this technology on the production of enterotoxins is not yet known.

The aim of this study was to assess HPP efficiency in three *Staphylococcus aureus* strains: non-enterotoxigenic ATCC 6538 and two enterotoxigenic strains 2065 MA and 2153 MA isolated from food.

Viable bacteria were enumerated by the pour plating method after *S. aureus* cells have been submitted to four HPP treatments: 450 MPa for 15 and 30 minutes; 600 MPa for 15 and 30 minutes. Staphylococcal enterotoxins were detected using the kit SET-RPLA Kit Toxin Detection Kit from Oxoid (Thermo Fisher Scientific).

With the treatment of 600 MPa for 30 minutes the non-enterotoxigenic strain had a survival rate 4.83 log of cells (reduction of 4.02 log), however, with the same treatment, the two enterotoxigenic strains were inactivated to the detection limit of the method (reduction of 9.3 and 8.6 log for 2065 MA and for 2153 MA, respectively), making these two strains much more sensitive to HPP inactivation than the ATCC 6538 strain ( $P < 0.05$ ). Thus, the presence of enterotoxins might have some influence on the resistance of *S. aureus* to HPP treatments, making this preservation technology appropriated for foods with potential risk of contamination by enterotoxigenic *S. aureus*.



## Food Microbiology & Biotechnology

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### HIGH PRESSURE PROCESSING TO INCREASE MICROBIAL SAFETY OF SERRA DA ESTRELA CHEESE

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Serra da Estrela Cheese (Protected Designation of Origin, PDO) is the most important and famous variety of cheese in Portugal, being manufactured from raw milk, salt and cardoon (*Cynara cardunculus*, L) flower extract. As other cheeses, particularly those made from raw milk, Serra Cheese might have in its composition several deteriorative and pathogenic microorganisms. High pressure processing (HPP) is capable of producing microbiologically safe products and causes negligible impairment in cheese characteristics, being a feasible possibility to render Serra Cheese free of microbial pathogens, with unmodified quality characteristics.

This work aimed to study the effect of HPP on Serra da Estrela Cheese after processing and during storage at 5 °C (100 days) on: total aerobic mesophilic (TAM) microorganisms, mesophilic lactic acid bacteria (LAB), *Enterobacteriaceae* (ENT), yeasts and moulds (YM), inoculated *Listeria innocua* (as surrogate for *L. monocytogenes*), and on physicochemical changes (pH, moisture content, water activity, titratable acidity (TA) and lipid oxidation). Cheeses with 45 days of ripening were treated at 400MPa/10min, 500MPa/5min and 600MPa/3min at 4 °C.

The results revealed that beneficial microorganisms – LAB, were the least affected by HPP, being reduced at maximum by ~0.8 Log CFU/g. The LAB decreased during storage, following a first order kinetics, being the higher rate found for cheese treated at 600 MPa/3min. TAM microorganisms were reduced by ~1.0 Log CFU/g (samples treated at 400 MPa/10min were the least affected). ENT counts were below the quantification limits in HPP samples, which remained unchanged during storage, while non-processed cheese showed a first order decrease throughout storage. YM counts exhibited ≥3.6 log cycle reductions after HPP. The samples inoculated with *L. innocua* at 8.58 Log CFU/g presented ≥4.8 log cycle reductions after HPP and levels below the detection limits after 14 days.

Physicochemical parameters (moisture content and activity, pH and TA) were generally not affected by HPP, while lipid oxidation increased during storage, with HPP samples showing lower values (50–66%) at 100 days of storage.

The results indicate that HPP improves cheese microbial safety and shelf-life, with lower lipid oxidation level than non-processed cheese.

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## Food Microbiology & Biotechnology

P043

### IDENTIFICATION OF FISH BACTERIA BY MID INFRARED SPECTROSCOPY USING MULTIVARIATE ANALYSIS

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The accurate and reliable detection and identification of microorganisms in food is critical to public safety. It is extremely important to develop rapid and inexpensive methods for the detection of food microorganisms to replace traditional analysis methods that are expensive and time consuming. The microbial growth and metabolism in foods result in deterioration conducting to the development of unacceptable off-flavours, discoloration or slime production and most important of all, can cause foodborne diseases (Gram & Dalgaard, 2002). It is particularly important to assess the microbiological condition for short shelf-life foods, as is the case of fish. As fish carries a variety of microorganisms from both aquatic and terrestrial sources, microbial spoilage can occur in a short period of time even under refrigeration.

Mid-infrared spectroscopy (MIR) is an established technique, which is a powerful, fast and non-destructive tool for food quality analysis and control. Hence, it is one of the most promising techniques for the food industry and has been successfully used to study microorganisms, since each bacterial specie has a complex cell membrane/wall composition which gives a unique spectral fingerprint (Helm, Labischinski, Schallehn, & Naumann, 1991). Therefore, it is an accurate method to assess the overall molecular composition of the microbial cells in a fast and non-destructive manner. The analytical information of the spectra can be interpreted using multivariate analysis relating the spectra obtained with the properties of the object of study, thus facilitating data interpretation (Brereton, 2003).

The aim of this work was to develop a rapid method to identify bacteria isolated from fish samples, in order to replace the traditional methods that are time consuming. For this purpose, fish bacteria (pathogenic and non-pathogenic) from hake and cod samples were isolated and the MIR spectra were obtained. Multivariate techniques, mostly pattern recognition procedures were applied in order to assess the feasibility of using this approach to address the problem of bacterial identification or bacterial classification. We conclude that MIR spectroscopy, in tandem with multivariate analysis tools, is an effective and rapid method that allows the identification of bacteria isolated from fish to the genus level. This could be of paramount importance to the food industry, allowing the microbial analysis of food samples within a time range that is worth and useful to decide of their suitability for consumption.

## Food Microbiology & Biotechnology

P044

### IN VITRO FERMENTATION OF ALOE VERA ACEMANNAN BY MIXED FECAL MICROBIOTA

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*Aloe vera* has been the object of several studies in the last two decades due to its reported health benefits. These health benefits include promotion of wound healing, antifungal activity, antidiabetic, anti-inflammatory, anticancer, immunomodulatory and gastro protective properties. However, to our knowledge, other activities such as prebiotic properties have not yet been investigated. As it is well known, the human intestinal microbiota has significant effects on host health, so that the interest in the maintenance of its balance and activity has fostered the research and industrial interest in identifying food components with prebiotic activities. Many oligosaccharides with prebiotic properties (such as fructooligosaccharides, inulin, lactulose and galactooligosaccharides) are commercially available, and many others are under study. Amongst these, acemannan, also known under the commercial name of “carrysin”, is one of them.

The goal of the present work was to assess the prebiotic potential of *Aloe vera* acemannan using fecal inocula (obtained from three healthy human donors) by *in vitro* fermentation. Changes in gut bacterial populations and their metabolic activities were monitored over 48 h by fluorescent *in situ* hybridization (FISH) and by measurement of short-chain fatty acid (SCFA) production using HPLC. The experimental results confirmed that *Aloe vera* acemannan selectively stimulated the beneficial gut microbiota, which promoted a favorable SCFA profile.

These results indicate that *Aloe vera* acemannan could be a new interesting prebiotic ingredient that, along with its other properties could open space for new applications in the food and pharmaceutical industries.

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## Food Microbiology & Biotechnology

### P045

#### INFLUENCE OF LACTIC ACID BACTERIA ADDITION ON SENSORY QUALITY OF TRADITIONAL SLICED SMOKED PORK PRODUCTS

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The bioprotective properties of native lactic acid cultures and their bacteriocins may be used for increasing microbiological safety and stability of traditional sliced smoked pork products. It is expected that this addition would not affect consumer's perception and consequently acceptability for these traditional smoked meat products. In order to evaluate the influence of lactic acid bacteria (LAB) addition on the sensory quality of sliced smoked pork products over shelf life, it is important to study the effect of different LAB cultures and their application method and the effect of the modified atmosphere (MA) surrounding the package.

A solution with  $10^9$  CFU/g LAB inoculum (*Lactobacillus sakei* ST153ch or BLC 35 commercial culture) was applied by immersion or spray on smoked pork slices that were produced at industrial scale, packed under MA (8% or 12 % v/v CO<sub>2</sub>) and stored at 5°C. A quantitative descriptive sensory test was performed by a sensory trained panel at 30, 90 and 120 days, involving previous sessions for main descriptors definition, their scale limits as well as verbal anchors by panel consensus. A final sheet with 9 descriptors (meat colour, greasiness, characteristic odour, off-odour, hardness, succulence, characteristic taste, acid taste, bitter taste), each one with a 13-point scale, was validated. Results' showed that meat colour, succulence and characteristic taste were the common attributes for both LAB cultures that varied over the shelf life. The application method had no significant effect on any sensorial attribute analysed. MA influence was only noticed in terms of greasiness and hardness. The samples with commercial culture BLC35 addition was evaluated as harder and less succulent than the ones with *Lactobacillus sakei* ST153ch addition.

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## Food Microbiology & Biotechnology

P046

### MICROBIAL DIVERSITY OF *AEROMONAS* SPP. USING MULTILOCUS SEQUENCE ANALYSIS

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The genus *Aeromonas* comprises ubiquitous bacteria regarded as emerging pathogens associated with a variety of human intestinal and extra-intestinal infections. In this bacterial genus taxonomy is a very controversial issue, as species distinction is often difficult to achieve, due to unreliable conventional biochemical tests and discrepancies between phenotypic and genetic groups.

In the present investigation multilocus sequencing analysis was applied to clarify the taxonomic relationships between 70 aeromonads isolated from clinical samples, a cheesemaking factory, a slaughterhouse and a supermarket. For comparison purposes an additional group of 42 strains, obtained from collaborating laboratories, representing various origins worldwide and a set of reference strains from a culture collection, was also included in the study.

Multilocus sequence analysis allowed genotyping of all aeromonads under study and demonstrated their high genetic variability, despite origin of isolation. Further analysis using the concatenated sequences for five housekeeping genes (*groL*, *metG*, *recA*, *gyrB* and *gltA*) and the BioNumerics software grouped the aeromonads in well-defined clusters, attributed to the species *A. hydrophila*, *A. caviae*, *A. media* and *A. salmonicida*.

In conclusion, the multilocus sequence analysis applied demonstrated to be applicable for evaluating the genomic diversity of aeromonads from distinct sources and countries. The results obtained highlighted the difficulty in clearly discriminating between *Aeromonas* species, nevertheless provided novel insights into the genetic structure of this bacterial group, which may contribute to identify relevant strain-dependent features and to increase the knowledge on potential spoilage and/or pathogenic properties.

## Food Microbiology & Biotechnology

P047

### MICROWAVE ASSISTED WATER EXTRACTION OF POLYSACCHARIDES FROM BREWER'S SPENT YEAST CELL WALL

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The second major by-product of brewery industry is the brewer's spent yeast (BSY). The yeast cell wall (YCW) structure is composed mainly of  $\beta$ -glucan, mannoprotein and chitin. As these polysaccharides have bioactive properties, the present study proposes their solubilisation in water using a microwave assisted extraction.

After an induced autolysis at 80°C, for 4 hours at 400 rpm, YCW was isolated by centrifugation. The YCW was submitted to a sequential extraction with superheated water at a ratio of 1:28 (m/v) in a microwave system. The first 2 extraction steps were designed to maintain 180°C for 2 minutes, followed by two extraction steps at 200°C. After each extraction cycle, the slurry was centrifuged and the residue was submitted to another extraction cycle. The resultant supernatants obtained after each extraction cycle were treated with 80% of ethanol in order to precipitate the polysaccharides.

The first two extraction steps at 180°C extracted mostly mannose-rich polysaccharides, with a yield higher than 60%. Glucose-rich polysaccharides were mainly obtained with the last two cycles at 200°C, yielding 50% of glucose. After the four extraction cycles, about 80% of mannans, and 70% of glucans were extracted. Gel permeation chromatography showed that the mannan-rich fractions had a molecular weight distributed in four populations, ranging from 60 kDa to 342 Da, and the molecular weight of the glucans was distributed in four populations between 96 kDa and 342 Da. The glycosidic-linkage analysis of the compounds obtained in each one of the four cycles confirms the presence of mannoproteins in the first two extraction cycles. For the 3<sup>rd</sup> extraction cycle it was observed the presence of 1,4-Glc and 1,4,6-Glc, which is indicative of the presence of glycogen (1, 2). In the fourth cycle a smaller amount of glycogen was also extracted, mixed with 30% of 1,3-linked glucose, which is characteristic of  $\beta$ -glucans (3).

In conclusion, it was possible to extract the polysaccharides from YCW using microwave assisted sequential extraction, allowing the separation of mannoproteins rich fractions from those rich in glucans, all of them soluble in water. Using this procedure, BSY can be a source of cold water soluble polysaccharides able to be used as food ingredients, to be incorporated in aqueous matrices as dietary fibre or even used as nutraceuticals with immunostimulatory activity.

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## Food Microbiology & Biotechnology

P048

### MONOCLONAL ANTIBODIES AGAINST EXTRACELLULAR POLYSACCHARIDES FROM *PLEUROTUS OSTREATUS*

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Several polysaccharides isolated from edible mushroom strains have been reported to exhibit anti-tumor activity as well as other medicinal effects. In order to quantify and characterize bioactive exopolysaccharides produced by *Pleurotus ostreatus*, the aim of this research work was to raise monoclonal antibodies (Mabs) against such polysaccharides which could be used as powerful probes for detection and structural analysis.

Exopolysaccharides were isolated from supplemented whey permeate medium after 10 days of *P. ostreatus* growth in an aerated 5 L batch reactor. Polysaccharides were characterized by protein and polysaccharide analysis and pseudo-superoxide dismutase (SOD) activity. A exopolysaccharide sample exhibiting high SOD-mimicking activity ( $8.16 \times 10^4$  U L<sup>-1</sup>) was used as antigen and Mabs were produced by using hybridoma technology. A mouse Mab named 3F8\_3H7 was selected for characterization purposes. Culture supernatants containing Mab 3F8\_3H7 were purified on Sephacryl S-300 HR column and the purified preparation of Mab obtained was apparently homogeneous on native PAGE. Ouchterlony double diffusion analysis of purified Mabs was carried out suggesting that Mab 3F8\_3H7 is of IgM class since a precipitin line was observed against rabbit anti-mouse immunoglobulin heavy chain  $\mu$ . Isoelectric focusing of purified samples of Mabs was carried out in polyacrylamide gels using an ampholine pH range of 3–10 and the protein bands were stained with silver nitrate.

In addition, the levels of exopolysaccharides produced by *P. ostreatus* in submerged cultures was quantified by indirect ELISA using IgM Mab. Daily samples of culture medium were collected and the highest concentration of exopolysaccharides was detected at the 7th day of culture growth.

The selectivity of the Mab to different commercial and non-commercial polysaccharides (75  $\mu$ g/well) was also investigated by using indirect ELISA. Studies carried out provided strong evidence that this Mab also recognizes intracellular polysaccharides isolated from *Ganoderma carnosum* and commercial  $\beta$ -1,3 glucan from *Euglena gracilllis*. However, this Mab revealed no reactivity for other tested samples of polysaccharides, such as starch, cellulose, chitosan from shrimp, chitin azure, laminarin (from *Laminaria digitata*) and  $\beta$ -D glucan from barley. Therefore, the data presented strongly suggest that this Mab could be used as a powerful tool to understand the structure and function of mushroom polysaccharides.

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### NOVEL ASSAY OF BETA-GLUCANS BY CONGO RED DYE IN A 96-WELL MICROTITER PLATE IN BASIDIOMYCETE MUSHROOMS

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Different species of mushrooms contain an enormous diversity of nutritional ingredients and biomolecules with a wide spectrum of therapeutic and prophylactic properties. Among these compounds, polysaccharides, which belong to  $\beta$ -glucans play an important role in immuno-modulating and antitumor activities. [1]

The present work involves a novel colorimetric assay method for  $\beta$ -glucans with a triple helix tertiary structure, with Congo red dye. The specific interaction that occurs between Congo red and  $\beta$ -glucans was detected by bathochromic shift from 488 to 516 nm (>20 nm) in UV-Vis spectrophotometer and therefore it has been used for quantification of the  $\beta$ -glucans in mushroom samples. A micro and high throughput method based on 96 wells microtiter plate was devised which requires only 2–40  $\mu$ g of polysaccharides in samples which presents several advantages over the published methods such as greater sensitivity, speed, assay of many samples and very cheap.

$\beta$ -glucans off several mushrooms (i.e. *Coriolus versicolor*, *Ganoderma lucidum*, *Pleurotus ostreatus*, *Ganoderma carnosum*, *Hericium erinaceus*, *Lentinula edodes*) were isolated by using a sequence of several extraction with cold water, boiling water, alkali (NaOH and KOH) and HCl [1,2] and quantified by this novel microtiter plate method. FTIR spectroscopy was used to study the structural features of  $\beta$ -glucans in these mushroom extractions samples as well as the specific interaction between these polysaccharides and Congo red dye. The effect of NaOH on triple helix conformation of  $\beta$ -glucans was investigated with several different polysaccharides namely *C. versicolor*, *G. lucidum*, *P. ostreatus*, *G. carnosum*, *H. erinaceus*, *L. edodes*). [3]

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## Food Microbiology & Biotechnology

P050

### NUTRITIONAL AND FUNCTIONAL AUDIT TO THE PROCESS OF FRESH-CUT STRAWBERRY

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From a nutritional and functional point of view it is important to understand the consequences of processing and storage in fresh-cut products composition in order to select the best technological conditions required for the preservation of fruits and vegetables health benefits. A nutritional and functional audit of postharvest handling systems and processing lines of a fresh-cut fruits enterprise was performed in order to generate recommendations that can optimize phytochemical preservation during processing. The effects of the production process upon the nutritional and phytochemical composition of strawberries was determined in order to validate the results obtained in real conditions and identify the critical points involved in the deterioration of the functional quality. Three replicated samples were collected at each step of the production flowchart of fresh-cut strawberries: at fruit reception, after calyxes removal, after decontamination and after processing. All samples were frozen with liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  until analyzed for nutritional characterization. Total antioxidant activity was assessed by the ABTS method, total phenolics by Folin Ciocalteu's method and phenolic compounds and anthocyanins were analyzed by high performance liquid chromatography (HPLC-DAD). According the main processing plan for strawberries the effect of processing steps on the main nutritional and functional quality markers showed that total antioxidant capacity, total phenolic compounds and total anthocyanins contents were significantly affected at least by one of the steps. As processing advanced from reception to cutting, antioxidant capacity increased, with clear differences between processing phases. Intact and processed strawberries showed similar total phenolic compounds content while decontaminated strawberries showed the lower value for phenolic compounds level. Catechin was not significantly affected during different processing phases but epicatechin, rutin and ellagic acid were affected mainly after calyxes removal and cutting. Total anthocyanins were also affected by all processing steps with cutted strawberries showing the lowest values. Cyanidin-3-glucoside and pelargonidin-3-rutinoside were not affected by processing while pelargonidin-3-glucoside changed after decontamination and cutting phases.

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### P051

#### NUTRITIONAL COMPOSITION AND QUANTIFICATION OF FUNGI AND YEASTS OF FIG (*FICUS CARICA*) MISSION AND KADOTA VARIETIES UNDER HIGH HYDROSTATIC PRESSURE

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In the last decades consumer demands in the field of food production has changed considerably. Consumers more and more demands products with a high nutritional value. However, the traditional techniques in the food processing may cause losses in the nutritional composition in the final product. An alternative in the food processing to preserve the majority of nutrients and properties is the application of non-thermal technologies. The application of non-thermal treatments such as high hydrostatic pressure (HHP) affects the viability of microorganisms and minimally affects nutritional and sensory characteristics of food being similar to those of fresh or minimally processed.

The aim of this study was to evaluate the effect of the application of 350 MPa of hydrostatic pressure for 5 min at 0, 45 and 60 °C, on the nutritional composition and microbiological quality of two fig varieties, Mission and Kadota, respectively.

The moisture, carbohydrates, fats, proteins and ash, tests were determined according to AOAC methods while the fungal and yeast counting were carried out according to the Mexican official standard. A single-factor design was selected as experimental design, where the process temperature was taken as the independent variable. The Duncan's statistical test was used to analyze the means difference with a significance level of 0.5%.

The results show that the interaction between the process temperature and the process pressure promotes a significant decrease on the microbial load. On the other hand, the nutritional analysis indicates that the HHP processing did not affect the moisture, protein, free-nitrogen extract of two fig varieties. However, the fats and ash content were affected for the HPP process. The application of high hydrostatic pressure affects positively to the microbiological quality of fig, without affecting the nutritional quality.

#### Acknowledgements

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## Food Microbiology & Biotechnology

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### OCCURRENCE OF *ACINETOBACTER* SPP. IN VEGETABLES

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*Acinetobacter* spp. have emerged in recent years as pathogens of major public health concern. Despite hospital-acquired infections being the most common form, these organisms are also recognized as responsible for community-associated infections. *Acinetobacter* spp. seem to be widespread in the environment and frequently isolated from water and soil, and have been recovered from various foods, including: vegetables fruits, meat, fish, cheese, milk, soft drinks and natural mineral water. *Acinetobacter lwoffii* and *A. johnsonii* are the predominant species recovered from food. Information about the presence of these organisms in a variety of food items is scarce and in Portugal does not exist. For effective infection control, it is of major importance to collect data about the occurrence of the various *Acinetobacter* species apart from medical sources, in order to understand their mechanisms of transmission and spread between community and hospital.

In this study, samples of different raw vegetables were tested for the presence of *Acinetobacter* spp. using a selective enrichment procedure followed by selective plating for isolation. Samples analysed included lettuce (n=4), parsley (n=1), carrot (n=1), and tomato (n=1). Presumptive colonies of *Acinetobacter* spp. were picked and further confirmed by phenotypic (Gram-stain, catalase and oxidase tests) and genotypic (16S rDNA sequencing) tests. All samples were contaminated with *Acinetobacter* spp. and a total of 54 isolates were confirmed to be *Acinetobacter* spp. These isolates were typed by REP-PCR (Repetitive Extragenic Palindromic sequence-based – Polymerase Chain Reaction), and 28 isolates presenting different typing patterns were selected for molecular identification of *Acinetobacter* species by *rpoB* gene sequencing. Species *A. johnsonii* and *A. calcoaceticus* were the most common with the frequency of 35.7% each. Other species were also found at lower frequencies, namely: *A. guillouiae* (10.7%), *A. tjernbergiae* (3.6 %), *A. beijerinckii* (3.6 %), *A. pittii* (3.6 %), *A. lwoffii* (3.6 %), and *A. bouvetii* (3.6 %).

Vegetables may therefore be a natural habitat of *Acinetobacter* spp. and provide a route by which these bacteria can be introduced into community and health care units and cause infections.

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### PEACH PHYTOCHEMICALS AVAILABILITY DURING YOGHURT SHELF-TIME

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Fruit preparations can be added to yoghurt to create new products and combine the nutritional value of dairy and fruit matrices. Interactions of plant phenolics with proteins may lead to the formation of soluble or insoluble complexes. These interactions may have a detrimental effect on the *in vivo* bioavailability of both phenolics and proteins. The aims of this study were to evaluate the protein profiles of yoghurt before and after the addition of peach and to assess the phytochemical availability of fruit in presence of yoghurt, in order to evaluate the possible interaction between protein and phenolic compounds therein. Industrial peach preparates containing 50% of fruit, 0.19% aroma, 0.17% sweeteners, 2.3% starch, 0.07% citric acid and 0.15% hydrocolloids were incorporated in natural yoghurt and kept during 28 days at 2 °C. Extracts were obtained with methanol: formic acid (9:1 v/v) and stored at -20 °C for 1 h to facilitate protein precipitation. Extracts was centrifuged and supernatant filtered with 3 kDa membrane. Individual phenolics and carotenoids as well as proteins were analysed by HPLC-DAD; complementary, proteins profile was analyzed by SDS-PAGE and Urea-PAGE. The polyphenols identified in peach pieces used in formulations were (+)-catechin, *p*-coumaric acid, chlorogenic acid, neochlorogenic acid and rutin and all the compounds do not present significant differences between control peach formulations and peach enriched yoghurt. Carotenoids detected were zeaxanthin,  $\beta$ -cryptoxanthin and  $\beta$ -carotene, whose levels increased in control samples after 7 days at 2 °C by 128, 114 and 74%, respectively and then remained constant without significant changes. When the peach preparation was in contact with the yoghurt matrix, zeaxanthin levels increase 54% during the 28-day shelf-life period at 2 °C and  $\beta$ -cryptoxanthin and  $\beta$ -carotene concentration increased 78 and 100%, respectively during first 14 days and then decreased until day 28. After 28-day shelf-life period peach enriched yoghurt had less zeaxanthin,  $\beta$ -cryptoxanthin and  $\beta$ -carotene (61, 42 and 39% respectively) than the initial concentrations detected at day zero of yoghurt shelf-life time.

The decrease in carotenoid suggests a possible interaction of these compounds with the dairy matrix. The soluble proteins detected were  $\alpha$ -lactalbumin present at 0.26 mg/ml that showed to be very stable throughout storage and even after peach addition. The  $\beta$ -lactoglobulin was quantified in control at 0.07 mg/ml and presents a significant decrease of 88% in peach yoghurt between days 14 and 28 of storage.

Free  $\beta$ -lactoglobulin and peach carotenoids availability decreased during peach yoghurt enriched shelf-life.

These results suggest that interactions between peach and yoghurt components may affect nutritional availability.

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### P054

#### POTENTIAL OF TRADITIONAL PULSES FROM PORTUGAL TO PROMOTE THE GROWTH OF PROBIOTIC STRAINS

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Pulses contain carbohydrates, proteins, minerals and vitamins which are essential requirements in the human diet. Therefore, these ingredients can constitute a very good source of growth factors and prebiotic components for probiotic strains. The main objective of this research work was to evaluate the ability of two traditional pulses in Portugal (broad beans and lupins) to support the growth of two probiotic strains: *Lactobacillus acidophilus* L10 and *Bifidobacterium animalis* subsp. *lactis* Bb12.

To achieve the objective of this work, a culture medium (Man Rogosa Sharpe (MRS) without glucose) was supplemented with different percentages of pulse flour (2, 3, 4 and 6%) and the growth and acidification profiles of the two probiotic strains was observed. MRS medium with glucose was used as positive control. Each medium was inoculated with 2% of each strain, homogenized and incubated at 37 °C for 48 hours. Samples were taken at 0, 4, 8, 12, 24, 36 and 48 hours of incubation and evaluated in terms of physico-chemical and microbiological properties.

The results obtained showed that the MRS supplemented with broad bean flour at different percentages supported the growth of *B. animalis* Bb12, but to less extent than the MRS with glucose. For MRS supplemented with lupin flour, the growth of *B. animalis* Bb12 was similar to that obtained in MRS with glucose regardless of the different percentages assayed. The observed growth with this strain was accompanied by a decrease in medium pH.

In the case of *L. acidophilus* L10, in both medium supplemented with broad bean, as well as that supplemented with lupin flour, independently of the percentages of flours, bacterial growth was maintained in the first hours of fermentation but then it decreased steadily thereafter; similar behavior was observed for MRS with glucose. A decrease of pH was scarcely observed and this is in agreement with the limited growth.

In conclusion, the preliminary results obtained in this work have shown that the pulses used may offer the possibility of improving the formulation of fermented beverages from both a nutritional and a bacterial growth enhancement perspective, giving an opportunity for innovation in the fermented product field as well as contributing to a more balanced diet given the high nutritional value of pulses.

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### POWER ULTRASOUND PRETREATMENTS OF CARROT: EFFECT ON THE CARBOHYDRATE COMPOSITION

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In recent years has increased the interest by the use of ultrasounds as an alternative to improving the efficiency of traditional processes. The forces involved by the ultrasonic waves can create microscopic channels that may ease moisture removal. It has been reported that cell rupture is caused by power ultrasound (US) application, as well as enzyme denaturation and microorganism inactivation can also be achieved.

The aim of this study was to evaluate the effect of pretreatment assisted with power ultrasound on carbohydrates extractability and composition of carrot tissue after a convective drying.

Carrot slabs (*Daucus carota* var. Nantes) of 1.9 x 1.9 cm and the slice thickness of 0.5 cm were used. The carrot slabs were pretreated with the power ultrasound application (US) and another batch of carrot slabs were immersed in water but without power ultrasound application (S). All samples were dried at 60°C and 2.6 m/s. Lyophilized untreated carrot slabs were used as control sample (L). The polysaccharides were sequentially extracted with water and then with aqueous solutions of imidazole, carbonate and KOH to leave a final residue rich in cellulose (cellulosic residue). The monosaccharides content and linkages analysis were determined in the fractions to evaluate the influence of the ultrasound treatments.

The pretreatment assisted with US caused the decrease of the drying time of ca. 18%. These samples showed higher content of polysaccharides in the carbonate and KOH extracts comparing with control. The carbonate extracts were mainly composed by pectic polysaccharides, inferred by the presence of high quantity of uronic acids, arabinose, and galactose. Conversely, the KOH extracted contained mainly xylose and glucose. The methylation analysis of the carbonate extracts revealed the presence of type I arabinogalactan and arabinans whereas KOH extracts presented xylans and xyloglucans.

The carrots dried without pretreatment of power ultrasound (S) had, in relation to US pretreated samples, higher content of sugars in the water extract, constituted mainly by pectic polysaccharides. These results may be explained by the mild drying temperature used in this study that could allow the enzymes to retain their activity, degrading partially the polysaccharides and rendering them more extractable in water and, consequently, absent from the following extractions performed with carbonate and KOH. The residue left was mainly composed by cellulose, inferred by the presence of (1→4)-Glc. The residue from the control sample was the one with higher amount of polysaccharides, explained by the lower extractability of these polysaccharides, not promoted by the drying conditions (as observed in S sample) neither by the ultrasounds (as observed in US sample).

The glycosidic-linkage analysis of the different extracts also showed that the occurrence of different new linkages in the US sample, allowing to infer that the carrot US pretreatment also affects non-specifically the more labile structures.

These results show that the US is a suitable technique to be applied as pretreatment as it allows a decrease of the drying time, explained by the non-specific modification of cell wall polysaccharides and by enzyme inactivation.

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## Food Microbiology & Biotechnology

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### PRELIMINARY CHARACTERIZATION OF AN EXTRACELLULAR PROTEASE PRODUCED BY A MARINE PROTEOLYTIC STRAIN

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Microbial proteases constitute one of the most important groups of industrial enzymes due to the wide spectrum of applications in different industries such as detergent, food, bioremediation, pharmaceutical, leather, etc. A number of bacteria, fungi and yeast have been reported for protease production. Additionally, proteases from marine microorganisms have received increased attention due to their inherent stability at different pH values, temperature and salinity.

The objective of this present work was the preliminary characterization of an extracellular protease produced by a sporulated, Gram and catalase positive bacteria isolated from estuarine bivalves, presenting proteolytic activity in casein.

The bacterial growth was performed in a liquid culture media, containing bacteriological peptone (2 %), yeast extract (0.5 %) and saccharose (0.5 %), with continuous shaking at 30°C and followed for 50 h. Broth was centrifuged at 10000 g for 15 min and the supernatant was filtrated using filters with 0.45 µm diameter pore. The filtrate was used for the characterization of protease produced.

Azocasein was used as substrate for the measurement of proteolytic activity and pH and temperature of maximum activity were determined. The effect of several inhibitors and cations was tested. The thermal and pH stability was also evaluated.

The optimum pH and temperature were recorded at 6.5 and 60 °C, respectively. The enzyme activity was only inhibited by EDTA and activated by Ca<sup>2+</sup> and Mg<sup>2+</sup>. The protease was stable at 40 °C for 15 h but quickly denaturated at 60 °C. The incubation of the protease at different pH at room temperature for 24 h showed that it was unstable at pH below 6.0 and stable until pH 11. However, the activity was only 25 % of its initial value.

These results suggest that this protease may be further exploited for various industrial applications.

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### PREPARATION OF CHITOSAN OLIGOMERS BY OXIDATIVE DEGRADATION WITH HYDROGEN PEROXIDE AND MICROWAVE IRRADIATION – ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES

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Chitosan oligomers (COs) have deserved particular attention due to their low viscosity and high solubility at neutral pH. Different studies have emphasized their ability to improve food quality and human health. Chemical and enzymatic methods have been widely used for the production of COs but the chemical hydrolysis is more commonly used in the industrial-scale. The objective of this work was to prepare COs from brown crab chitosan by an oxidative degradation method involving hydrogen peroxide and microwave radiation and to evaluate their antioxidant and antibacterial properties.

Chitin was extracted from brown crab exoskeleton and deacetylated to chitosan. COs with high molecular weight were prepared by dissolving 1 g chitosan in 50 mL 2% acetic acid, 5 mL 30% H<sub>2</sub>O<sub>2</sub> was added and the reaction mixture was irradiated with microwave for different periods. COs with low molecular weight were prepared by adding directly 2 g chitosan in 50 mL 15% H<sub>2</sub>O<sub>2</sub> and microwave irradiated as previously. COs were recovered by ethanol precipitation and dried. The reducing power and antiradical activity (DPPH and ABTS) of COs were measured. The antibacterial activity of COs was measured using the optical density method during the growth of seven of Gram negative and five Gram positive strains with different concentrations of high (3000 Da) and low (< 600 Da) molecular weight oligomers (1%, 0.5% and 0.1%).

The highest radical scavenging ability was recorded in COs with 600 Da. Conversely the highest antibacterial activity was achieved by COs with the highest molecular weight. Differences were registered among strains and *Escherichia coli*, *Salmonella typhimurium*, *Shewanella putrefaciens*, *Vibrio vulnificus*, *Pseudomonas putida* were those more effectively inhibited. In general, Gram positive strains were less affected by the presence of COs.

It may be concluded that COs can be conveniently and effectively obtained by oxidative degradation of chitosan with H<sub>2</sub>O<sub>2</sub> and microwave irradiation. The lowest molecular weight COs exhibited the highest antioxidant activity whereas the highest antibacterial activity was achieved by the COs with higher molecular weight.

The research leading to these results has received funding from ERDF through Atlantic Area Transnational Cooperation Programme Project MARMED (2011-1/164) and Project ACRUNET.



## Food Microbiology & Biotechnology

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### PREPARATION OF COLOURED AND ANTIOXIDANT FOOD INGREDIENTS FROM INSTANT COFFEE AND THEIR USE IN COOKIES

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Coffee has been shown to be a source of compounds with potential health benefits, namely the antioxidant properties of chlorogenic acids and the brown polymeric compounds known as melanoidins. As instant coffee is a rich source of these compounds, it was used to obtain melanoidin and/or chlorogenic rich fractions.

In this work, instant coffee fractions were obtained using ultrafiltration and ethanol precipitation methodologies. The brown compounds were mainly retained by the membrane of 100 kDa (HMWaq) and tend to precipitate in 75% ethanol solutions (PpEt). The fraction not retained by the membrane (LMWaq) and the fraction that was soluble in 75% ethanol (SnEt) presented both a light brown colour. The compounds with higher antioxidant activity remained in SnEt (1149 mg<sub>CGA</sub>/100 g<sub>Extract</sub> and 12.3 µg/mL<sub>ABTS</sub>), whereas PpEt, HMWaq, and LMWaq fractions presented intermediate antioxidant activity (16.0–19.1 µg/mL<sub>ABTS</sub>). These fractions were incorporated in dough to prepare cookies with different brown colours and enhanced antioxidant activity. Concentrations of 1, 5, and 10% to the flour content were tested.

The addition of the coffee fractions did not show significant changes in dough pH and water activity neither in cookies hardness and flexibility. On the other hand, the addition of all coffee fractions had a significant impact in colour, showing darker/browner cookies. This was more visible for the browner samples. The addition of 1% of SnEt and HMWaq fractions increased 78% and 73%, respectively, the antioxidant activity of the cookies when compared to the cookies used as control, with no addition of coffee fractions.

Coffee is also a known source of 5-hydroxymethylfurfural (HMF) and acrylamide, and these potential carcinogenic compounds has been associated with cereal-based foods submitted to high temperatures under conditions similar to those used for the baking of the cookies. In order to observe the variation of their concentrations when the coffee fractions were used in different concentrations, the contents of these two compounds were measured. It was observed that the amount of acrylamide was similar in all extracts with those obtained in the cookies used as control (0.1 mg/kg<sub>cookies</sub>) whereas the levels of HMF was 0.2 mg/kg<sub>cookies</sub> in control cookies and increased in accordance with the content of the coffee fraction added. Anyway, both acrylamide and HMF contents were, in all cases, below the recommended maximum (500 µg/kg<sub>cookies</sub> and 74.5 mg/kg<sub>cookies</sub>, respectively for acrylamide and HMF), described as safe for baked cookies.

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## Food Microbiology & Biotechnology

P059

### PREPARATION OF FISH PROTEIN HYDROLYSATES BY A FERMENTATIVE PROCESS

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The fermentative processes occurring in several types of proteinaceous food products lead to the formation of a complex mixture of peptides. This results from a wide spectrum of extracellular proteases produced by microorganisms. The production of fish protein hydrolysates has been considered a valuable alternative for the upgrading of fish by-products. They are usually prepared by adding commercial proteases but the fermentative process has not been used in their production. Thus, the objective of this work was to prepare fermentative protein hydrolysates (FPH) from a commercial protein hydrolysate (CPH) using an indigenous marine proteolytic strain.

Bacterial growth was performed in liquid culture media, containing three levels of CPH (1, 2 and 4%), yeast extract (0.5%) and saccharose (0.5%), with continuous shaking at 30°C for 24, 50 and 72h. Bacterial growth was followed by measurement of the absorbance and bacterial counts. Broths were centrifuged (10000g, 15min) and the supernatants were filtrated and identified as FPH1%, FPH2% and FPH4%. Aliquots of FPH were used for the determination of the enzymatic activity and peptide profile. Liquid FPH were concentrated and lyophilized and their antioxidant and chelating activities were determined.

The maximum bacterial counts and enzymatic activity was achieved after 24h. The achieved protein yields were 29%, 28% and 58% respectively for FPH1%, 2% and 4%. The highest increase (6%) of the degree of hydrolysis was recorded in FPH2% after 72h. The peptide profile of FPH showed important differences relatively to the initial peptides, as the disappearance of peptides in the range of 200–400Da and formation of dipeptides and free amino acids. The DPPH radical scavenging activity of FPH increased with the fermentative period as indicated by the decrease of EC50. The ABTS antiradical activity of the different FPH didn't show a clear trend with the fermentative period and CPH level. Within each FPH the highest reducing power was achieved after 72h. However, the reducing power decreased with increasing level of CPH. In general the FPH obtained after longer periods showed lower Fe chelating activity than the initial peptides. The Cu chelating activity didn't present differences among FPH samples.

This work was developed in the frame of the project SECUREFISH – “Improving Food Security by Reducing Post Harvest Losses in the Fisheries Sector”. Collaborative project of 7<sup>th</sup> framework programme

## Food Microbiology & Biotechnology

### P060

#### PRODUCTION OF ANTILISTERIAL BACTERIOCIN BY *PEDIOCOCCUS ACIDILACTICI* HA6111-2 UNDER DIFFERENT STRESS CONDITIONS

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Nowadays, the application of lactic acid bacteria (LAB) and their bacteriocins are widely used in food industry. Bacteriocin producer LAB cultures are commonly used as biopreservatives in fermented food products; thus the food industry is interested in stable cultures with stable bacteriocin activity. *Pediococcus acidilactici* are usually isolated from different fermented meat products. The aim of our study was to investigate how the *P. acidilactici* HA6111-2 (isolated from alheira) produce bacteriocins under different stress conditions: pH, NaCl and temperature. The modified MRS broth was inoculated with the LAB strain and incubated at 30 °C (except for the stress conditions of temperature). Changes in pH and optical density were recorded every hour, for 48 h. Bacteriocin activity in the cell-free supernatant was recorded every 3 h for 48 h. *Listeria monocytogenes* serogroup IIb (from cheese), *L. monocytogenes* serogroup IVb (from cheese), *L. monocytogenes* serogroup IIb (from hamburger) and *L. innocua* NCTC 11288 were used as target strains. *Pediococcus acidilactici* HA6111-2 could not grow well at low pH and at 10 °C environment, but it was able to produce low amount of bacteriocin. In case of pH 8.5 the alkaline adaption was clearly observed and it took ~ 28 hours. After adaption, the pH rapidly dropped and the stationary phase with highest bacteriocin production started. At 7.5% NaCl, viable cell number of *P. acidilactici* HA6111-2 slightly increased, the pH poorly decreased and the highest bacteriocin activity was achieved after 30-33 hours. At 50 °C, the cells were damaged; after 24 hours we could not detect viable cells, although the bacteriocin activity was detected at low level until 40 hours. The results also indicated that *L. monocytogenes* serogroup IIb (from hamburger) was more sensitive, while *L. innocua* showed to be more resistant. It was proved that under stress conditions, *P. acidilactici* HA6111-2 could produce antilisterial bacteriocins, however the bacteriocins activity was different for the *Listeria* strains.

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## Food Microbiology & Biotechnology

P061

### PRODUCTION OF SQUALENE AND DHA BY *AURANTIOCHYTRIUM* SP. ATCC PRA-276

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Some heterotrophic strains of the Thraustochytrids genus have been used for the production of high added-value biomolecules such as squalene and polyunsaturated fatty acids (PUFA). Squalene has many different applications, both in cosmetics and pharmaceutical products. PUFA are involved in several important biochemical mechanisms, especially in the prevention of cardiovascular diseases. The production of these biomolecules by these strains represents an alternative to the common sources as shark liver oil and fish muscle. Thus, the purpose of this work was to study the effect of salinity (1.5 and 3.5 % S), temperature (26 °C and 30 °C) and glucose concentration (20, 30 and 40 g/L) on the production of squalene and PUFA by *Aurantiochytrium* sp.

*Aurantiochytrium* sp. ATCC PRA-276 cultures were grown in Erlenmeyer flasks (250 mL) with an orbital agitation of 150 rpm and with 100 mL of growth medium used by Chen et al. (2010) prepared with seawater (1.5 and 3.5 %S), three levels of glucose (20, 30 and 40 g/L) at two temperatures (26 and 30 °C). Daily sampling was done until the seventh day of growth. It was evaluated by optical density at 600 nm and cell dry weight. The biomass was recovered by centrifugation and lyophilized for the determination of squalene and fatty acid content. The highest biomass production was achieved in the culture medium with 1.5% salinity.

Regarding the effect of glucose concentration, the highest squalene production was achieved with 20 g/L and 1.5 % salinity. The squalene production was also higher at lower temperature having been attained a maximum yield of 248 mg/L in the growth medium with 30 g/L of glucose, 1.5 %S after 48 h.

The highest production of DHA (1.08 g/L) was achieved in the growth medium with 30 g/L of glucose and 1.5 % salinity at 30 °C. The DHA production was not significantly different when the temperature growth increased from 26 °C to 30 °C.

The results obtained may conclude that the growth conditions of *Aurantiochytrium* sp. leading to the highest production of squalene after 48 h were: 1.5 % salinity, 20 g/L glucose and 26 °C. The production of DHA generally increases until 96 h of growth.

Chen G., Fan K-W., Lu F-P, Li Q., Aki T., Chen F. and Jiang Y. 2010. *New Biotechnology*, 27, 4: 382-389.

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## Food Microbiology & Biotechnology

### P062

#### PROTEIN-BOUND POLYSACCHARIDES FROM *AMANITA PONDEROSA* CULTURES: CHARACTERISATION, TOXICOLOGICAL ASSESSMENT AND ANTIOXIDANT PROPERTIES

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In recent years, protein-polysaccharide complexes extracted from mushrooms have received great attention from the scientific community, due to their medicinal properties, namely antioxidant, antitumoral, antimicrobial, immunomodulatory, antiatherogenic and hypoglycemic properties [1, 2]. The southern of Portugal, due to its Mediterranean climate and flora diversity, is a region with a high prevalence of wild edible mushrooms *Amanita ponderosa* [3].

The aim of this work was to produce and characterize protein-polysaccharide complexes, extracted from cultures (mycelia or supernatants) of different strains of *A. ponderosa* in order to evaluate their antioxidant properties.

Batch cultures were performed during 15 days, and polysaccharides content were determined by the phenol-sulphuric method. A combined FTIR-ATR (Fourier-transform infrared using the attenuated total reflection) and Raman spectroscopy was used for the screening of bioactive protein-polysaccharides, showing bands profile compatible with this type of compounds. The characterization and separation of different polysaccharide-protein complexes using SEC UV-IR-HPLC (HPLC Size Exclusion Chromatography, coupled to UV (280nm) and RI detectors), showed the presence of four major complexes, with different molecular weight. Extracts showed no toxicity against *Artemia salina* (lethality < 5%). The polysaccharide-protein complexes presented antioxidant activity by DPPH and  $\alpha$ -carotene/linoleic acid methods and showed ability to mimitize the catalase enzymatic activity.

Therefore, based on the observed biological properties, extracts of *A. ponderosa* cultures, could be an important source of bioactive compounds, with potential medicinal value.

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## Food Microbiology &amp; Biotechnology

P063

**SACCHAROMYCES CEREVISIAE AND DEKKERA BRUXELLENSIS INTERACTIONS IN ALCOHOLIC FERMENTATIONS: GROWTH AND 4-ETHYLPHENOL PRODUCTION**Rute Coutinho<sup>1</sup>; Patrícia Branco<sup>2</sup>; Margarida Monteiro<sup>2</sup>; Manuel Malfeito-Ferreira<sup>1</sup>; Helena Albergaria<sup>2</sup><sup>1</sup>Instituto Superior de Agronomia, Technical University of Lisbon; <sup>2</sup>Laboratório Nacional de Energia e Geologia (LNEG)

The yeast *Dekkera/Brettanomyces bruxellensis* can cause enormous economic losses both in wine industry and fuel-ethanol processes due to production of phenolic off-flavour compounds and low ethanol productivities. In winemaking this microbial hazard is usually tackled by the use of chemical preservatives such as sulphur dioxide. In spite of this, *D. bruxellensis* strains are frequently found in wines at low levels (*ca* 10<sup>3</sup> cells/ml) where they can metabolise residual sugars producing phenolic off-flavours compounds, such as 4-ethylphenol.

In the present work we investigated *S. cerevisiae* and *D. bruxellensis* interactions during alcoholic fermentations and evaluated the effectiveness of antimicrobial peptides secreted by *S. cerevisiae* to prevent growth of the main wine spoilage yeast and the production of 4-ethylphenol. Several fermentations were performed with single cultures of *D. bruxellensis* and mixed cultures of *S. cerevisiae* and *D. bruxellensis*, both in synthetic grape juice (SGJ) and grape must. Yeast growth (culturability and viability) and fermentation performance (i.e. sugars consumption, ethanol and 4-ethylphenol production) of those fermentations was accessed by different methods, namely by fluorescence *in situ* hybridization and flow cytometry. Results showed that *S. cerevisiae* significantly reduced the growth of *D. bruxellensis* and the production of 4-ethylphenol both in SGJ and grape must fermentations performed with mixed cultures. Moreover, our work also showed that antimicrobial peptides secreted by *S. cerevisiae* are effective to prevent growth of *D. bruxellensis* and production of phenolic off-flavor compounds in wine.

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## Food Microbiology & Biotechnology

P064

### SUITABILITY OF BUTTERMILK AS CULTURE MEDIUM TO SUPPORT THE SURVIVAL OF PROBIOTIC STRAINS

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Buttermilk is the liquid phase released during churning of cream in the process of butter making. This liquid phase contains most of the water-soluble components of cream. Buttermilk finds applications in various food products. This subproduct represents an important volume for the dairy industry and a way to use it and give it value is to study its suitability to support the growth of probiotic strains in order to use it to formulate synbiotic dairy drinks. With this idea in mind, in this work we proposed to evaluate the growth and survival of several probiotic strains, namely *Lactobacillus paracasei* L-26, *Lactobacillus acidophilus* L-10, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* 01, *Lactobacillus casei* 431, *Lactobacillus plantarum* 226v PROBI AB, *Lactobacillus brevis* D24, *Bifidobacterium lactis* B94 e *Bifidobacterium lactis* BB12 when inoculated in buttermilk at 2% inoculum. Growth was performed at 37 °C for 24 h. Based on the results obtained, we carried out a further study to determine the optimal conditions to formulate a synbiotic drink where fructooligosaccharides were used as prebiotic compounds. Results achieved enable us to conclude that buttermilk is a suitable matrix to maintain the viability of probiotic strains and to formulate a drink with appropriate sensory properties. The use of buttermilk has a double benefit, on the one hand we eliminate an important sub product and on the other hand we obtain an added-value product that can be marketed.

## Food Microbiology & Biotechnology

P065

### SURVIVAL OF A PROBIOTIC CULTURE INCORPORATED INTO ORANGE JUICE AFTER SPRAY-DRYING

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Probiotic bacteria such as lactic acid bacteria (LAB) have been linked to several health benefits to humans, especially in the prevention of intestinal disorders. The production of a beverage, such as orange juice, would be more valuable with a probiotic culture incorporated.

The objective of this work was to spray-dry orange juice incorporating a probiotic (*L. plantarum* deposited in ESB Culture Collection) and assessing survival after drying and during storage. The probiotic culture was grown in a culture medium with one of 5 different sugars (glucose, fructose, sucrose, sorbitol and lactose), incorporated into orange juice, and the mixture dried by spray-drying. As a control, the probiotic culture was incorporated into skim milk and all experiments were done in triplicate. The powders obtained were stored at two different water activities ( $a_w$ ) – 0.06 and 0.11, in the presence/absence of light and also at two temperatures – room temperature and 4 °C.

After 90 days of storage, there was no log reduction of the probiotic dried in skim milk for all the storage conditions. For all the sugars used in the growth of the probiotic culture, the log reductions were minimal at a temperature of 4 °C and  $a_w=0.06$ . Similar results were obtained at room temperature, in the absence of light, with the exception of the probiotic grown in the medium with sucrose. In the case of storage conditions of  $a_w=0.06$  at room temperature, in the presence of light, except for the probiotic cultures grown in the presence of glucose or lactose, the organism were reduced to below the level of detection limit of the technique in 90 days of storage.

As a conclusion of this study, differences were obtained in the survival of probiotic culture, dried in orange juice by spray-drying, considering the different storage conditions and the various sugars incorporated in the culture medium for its previous growth.



## P066

## SYNERGISTIC EFFECTS BETWEEN WILD MUSHROOM EXTRACTS AND STANDARD ANTIBIOTICS AGAINST MULTI-RESISTANT BACTERIA

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The indiscriminate use of antibiotics and chemotherapeutic agents, among other factors, has been contributing for the development of resistant species (Andrade et al. 2006). Bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and Extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* are microorganisms of concern with regard to multi-resistances. Due to this problem, there is a need to investigate new strategies to reverse this tendency in order to achieve the appropriate and effective treatment against infections by such microorganisms. Natural matrices, in particular wild mushroom extracts emerge as interesting possibilities to be explored as antimicrobial drugs (Alves et al. 2012a).

Our previous results proved the antibacterial activity of extracts from specific wild mushrooms (*Fistulina hepatica*, *Leucopaxillus giganteus*, *Mycena rosea*, *Russula delica*, *Sarcodon imbricatum*) (Alves et al. 2012b). Those extracts were applied upon different multi-resistant microorganisms (*Escherichia coli*, Extended-spectrum beta-lactamase-producing (ESBL) *Escherichia coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA), combined with commercial antibiotics (penicillin, ampicillin, amoxicillin/clavulanic acid, cefoxitin, ciprofloxacin, cotrimoxazol, levofloxacin). The main objective was to evaluate the capacity of natural extracts to potentiate the action of standard antibiotics, through synergisms that allow a decrease in their therapeutic doses and ultimately contribute to the reduction of resistances. Microdilution method was used to determine minimum inhibitory concentrations (MICs).

The results obtained showed higher synergistic effects against MRSA than against *E. coli*. *Mycena rosea* and *Fistulina hepatica* were the best mushroom extracts for synergistic effects against MRSA. The efficiency of *Russula delica* extract against *E. coli* 1 (resistant to ampicillin, ciprofloxacin and trimethoprim/sulfasoxazole) and *E. coli* 2 (resistant to amoxicillin/clavulanic acid and ampicillin) was higher than that of *Leucopaxillus giganteus* extract; nevertheless the latter extract exhibited better synergistic effects against ESBL *E. coli*.

This study shows that, similarly to plants, some mushroom extracts can potentiate the action of antibiotics extensively used in clinical practice for Gram-positive or Gram-negative bacteria, with positive action even against multi-resistant bacteria. Therefore, mushroom extracts could decrease therapeutic doses of standard antibiotics and reduce microorganism's resistance to those drugs.

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## Food Microbiology & Biotechnology

P067

### THE ANTIMICROBIAL EFFECT OF PULSED ELECTRIC FIELDS (PEF) AGAINST *BRETTANOMYCES/DEKKERA* YEASTS

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PEF is known to provoke a dramatic increase in cell membrane permeability caused by externally applied short and intense electric pulses (1). One possible application of PEF is on the microbiological control of food by the inactivation of pathogenic and spoilage organisms in food matrixes including liquids (2). Puertolas et al (3) applied this technology on several wine spoilage microorganisms. *Brettanomyces / Dekkera* are well known to be involved in the production of volatile phenols in wines imparting aroma defects that may affect wine quality (4). The monitoring and control of these organisms is of major importance for wine producers. This work aimed to evaluate the effect of PEF on the survival of *Brettanomyces / Dekkera* in a continuous system, taking into account the energy efficient needed for industrial scale. Cell suspensions prepared by diluting YM culture medium with sterile water (1:10) were submitted to the following PEF parameters: electric field 10 kV/cm, pulse width 50 µs, pulse frequency 50 Hz, pulse current 13 A, using a EPULSUS-PM1-10<sup>®</sup> modulator. A cell inactivation effect was observed leading to a reduction of 3 logarithmic cycles of the initial population in 2 min for both strains tested (*Dekkera bruxellensis* PYCC 4801 and *Dekkera/Brettanomyces* 2). When only distilled water was used as cell suspension medium exposed to 100 µs, 100 Hz and 3 A of pulse width, frequency and current respectively, for 5 min, cell survival was almost unaffected. The electroporation medium composition and the way it influences properties such as medium conductivity seems to play an important role on the efficiency of the method. This is a preliminary study approaching for the first time the application of a continuous PEF system to wine spoilage organisms. Future work will focus on the influence of PEF parameters, medium composition and strain variability on PEF efficiency, along with the minimum energy needed for the microbial inactivation.

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## Food Microbiology &amp; Biotechnology

P068

## TOXICOLOGICAL EVALUATION AND HEPATOPROTECTIVE EFFECT OF AMANITA PONDEROSA

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Edible mushrooms, moreover to their gastronomic characteristics, are a source of valuable nutrients and bioactive compounds with potential beneficial effects on human health. Currently mushrooms become attractive as functional foods due to medicinal properties and useful in preventing diseases shown antitumoral and immunomodulating properties, cardioprotective, hepatoprotective, antibacterial, antiviral and antifungal effects. In the near future, could play an important role as a nutraceutical and/or therapeutic agents [1, 2].

The southern of Portugal, namely in Alentejo region, due to its Mediterranean microclimate and flora diversity, is one of the European regions with a high predominance of wild edible mushrooms *Amanita ponderosa*. Although there are many studies about the beneficial properties of constituents and some edible wild mushrooms, there are no studies on the biological activity of *A. ponderosa* [3].

The main objective of this study was to evaluate the hepatoprotective activity of fruiting bodies and mycelia of liquid cultures of some strains of *A. ponderosa*. Assay was performed using *Wistar* rats as an experimental model and serum enzymatic activities, ALT, AST, ALP and GGT were determined. Toxicological evaluation was assayed in *Artemia salina* (LC<sub>50</sub>) and in *Swiss* mice (LD<sub>50</sub>), according to OECD guidelines [4].

The lyophilized fruiting bodies and mycelia showed no lethality against *brine shrimp* and also no acute toxicity to *Swiss* mice. Additionally the oral administration of *A. ponderosa* samples in *Wistar* rats reduce the liver damage induced by ethanol, with a significant decrease of hepatic serum enzymes. This result was equivalent to the observed for silymarin, used as standard drug, showing a hepatoprotective effect of *A. ponderosa*.

Therefore, mushrooms and mycelia of liquid cultures of *A. ponderosa* may be an important source of bioactive compounds with potential medicinal value, as co-adjuvants in treatment of hepatic diseases.

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## Food Microbiology & Biotechnology

P069

### VIABILITY THROUGHOUT STORAGE OF POTENTIAL PROBIOTIC STRAINS, WHEN IN CONTACT WITH FRUIT PULPS

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Production of functional food products has been, over the past years, a trend in the food industry. Functional foods are foods that, besides the nutritional value, promote beneficial effects in one or more functions of the human organism. The beneficial effects can be promoted by several different components of the food products, like antioxidant compounds, prebiotics, probiotics, among others. Probiotics are “live microorganisms, which when consumed in adequate amounts, confer a health effect on the host”. A daily intake of  $10^8$  colony forming units (CFU) of probiotic bacteria has been suggested for a beneficial effect to be observed. The majority of the food products containing probiotics have been dairy and dried products, since they provide a favourable environment for the maintenance of viability. However, other food products have been tested/studied as vectors for probiotic delivery. Among such products are fruit based products such as juices. In this research work, the viability throughout storage of 6 potential probiotic strains (4 *Leuconostoc* and 2 *Lactobacillus*) isolated from different fruits, when in contact with fruit pulps, was studied. Eleven fruit pulps were inoculated with each of the probiotic strains and stored at 37 °C for 7 days, and at 4 °C for 28 days, and viability was assessed. Results showed that, when the pulps were stored at 37 °C, after 7 days, only avocado was capable of sustaining viable cell numbers high enough for the production of a functional food product. Storage at 4 °C showed, as expected, much better results. In almost every fruit pulp, every potential probiotic strain presented, after 28 days, high survival percentages, and some fruit pulp/probiotic strain combinations presented percentages above 95%. These results show that storage at 4 °C is, between the two temperatures tested, the most adequate for maintenance of probiotic viability. Results also showed that fruit pulps, when stored at refrigerated temperature, are capable of sustaining probiotic viabilities in the levels needed for the production of functional food products.

## Food Microbiology & Biotechnology

P070

### VIRULENCE TRAITS AMONG AEROMONAS ISOLATED FROM WATER, FOOD AND FOOD-RELATED ENVIRONMENTS

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*Aeromonas* are recognized emerging pathogens responsible for a variety of infectious diseases in humans, gastroenteritis being the most common clinical manifestation. *Aeromonads* ubiquitous nature, and their presence in water and raw/unprocessed food, leads to the assumption that the majority of *aeromonas*-zoonoses are waterborne, with the main risk of acquiring infections being by consumption of contaminated water or food products.

The pathogenesis of *aeromonads* is multifactorial and poorly understood, but the presence of virulence traits has already been reported in environmental and food isolates; turning the safety of such niches/products a matter of concern. Hence, the present investigation aimed to assess the pathogenicity potential of 64 *aeromonads* isolated from water, food and food-related samples at cheese factory, a slaughterhouse and a supermarket; for comparison purposes 25 clinical strains were also analyzed.

The dissemination of virulence factors among the *Aeromonas* spp. was assessed by phenotypic assays, PCR-amplification and dot-blot hybridization under high-stringency conditions. Results showed that the isolates have high ability to produce extracellular enzymes and toxins, namely DNases (86%), lipases (61%), proteases (93%) and hemolysins (99%); with the comparison between non-clinical and clinical isolates showing no statistically significant differences ( $p < 0.05$ ). Furthermore, molecular screening revealed a high prevalence of virulence determinants: *ahyB* (elastase-81%), *alt* (heat-labile cytotoxic enterotoxin-88%), *ascV* (structural protein of Type III Secretion System-85%), *ast* (heat-stable cytotoxic enterotoxin-91%) and *lip* (lipases/phospholipase-86%); while lower prevalences were observed for *act/hlyA* (cytotoxic enterotoxin/hemolysin-73%), *aexT* (ADP-ribosylating toxin of Type III Secretion System-61%) and *flaA/B* (flagellin A/flagellin B-66%). The genes *flaA/B* and *lip* were significantly more prevalent in non-clinical isolates, probably due to niche-specific adaptations.

In conclusion, our results showed a high dissemination of putatively pathogenic *aeromonads* in the environments under investigation. *Aeromonas* spp. carrying distinct combinations of genetic determinants for enterotoxins, hemolysins and other traits associated with adherence, tissue invasion and spread in the host were identified in non-clinical *aeromonads*; pointing towards the need for a more accurate monitoring of the presence of this underestimated pathogen in food and water.

## Food Microbiology & Biotechnology

P071

### WINE AUTHENTICITY – SSR MARKERS FOR MUST AND WINE VARIETAL DETECTION

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Improved quality control in the wine industry demands for the possibility to identify grape varieties in wine. DNA markers have become powerful tools used for cultivar identification, conducted with genomic DNA that is easily extracted from leaves. However, it is rather difficult to obtain high quality DNA from must and wine samples. The main problems are the low DNA concentration extracted, the low reproducibility of DNA analysis and the DNA degradation during vinification processes. The aim of this work was to test two DNA extraction methods developed by our teams [1, 2]. SSR analysis was evaluated in terms of quality and reproducibility between 2 different laboratories. Experimental microvinifications were performed from white and red grapes. High quality DNA enabling PCR amplification for SSR sequences was achieved. Cultivar identification could be performed based on the analysis of the six nuclear microsatellites (SSR) recommended by OIV (VVMD5, VVMD7, VVMD27, VvZAG62, VvZAG79, and VVS2). The SSR molecular markers can be efficiently used for must and wine varietal identification for quality control, certification and traceability purposes protecting consumers against misleading information as well as assuring an authentic advertising and a fair trade.

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## Food Microbiology & Biotechnology

P072/F03

### IMMOBILIZATION OF LIPOXYGENASE INTO CELLULOSE DERIVATIVES FOR MYCOTOXINS DETECTION IN FOOD

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A simple and effective method for mycotoxins detection in food, *e.g.* by using a biosensor, is welcome. Aflatoxins are particularly toxic and carcinogenic. The enzyme lipoxygenase (E.C. 1.13.11.12) enables the bioactivation of aflatoxins, and in the presence of linoleic acid it converts the aflatoxin B<sub>1</sub> into epoxide, which can be measured by spectroscopy. The covalent immobilization of lipoxygenase into polymeric matrices is an essential step. The use of cellulose derivatives as support is promising because they are inert materials, biocompatible and allow modeling their hydrophilic/hydrophobic properties.

Cellulose acetate (CA), cellulose acetate butyrate (CAB) and ethyl cellulose (EC) were prepared and used as membranes. The lipoxygenase was covalently linked to these membranes, previously activated: *a*) by sodium periodate (NaIO<sub>4</sub>); *b*) by (NaIO<sub>4</sub>), insertion of an extensor arm of hexamethylene diamine (HMD), followed by activation of amine groups with glutaraldehyde. The immobilization process was optimized for 3 factors: i) NaIO<sub>4</sub> concentration; ii) contact time and iii) concentration of lipoxygenase. The lipoxygenase activity (free and immobilized) was evaluated by the velocity of linoleic acid peroxide release in the hydroperoxidation reaction of linoleic acid with O<sub>2</sub>, by spectrophotometry at 234 nm, in maximum activity conditions of lipoxygenase (25°C e pH 9.0). The protein content of immobilized lipoxygenase was estimated by Bradford assay.

The best activity was 16.1 U/mg<sub>support (dry basis)</sub> for CAB activated with NaIO<sub>4</sub> (1 M) for 3 h, the insertion of an extensor arm of HMD (1% (w/w)), and activated with glutaraldehyde (5% (v/v)), followed by the immobilization of lipoxygenase (20 000 U/mL) in phosphate buffer (0.1 M), pH 7.4. Under these conditions the enzymatic activity retention after immobilization was 35%. The kinetic parameters for the immobilized and free enzyme were  $K_m = 2.8 \mu\text{mol/mL}$ ,  $V_{\text{max}} = 94.4 \text{ U/mg}_{\text{lipoxygenase}}$  and  $K_m = 2.3 \mu\text{mol/mL}$ ,  $V_{\text{max}} = 272.7 \text{ U/mg}_{\text{lipoxygenase}}$ , respectively. Immobilization did not affect enzyme affinity to the substrate. Shelf stability at 9 °C of immobilized lipoxygenase was much greater than of free enzyme after 7 days (82% *vs* 6%). Contact angle goniometry and water sorption capacity assays have shown that CA membranes are more hydrophilic for lipoxygenase immobilization. Also this characteristic increases with the activation procedures.

## Food Microbiology & Biotechnology

P073/F04

### FOOD SAFETY EVALUATION FOR CASSAVA AND YAM PRODUCERS IN NIGERIA

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Cassava and yam are two of the most important crops in Nigeria; their cultivation and processing can affect the lives of millions of people. Both crops are produced and sold either by SMEs or in household environments.

Here we report a preliminary baseline assessment for food safety in the processing and sale of cassava and yam-derived products.

Sixteen producers, both SMEs and households, were visited and the possible elements of risk for safety were evaluated. Products such as high quality cassava flour, gari fufu (cassava-derived) and gbodo and elubo (yam derived) were considered in the assessment. Some of the elements considered were microbial contamination (with pathogen bacteria) and chemical pollution (pollutants in the water, from the workers and cyanide compounds in the case of cassava). Moreover, the safety level in 120 food street vendors was also examined.

Results showed that SMEs have a higher awareness level on food safety than the household producers; one reason for this is that people working in household producers do not have any training in food safety. At the same time, however, often for logistic reasons the production area is close to the living area; consequently the layout of the production line may not conform to the prerequisites required by proper food safety programme.

These results showed that to improve the safety level it would be crucial to give a proper training to the people involved in the processing of these crops and, at the same time, to implement some good manufacturer and hygiene practices according to the needs.



## Food Microbiology & Biotechnology

P074/F28

### TRIAL PRODUCTION OF DIABETIC BISCUITS WITH REDUCED ACRYLAMIDE LEVEL

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A special production of biscuits dedicated for diabetics by replacement of saccharose with fructose is associated with more intense formation of undesirable acrylamide – food-borne contaminant releasing from the reaction of amino acid asparagine and reducing sugars. Especially fructose is a strong activator of acrylamide formation. Keeping acrylamide below an indicative value, which is set at 500 µg.kg<sup>-1</sup> for this category, the Slovak producer of biscuits tried to incorporate the enzyme L-asparaginase treatment of dough before baking in order to prevent biscuits from acrylamide formation. The trial was successfully completed in the production of three kinds of diabetic biscuits enriched with folic acid in which acrylamide was reduced up to 50%. L-asparaginase was applied as an ingredient of flour while no other changes (time/temperature adjustment, pH setting, ammonium salt substitution, prolongation of dough making, dilution etc.) were introduced into the processing. The aforementioned settings could potentially result in alterations of organoleptical properties, increasing costs or lowering effectiveness of biscuit production. Otherwise, enzymatically treated products were not significantly altered neither in appearance, sizes, colour, taste, smell, nor texture.

This approach can be applied also in a novel biscuit processing exploiting valuable residues from coffee and wine production which are associated, besides their health beneficial effect, with increasing acrylamide level. Based on our successful Slovak-Portuguese laboratory trials, the incorporation of these residues in experimental samples is promising and its scale-up to the industrial production is a challenge for an attractive innovation of the portfolio.

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## Industrial Microbiology & Biotechnology

P075

### BIOPLASTIC PRODUCTION FROM CHEESE WHEY WASTEWATER

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The current emphasis of sustainability brings a growing interest for the research and production of bioplastics. Polyhydroxyalkanoates (PHA) are biopolymers with physico-chemical properties similar to polypropylene but with the advantage of being biodegradable and biocompatible.

These biopolymers are produced from non-fossil feedstocks and involve microbial cultivation of non-toxic waste, unlike the ones resulting from petroleum-based materials.

The proposed work aims to develop and evaluate a three-step process for polymer production using microbial mixed cultures (MMC) fed with cheese whey and their downstream process.

In the first step an acidogenic fermentation process was developed in a MBBR in order to produce volatile fatty acids (VFA) from cheese whey. These VFA serve as substrates for aerobic bacteria in SBR where the selection of cultures with high storage capacity occurred under a “feast/famine” regimen (second step). The last step was a PHA accumulation batch process by the selected MMC from the VFA produced. Different extraction methods were tested to separate the intracellular PHA granules from biomass.

In the acidification step, the influence of three organic load rates (ORL) of 20, 30 and 50 gCOD/L.d was studied. Results showed that increasing values of ORL resulted in the increase of the content of VFAs in 110% from 20 to 50 gCOD/L.d. Regarding the VFAs products, the predominant acids were acetic and propionic acid at an OLR 20 gCOD/L.d, but higher ORLs resulted in the shift from propionic acid to butyric acid production. The observed decrease on pH values may be the explanation for that shift, since it favours the production of longer chain acids.

In a second stage, the selected cultures were obtained in the SBR operating at a 1.5 gCOD/L.d and a C: N ratio of 100:11. The chemical oxygen demand (COD) removal was 90±3% and the substrate was fully exhausted in 2 to 3 hours. The occurrence of PHA inside the selected microorganisms was confirmed through microscope observations with the Nile Blue staining.

Selected cultures were used for the PHA accumulation assay with fermented whey (about 30 Cmmol/L) under ammonia limitation. The results showed a maximum PHA content of 23% of cell dry weight. The PHA produced consisted of a co-polymer of hydroxybutyrate and hydroxyvalerate (PHB/HV) with molar composition of 87:13.

For the extraction methodology, three solvents were tested: chloroform, propylene carbonate (PC), and sodium hypochlorite. Results showed process yields of 95% for chloroform method and 60% for PC methodology. The sodium hypochlorite did not allow for polymer extraction.

The process presented in this work showed that wastewater components can be used as a viable solution to produce biopolymers by using simple wastewater treatment technologies.

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## Industrial Microbiology & Biotechnology

P076

### CELLULASES AND HEMICELLULASES PROFILE OF *KITASATOSPORA* SP. ISOLATED FROM THE GUT OF THE MILLIPEDE *TRIGONIULUS CORALLINUS* (GERVAIS)

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The ability decomposing of invertebrates saprophytes the soil fauna results from the capacity to associate with microorganisms. This interaction allows saprophages millipedes to feed on lignocellulosic wastes, degraded by enzymes provided by their symbionts. The objective of the present study was to detect, identify and characterize the cellulolytic enzymes from Actinomycetales isolated directly from the gut of the millipede *Trigoniulus corallinus* (Gervais). From the isolated strains, 3 selected strains highly cellulitic, identified as *Kitasatospora* sp., were grown in residues coming from the waste water treatment plant from one industry of production of paper and pulp (Primary and Biological residues). In order to detect the cellulolytic and hemicellulolytic activity of the selected strains grown in the residues, supernatants were collected, concentrated dialyzed and analyzed by SDS-PAGE and zymography and finally the protein content was measured. Zymograms were performed with 0,05% Carboxi-metil-cellulose sodium for cellulases (CMCases) profiling, and with 0,05% locust bean gum for hemicellulases (mannanases) profiling. Nitrogen supplementation of the residues increased the hemicellulolytic activity of the strains without changing the profile of hemicellulases. The hemicellulolytic peak of activity vary between strains and was detected after fifteen days of incubation for strain C, and seventeen days for strains A and B. A lagged was observed between the maximum protein content obtained for each strain during cultivation and the highest hemicellulolytic activity of the strains, except for strain A which achieved the maximum protein and hemicellulolytic activity after 15 days incubation on the waste waster primary residue. Cellulases profile of strain A was defined by four bands. However, a complex profile was observed when considering the functional group of mannanases. Seven defined bands of mannanases were observed, with molecular weight between 20 and 96 KDa. These results suggest that these hemicellulolytic strains are potential sources of enzymes with high potential to be use in biotechnological processes, to generate byproducts for bioenergy production.

## Industrial Microbiology & Biotechnology

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### COMBINED EFFECTS OF NITROGEN AVAILABILITY AND CO-FERMENTATION WITH *HANSENIASPORA GUILLERMONDII* ON WINE AROMA PROFILE

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Non-*Saccharomyces* yeasts include different species which comprise an ecologically and biochemically diverse group capable of altering fermentation dynamics and therefore wine composition and flavour. In this study, single- and mixed-culture of *H. guilliermondii* and *S. cerevisiae* were used to ferment natural grape-juice, under two nitrogen regimes. In single culture the strain *H. guilliermondii* failed to complete total sugars breakdown even though the nitrogen available has not been a limiting factor of its growth or fermentative activity. In mixed culture, that strain negatively interfered with the growth and fermentative performance of *S. cerevisiae*, resulting in lower fermentation rate and longer fermentation length, irrespective of the initial nitrogen concentration. However, this co-inoculation had a positive impact on the volatile profile of the wines, particularly of those obtained from DAP-supplemented musts. The data obtained suggest that the strain *H. guilliermondii* used herein has potential to be used as adjunct of *S. cerevisiae* in wine industry, although possible yeast interactions still need to be elucidated.

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## Industrial Microbiology & Biotechnology

P078

### COMMERCIAL YEAST STRAINS STRESS TOLERANCE VARIATION TO SEVERAL INDUSTRIAL RELEVANT INHIBITORS

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<sup>1</sup>UTAD

In the fermentation industry, *Saccharomyces cerevisiae* is exposed to multiple stresses (high concentrations of sugar and ethanol, low pH, temperature fluctuation, potential toxic metabolites, among others). Therefore, yeast strains tolerant to all these agents are potentially interesting for alcoholic beverage production and for other fermentation-related industrial processes. In this study, a set 20 *S. cerevisiae* yeast strains from different geographic provenance was phenotypically screened against the following stresses: temperature variation (up to 40 °C), SO<sub>2</sub> (up to 360mg/l), ethanol (up to 15%), acetic acid (up to 150 mM), salinity (up to 2 m), H<sub>2</sub>O<sub>2</sub> (up to 2.5mM), as well as resistance to cerulenine (6µM) and TFL (1mM). Cells in exponential-growth-and three ten-serial dilutions were spotted onto YPD agar plates with and without the appropriate stress agent, followed recorded as the colony area after 4 days incubation. Yeast strains were grouped in five distinct clusters uncovering a significant phenotypic diversity. Some of the strains displayed very useful biotechnological features such as high resistance to temperature variation and oxidative shock, as well as moderate resistance to acetic acid. Moreover, fermentation performance was evaluated in eight yeast strains representative of each distinct cluster. The results showed no direct correlation between high tolerance to stress and better fermentative fitness. Overall, our results reinforce the need of using an integrated approach to select more suitable strains for a particular industrial process.

This work was supported by FEDER through COMPETE (FCOMP-01-0124-FEDER-014043) and by national funds by FCT through the project PTDC/AGR-ALI/111224/2009. CB is a recipient of a PhD grant from FCT (SFRH/BD/61881/2009).

## Industrial Microbiology &amp; Biotechnology

P079

DISTINCT BEHAVIOR BETWEEN MULTI-ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* STRAINS TOWARDS REACTIVE OXYGEN SPECIESVera M. Sousa<sup>1</sup>; Adélio Mendes<sup>1</sup>; Célia M. Manaia<sup>2</sup>; Olga C. Nunes<sup>1</sup>

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Antibiotic resistant bacteria have been implicated in a large number of nosocomial infections. This worldwide problem drew the attention to the development of new disinfection techniques, such as photoinactivation. During photocatalysis, the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radicals (•OH) and superoxide radical (O<sub>2</sub><sup>•-</sup>) are supposed to occur. Hence, photocatalysis has been used to inactivate organisms, through ROS attack. However, several studies reported that in *Escherichia coli* an increased tolerance to oxidative stress induced by ROS may occur, and that it may be related to antibiotic resistance. In this study, the susceptibility to photoinactivation of two antibiotic resistant *E. coli* environmental strains (A5EL5 and E5EL20) belonging to the same phylogenetic group and with similar antibiotic resistance phenotype was assessed.

After 40 min of UV/TiO<sub>2</sub> exposure, viability losses of 44.2 % and above 99.0 % were recorded for strains A5EL5 and E5EL20, respectively. Based on the hypothesis that these two strains have distinct tolerance to different ROS generators, the ROS formed after contact with H<sub>2</sub>O<sub>2</sub>, KO<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> + Fe<sup>2+</sup> (Fenton reaction) were quantified using a fluorescence method. The probe 2', 7'-dichlorohydrofluorescein diacetate (DCFH-DA) is widely used to measure the ROS content in cells. After hydrolysis by cellular esterases, DCFH is subsequently oxidized by ROS to highly fluorescent DCF. After 60 min of contact with DCFH-DA, cells were incubated in the presence of H<sub>2</sub>O<sub>2</sub> or KO<sub>2</sub> for O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> + Fe<sup>2+</sup> (Fenton reaction) for •OH. Fluorescence units were measured in the crude cell extracts and converted into ROS concentration and the data was normalized by total protein content. Significant differences (p<0.05) between ROS content in A5EL5 and E5EL20 crude cell extracts were found for H<sub>2</sub>O<sub>2</sub> (1.4x10<sup>-5</sup> ± 2.9x10<sup>-6</sup> and 1.1x10<sup>-4</sup> ± 1.1x10<sup>-6</sup> μmol ROS.μg<sup>-1</sup> total protein, respectively) and Fenton reaction (9.6x10<sup>-5</sup> ± 5.6x10<sup>-6</sup> and 1.5x10<sup>-4</sup> ± 5.2x10<sup>-6</sup> μmol ROS.μg<sup>-1</sup> total protein, respectively). No significant difference was found for KO<sub>2</sub>. These preliminary tests, suggest that, in fact, the different tolerance to photocatalysis of the environmental *E. coli* may be related to oxidative stress response, but not to antibiotic resistance.

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## Industrial Microbiology & Biotechnology

P080/F24

### ELECTROINDUCED RELEASE OF RECOMBINANT LACCASE FROM *SACCHAROMYCES CEREVISIAE* BASED ON CELL ELECTROPERMEABILIZATION

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Laccases are copper-containing oxidase enzymes that are found in many plants, fungi, and microorganisms. They have the potential to cross link food polymers such as proteins and non starch polysaccharides in dough, thus provoking changes in dough properties. Laccases play an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant and removal of endocrine disruptors. These enzymes are used for pulp delignification, pesticide or insecticide degradation, organic synthesis. The darkening and the cloudiness are quality defects in beer. Laccase can be added to the wort or at the end of the process to remove the polyphenols that are responsible for these defects.

Laccases secreted from native sources are usually not suitable for large-scale purposes, mainly due to low production yields and high cost of downstream processing. Heterologous expression may provide higher enzyme yields and may permit to produce laccases with desired properties (such as different substrate specificities, or improved stabilities) for industrial applications.

Recombinant laccases from different sources have been expressed in *Saccharomyces cerevisiae* as secreted proteins. The reported yields differ greatly, ranging from 0.035 mU to 1.1 U per milliliter growth medium. The following procedures, applied for concentration and purification of the enzyme could lead to a significant reduction of the recovery yield.

*Bacillus subtilis* endospore coat protein CotA (65 kDa) showing laccase activity was expressed as intracellular protein in *S. cerevisiae* W303 – 1A [pMAB39–CotA–9]. Pulsed electric fields (PEF) have been applied in order to increase the membrane permeability, thus facilitating the release of proteins. Cell membranes were irreversibly permeabilized by application of monopolar square wave pulses with field intensities in the range of 5–6 kV/cm and pulse duration of 0.8 – 1 millisecond. The electric treatment was performed in continuous flow system at flow rate of 6 ml/min. Immediately after pulsation cells were diluted in potassium phosphate buffer with pH=5–8,5. At definite intervals of incubation, the cells were centrifuged and the activity of released laccase was determined in the supernatant. The results showed that the enzyme was retained inside the cell at low pH, and efficiently liberated at pH over 7. The enzyme is stable for at least 14 days in buffer without protease inhibitors. The specific activity of laccase liberated from electroporated cells is about eightfold higher than that in cell lysate obtained by mechanical disintegration. An interesting observation was that the efficiency of electroinduced enzyme release depends strongly on the composition of cultivation medium. The recovery yield (up to 0.5 U/ml growth medium) is comparable with the most efficient *S. cerevisiae* systems, designed for secretion of laccases of different origin.

The data obtained let us to assume that similar protocol of electroporation and postpulse incubation could be successfully applied for recovery of other recombinant enzymes from *S. cerevisiae*.

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### ENHANCED OIL RECOVERY UNDER SIMULATED RESERVOIR CONDITIONS USING AN INDIGENOUS *BACILLUS SUBTILIS* STRAIN

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Microbial Enhanced Oil Recovery (MEOR) is potentially useful to increment oil recovery from reservoirs beyond primary and secondary recovery operations using microorganisms and their metabolites. *In situ* stimulation of microorganisms that produce biosurfactants and degrade heavy oil fractions reduces the capillary forces that retain the oil inside the reservoir and decreases oil viscosity, thus promoting its flow and increasing oil production. *Bacillus subtilis* #573, isolated from crude oil samples obtained from a Brazilian oil field with a moderate temperature (40°C), was selected for further use in MEOR. This isolate can grow at temperatures up to 55°C and salinities up to 100 g/l, and produces extracellular biosurfactants under both aerobic and anaerobic conditions in the presence of hydrocarbons. The biosurfactants produced reduced the surface tension to 30 mN/m, decrease the interfacial tension oil–water and exhibited a high emulsifying activity, as well as thermo– and salt–tolerance. The microbial isolate also showed the ability of degrading long–chain *n*–alkanes under aerobic and anaerobic conditions. Mobilization of heavy crude oil by this isolate was evaluated using sand–pack columns at 40°C. Growing *in situ* *B. subtilis* #573 for 14 days allowed a 17% recovery of the entrapped crude oil. The recovered crude oil showed a decrease in the percentage of *n*–alkanes higher than C25 and its viscosity was reduced about 32%, which contributed to enhance its mobility. A core flooding equipment was used for a better simulation of the oil reservoir conditions (40°C and 32.4 bars). Preliminary results showed an additional oil recovery of 4%. The results obtained demonstrated that the selected isolate can be useful to recover residual oil from mature reservoirs.



## Industrial Microbiology & Biotechnology

P082

### EXPRESSION OF HETEROLOGOUS PROTEINS BY *PICHA PASTORIS* UNDER INCREASED AIR PRESSURE

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The *P. pastoris* expression system has gained acceptance as an important host organism for the production of heterologous proteins. Its expression system presents the advantage of the organism can grow to the desired cell density on glycerol as the carbon source and then on methanol for high level heterologous production. However, the oxygen limitation generally has a detrimental effect on the expression of foreign genes. The increased air pressure could be used to improve the oxygen solubility in the medium and to reach the high oxygen demand of methanol metabolism.

For the first time, two recombinant *P. pastoris* strains (KM71H/ pPICZ $\alpha$ A/frutalin and GS115/pPICZ/lacZ) producing frutalin and  $\beta$ -galactosidase, respectively, were used to investigate, the effect of increased air pressure on yeast growth and heterologous protein expression. Several trials were carried out in a stainless steel bioreactor (Parr 4563, Parr Instruments, USA) under total air pressure of 1 bar and 5 bar. The increase of air pressure up to 5 bar had a small effect on biomass production, but led to a 9-fold improvement in  $\beta$ -galactosidase specific activity compared to 1 bar. Also, the recombinant frutalin secretion was enhanced by the increased air pressure up to 5 bar.

The protease specific activity reached at 5 bar was 1.5 times lower than that obtained at 1 bar. This result revealed that the use of increased air pressure up to 5 bar provided optimal conditions for reduction of the proteolysis that occurred on frutalin secretion at atmospheric pressure.

## Industrial Microbiology & Biotechnology

P083

### IDENTIFICATION OF GENES INVOLVED IN THE RESPONSE OF *SACCHAROMYCES CEREVISIAE* AGAINST 2-PHENYLETHANOL

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The aromatic alcohol, 2-Phenylethanol (2-PE), with a rose-like odour, is widely applied in cosmetics, perfumery and food industries. So far has been mainly produced by chemical synthesis, using potential carcinogenic substrates. Due to increasing consumers' demand for natural flavours, the microbial transformation process has been receiving special attention as an alternative production method. Yeasts have been pointed to be the most prominent microorganism to carry out this process. Indeed, several yeasts species have proven to be able to produce 2-PE. The most efficient biotechnological approach is the growth associated bioconversion of L-phenylalanine (L-Phe) via the Ehrlich pathway. However, the drawback of this biocatalytic process is the toxic effect that it has on yeast cells demanding its removal from the fermentation medium rendering the production biotransformation process more intricate and costly. Therefore, the aim of this study was to systematically identify, using a chemogenomic approach, the genes required for *Saccharomyces cerevisiae* tolerance to 2-PE. To this end, the complete EUROSCARF haploid mutant collection (approximately 5100 mutants individually deleted for non-essential genes) was screened. For the phenotypic screening, deletion strains were grown in 96-well plates in YPD medium, for 12 h. Using a 96-pin replica platter, the cells were spotted onto the surface of solid YPD medium supplemented with 2-PE at final concentrations of 4, 4.75 and 5.5 g/L, and incubated at 30° C for 2 or 3 days, depending of the severity of growth inhibition. This screening uncovered a set of genes required for yeast resistance to 2-PE that could be used for the construction of more suitable strains for application in industrial production.

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## Industrial Microbiology & Biotechnology

P085

### MICROBIOLOGICAL EVALUATION OF CONTINUOUS WASHING SYSTEMS IN FLOOR MAT OF POULTRY PROCESSING

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The poultry processing involves a sequential set of different processing steps, many of which have great microbiological importance, in view of the quality in the final product. Microbiological analyzes are intended to monitor the hygiene of slaughterhouses, coating it is of great importance when trying to prove the effect of changes in work techniques or the use of new equipment. Through comparative analyzes on samples of products (frozen cuts of chicken – thighs and drumsticks) processed that passed through sanitary mats with and without a continuous washing system, there was, in the end, that products with higher microbial loads were from the treadmill that had the auto washing system. The data also showed that all samples remained accordance with the standards established by law. Microbiological analysis of the surfaces of the mats showed better performance on the treadmill without washing system and can observe statistical differences according to the Tukey test ( $p < 0.05$ ) when compared with the conveyor system.

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## Industrial Microbiology & Biotechnology

**P086**

### OLIVE MIL DRY RESIDUE VALORIZATION THROUGH BACTERIAL CELLULOSE PRODUCTION

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Cellulose is one of the most abundant natural polymers on Earth, most of which produced by plants. However cellulose can also be produced by microorganisms such as algae and bacteria. Bacterial Cellulose (BC), produced by several bacteria, and especially those belonging to the *Gluconacetobacter* genus, is a very peculiar form of cellulose with unique mechanical properties, due to its 3D nanofibrillar structure that can be exploited in numerous applications, namely in the biomedical field. Bacterial cellulose presents high purity, since it is not associated with other components (has hemicelluloses and lignin in plants cellulose), and shows high water absorption capacity and excellent mechanical properties. However, the production costs of BC are very high because of the use of quite expensive culture media. Following the interest in searching for more economically viable conditions to produce BC, namely using agro-forest residues as carbon and nutrients sources, in this work the possibility of using residues from the olive oil production industry as carbon and nutrients source for the production of BC by *G. sacchari* was evaluated. The dry olive mill residue (DOR) was submitted to water extraction at 40 and 100°C (DOR40 and DOR100) and to hydrolysis with H<sub>2</sub>SO<sub>4</sub> 1M (DOR100H) in order to obtain sugar rich aqueous extracts to be used in BC production. The sugars present in DOR40 and DOR100 were glucose, fructose and xylose, while in DOR100H, arabinose was also detected, accounting for ~11.8 gL<sup>-1</sup>. The amount of BC produced was 0.81 g/L from DOR40 and 0.85 g/L without nutrient supplementation. These results corresponded to 32 and 34 % of the production previously achieved with conventional and rich HS medium (around 2.5 g/L). In order to enhance the production of BC, the next step was the supplementation of DOR40 and DOR100 with N and P sources in order to overcome possible nutritional limitations. The presence of salts led to an increase on BC production between 21.5 % and 43.2% when comparing with the first tests. The obtained results seem to be promising to overcome high BC production costs.

## Industrial Microbiology & Biotechnology

P087

### PHA PRODUCTION FROM WASTES OF PULP INDUSTRY

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The increasing use of petroleum-based materials in modern lifestyle has raised several concerns. Among these materials, plastics are the most important, since they are generally used around the globe for a wide variety of applications. Following several environmental concerns, bio-based and biodegradable polymeric materials, like polyhydroxyalkanoates (PHAs), may be among the most suitable alternatives.

The effectiveness of PHAs production process includes a first stage of selection of a mixed microbial culture (MMC) with a stable PHAs-producing capacity, which determines the success of subsequent PHAs accumulation step. This could be done using hardwood spent sulphite liquor (HSSL), which is a complex feedstock originated from the pulp industry and meets the concept of a lignocellulosic-based biorefinery. To complement the selection of the PHAs-storing populations, the evolution of microbial community must be evaluated in order to identify the best producers and their individual relative abundance, allowing the design of optimal operating.

In this project, a MMC collected in a wastewater treatment plant was submitted to ADF conditions in a SBR in order to select PHA-accumulating organisms using HSSL. The maximum PHA cell content obtained was 67.6 %. The GC analysis showed that the PHA produced was a homopolymer of 3-hydroxybutyrate. The microbial culture showed a clear preference for acetic acid (the main substrate for PHB production) but was also able to consume a small part of the lignosulphonates and some xylose. The identity of PHA-storing bacteria was obtained by FISH. Bacteria belonging to the three main classes were identified: *Alpha*- (72.7 ± 4.0 %), *Beta*- (11.1 ± 0.37 %) and *Gammaproteobacteria* (10.3 ± 0.3 %). PHA accumulators were not affiliated to species already known and described so far. Pure cultures of PHA accumulating bacteria were also obtained and analyzed for their storage capabilities and application potentialities.

## Industrial Microbiology & Biotechnology

P088

### PRELIMINARY CHARACTERIZATION OF HYDROPHOBINS FROM A *BJERKANDERA* SP.

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Hydrophobins are fungal proteins that have the ability to change the hydrophobic or hydrophilic nature of a surface once they self-assemble into an amphipathic membrane at the surface interface. Their potential for industrial applications is high and since their discovery in a *Schizophyllum commune* isolate, hydrophobins from different species have been isolated and sequenced.

As a general characteristic they present similar hydropathy patterns, high percentage of cysteine residues but low sequence homology even within same genera.

Three strains, two fungal unidentified isolates and *Bjerkandera* sp. BOS55 from the laboratory collection were tested for hydrophobin production. Fungal ITS amplification and sequencing allowed the identification to species of the isolates, respectively, *Mucor circinelloides* and *Trichoderma harzianum*. Hydrophobins from all three strains were produced, extracted and preliminarily characterized.

A never described antimicrobial activity, as well as biofilm formation inhibition for *Candida albicans* was detected for hydrophobin solutions. Further more, isolation and preliminary characterization of hydrophobins from *Bjerkandera* sp. is also described for the first time. Purification and isolation of these proteins was performed by HPLC and MALDI-TOF sequence characterization is under way.

## Industrial Microbiology & Biotechnology

### P090

#### PRODUCTION OF BIOPLASTICS BY *CUPRIAVIDUS NECATOR*: APPLICATION OF THE BIOREFINERY CONCEPT TO THE VALORIZATION OF A WINERY BY-PRODUCT

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Polyhydroxyalkanoates (PHAs) are intracellular aliphatic polyesters produced and accumulated by microorganisms as carbon source, which are biocompatible, completely biodegradable, and synthesized from renewable resources. Also, as they exhibit thermoplastic properties similar to those of synthetic plastics, PHAs are considered a promising alternative to the petroleum-based plastics. However, the current PHA production is still expensive, and thus the use of inexpensive carbon sources like industrial by-products could make PHA production sustainable and profitable.

The aim of this work was the valorization of grape skins, a winery by-product whose aqueous extract is extremely rich in simple sugars, through the production of PHAs by the well-known producer *Cupriavidus necator*.

Batch tests were initially performed in defined medium supplemented with glucose (23.42 g/L) and mannose (2.17 g/L), which were indicated as the main sugars of the red grape skins aqueous extract in preliminary tests. Afterwards, experiments were conducted using grape skins aqueous extract supplemented with the defined medium components. For all tests the biomass and sugar concentration were evaluated along the experiments as well as polymer accumulation by fluorescent microscopy. *C. necator* was able to grow and consume sugars from grape skins aqueous extract showing a maximum specific growth rate of  $0.0323 \text{ h}^{-1}$  and a sugar consumption rate of  $0.0911 \text{ g}_S/(\text{g}_X \cdot \text{h})$ . Along the tests, it was verified microscopically the occurrence of PHA accumulation. Moreover, FTIR analysis revealed a polyhydroxybutyrate (PHB) characteristic pick profile, confirming the PHB nature of the film extracted from biomass. The PHB cell content was estimated and a value of 30.38% of cell dry weight was obtained.

These results proved that the red grape skins aqueous extract can be considered as a prominent substrate for *C. necator* growth and PHA production towards the valorization of this winery by-product, while lowering the cost requirements of the PHA production.

## Industrial Microbiology & Biotechnology

P091

### SACCHAROMYCES CEREVISIAE STRAIN IMPLANTATION IN INDUSTRIAL MIXED STRAIN WINE FERMENTATIONS USING SSR MARKERS

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The use of pure cultures of selected yeasts is actually a current oenological practice. Advances in molecular biology allowed the development of several techniques to identify yeasts at the strain level, enabling the study of population dynamics and starter strain implantation during fermentation. Nevertheless, industrial assays are scarcely reported.

This study aimed to evaluate the implantation of two commercial active dry yeasts, ADY1 (which is a *Saccharomyces cerevisiae* starter yeast used to initiate fermentation) and ADY2 (which is a *S. cerevisiae* used to prevent or treat stuck fermentations), added in a sequential mode (ES1) or in co-inoculation (ES2), in an industry scale winemaking process (250 hL). Three samples were collected during fermentation at days one (T1), nine (T9), and seventeen (T17). Microsatellite analyses (SSR markers) were used for strain differentiation.

The microsatellite loci analysis revealed seven profiles for ADY1 and three profiles for ADY2. This genomic diversity inside each ADY seems to result from re-arrangements of the same strain as previously reported to occur in commercial wine yeasts [1].

SSR analysis of the isolates from ES1 allowed exclusive detection of ADY1 profiles, at T1 and T9 and the detection of nearly 5 % of other profiles at T17, probably corresponding to wild strains of *S. cerevisiae*. No ADY2 profiles were detected. In the ES2 experiment the profiles of both ADY were detected. Again the ADY1 profiles predominated with approximately 60% for the three sampling times. The detection of ADY2 profiles decreased from T1 (37.5 %) to T17 (12.5 %), while an increase in wild strains profiles was observed, from 4 to 25%.

Additionally, it was interesting to notice that the sequential inoculation (ES1) resulted in a slower fermentation, a stable number of viable cells (evaluated by culture) and a higher wine quality. The co-inoculation (ES2) resulted in a faster fermentation with a decline in the number of cells presenting culture viability and a low quality wine.

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## Industrial Microbiology &amp; Biotechnology

P092

## SUSTAINABLE GLYCOLIPIDS: YEAST CONVERSION OF LIGNOCELLULOSIC BIOMASS INTO MANNOSYLERYTHRITOL LIPIDS

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Microbial surfactants are recognized as fine chemicals with high-value applications and interesting characteristics such as low toxicity, high biodegradability, effectiveness at extreme temperatures or pH, and mild production conditions when compared to chemical surfactants. Mannosylerythritol lipids (MELs) are microbial glycolipids containing a 4-O- $\beta$ -D-mannopyranosyl-meso-erythritol as the glycosidic/hydrophilic moiety and two short-chain fatty acids (usually C8 to C12). The sustainable production of MEL can potentially be achieved by the use of lignocellulosic materials as substrates.

Yeasts from the genus *Pseudozyma* were previously described as MEL producers from glucose, glycerol or vegetable oils. In this work, *P. antarctica* PYCC 5048<sup>T</sup> and *P. aphidis* PYCC 5535<sup>T</sup> were characterized in terms of growth on D-xylose and L-arabinose and MEL production from these pentoses in comparison to the use of D-glucose as substrate. The yeast strains were able to grow in pentoses with similar growth rates to those obtained in D-glucose (around 0.2 h<sup>-1</sup>). *Pseudozyma antarctica* was identified as the best MEL producer with comparable MEL titers (4.8–5.4 g/l) and yields (0.11–0.14 g/g) from either glucose, xylose or their mixtures.

Moreover, the conversion of cellulosic materials into MEL was investigated. Commercial grade cellulose (Avicel<sup>®</sup>) and the cellulosic fraction from pretreated wheat straw were hydrolyzed by enzyme cocktails (Celluclast 1.5L and Novozyme188) and the hydrolysate converted by either by simultaneous saccharification and fermentation (SSF) or separated hydrolysis and fermentation (SHF). *Pseudozyma antarctica* showed higher MEL yields from both cellulosic substrates, reaching titres of 1.1 g/l and 2.9 g/l by SSF of wheat straw or Avicel<sup>®</sup> (4% glucan), respectively. Higher MEL titres were obtained using a fed-batch SSF strategy, reaching 2.5 and 4.5 g/l from Avicel<sup>TM</sup> and wheat straw, respectively.

This work demonstrates the ability of *Pseudozyma* yeast strains to produce a glycolipid (MEL) from pentoses (D-xylose and L-arabinose), as well as from (ligno)cellulosic materials, thus widening the substrates range for the sustainable production of this biosurfactant with multiple potential applications.

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## Industrial Microbiology &amp; Biotechnology

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## TRACKING THE EFFECT OF THE CULTURE DESIGN IN THE IN-SITU NIR MONITORING OF PLASMID BIOPROCESSES

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As a consequence of the growing interest in bioprocess monitoring for the production of plasmid-based preventive/therapeutic products, the development of *in-situ* plasmid bioprocess monitoring techniques turns out as a crucial step towards bioprocess optimization and control. Complex mechanisms occur inside the bioreactor in terms of the metabolic interrelationships involving the strain, nutrients, products and by-products formation. Being able to accurately predict all metabolic stages along the bioprocess, in a rapid and sterilized way, as shown by *in-situ* near infrared (NIR) spectroscopy [1], and independently of the choice of the strain, plasmid model and carbon source, is the natural next step towards plasmid bioprocess optimization.

It is very common in the pharmaceutical industry that the culture conditions in a given bioprocess can suddenly change, either intentionally or an operator's mistake. As it is known that slight changes in the choice of medium composition and culture strategy, batch or fed-batch, have a relevant impact on the course of the bioprocess, and consequently on the final biomass and plasmid quantities, a predictive model based on NIR spectra accounting for changes in the medium composition and predicting all key variables in a common system, in real time, was investigated.

Three *Escherichia coli* DH5 $\alpha$  cultures, producing the same plasmid model, pVAXLacZ, and grown on different media (glucose and/or glycerol) and culture conditions (batch or fed-batch) were monitored with a NIR fiber optic transfectance probe. Accurate partial least squares models were obtained for biomass and plasmid productions, yielding an R<sup>2</sup> of 0.99 and 0.98 and a RMSEP of 0.43 and 7.52, respectively. Good PLS predictions were also obtained for the carbon sources glucose and glycerol, with an R<sup>2</sup> of 0.93 and a RMSECV of 0.46 and 0.33, respectively. The by-product acetate could also be well estimated by a model yielding a R<sup>2</sup> of 0.96 and a RMSEP of 0.32.

This work has shown the possibility of *in-situ* monitoring *E. coli* bioprocesses for plasmid production with NIR spectroscopy, allowing a real-time acquisition of information concerning all key variables, with no use of reagents or risk for contamination. The implementation of such real-time monitoring technique in the industry also opens the door for screening NIR-based methodologies for searching for the best host microorganism and culture conditions for producing optimal plasmid-based therapeutical products.

This work was supported by PTDC/BIO/69242/2006 research grant, FCT, Portugal. M. Lopes was supported by the post-doctoral grant SFRH/BPD/73758/2010, FCT, Portugal.

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## Industrial Microbiology & Biotechnology

P094/F25

### OPTIMIZATION OF A SSF PROCESS USING INVERTASE APPLIED TO FOSSIL FUELS BIODESULFURIZATION

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Biodesulfurization (BDS) can be a complementary technology to the commonly used physical-chemical process. BDS is based on the use of microorganisms for the removal of sulfur even from the most recalcitrant compounds, such as dibenzothiophene (DBT), at mild operating conditions, making it cheaper and more eco-friendly. Knowing that our desulfurizing microorganism, *Gordonia alkanivorans* strain 1B, is a fructophilic bacterium, fact supported by an enhancement of DBT desulfurization rate during the desulfurization process using fructose or fructose-rich materials as carbon source [1, 2,3] the optimization of BDS requires the availability of fructose to the microorganism. In this context, the main goal of this work was the optimization of DBT-desulfurization by strain 1B using sucrose as carbon source and invertase through Simultaneous Saccharification and Fermentation (SSF) approach, a technique which permits the availability of fructose during the course of BDS.

The determination of optimal conditions for both sucrose hydrolysis by *Zygosaccharomyces bailii* Talf1 invertase, and *G. alkanivorans* 1B desulfurization in a SSF approach, was carried out through two experimental uniform designs accordingly to Doehlert distribution for two factors, namely: temperature (28–38°C) vs pH (5.5–7.5), maintaining the same enzyme concentration (1.16 U/g/l); and temperature (28–38°C) vs enzyme concentration (0–4 U/g/l), maintaining initial pH at 7.5. The *Z. bailii* enzyme extract production and BDS assays were carried out as described by Paixão et al. 2013 [3] but using 10 g/l sucrose as carbon source and 250 mM DBT as sulfur source.

The different surface responses obtained from both experimental designs showed the importance of the 3 factors studied (pH, enzyme concentration and temperature), with factor weight varying depending on the variable studied and fermentation time. Based on 2-HBP production results, the analysis of the response surfaces obtained point out for pH 7.5, 32 °C and 1.8 U enzyme per g/l of sucrose as the optimal conditions for BDS through SSF approach using Talf1 invertase.

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## Industrial Microbiology & Biotechnology

P095/F26

### INFLUENCE OF MOISTURE CONTENT, TEMPERATURE AND INOCULUM SIZE ON LIPASE PRODUCTION BY FILAMENTOUS FUNGI UNDER SOLID-STATE FERMENTATION OF OLIVE POMACE

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Pollution by olive mill wastes is a crucial problem in the Mediterranean area and proper management is necessary. Olive pomace, a solid by-product generated by the olive oil two phase extraction process, is an acidic and very humid material, rich in organic matter, potassium, nitrogen, carbohydrates, phenols and also contains residual fats, which make an interesting substrate for lipase production under solid-state fermentation.

The aim of this work was to optimise moisture content (MC), temperature and inoculum size, in order to improve lipase production by *Aspergillus ibericus* MUM 03.49, *Aspergillus niger* MUM 03.58 and *Aspergillus tubingensis* MUM 06.152, under solid-state fermentation (SSF) of olive pomace mixed with wheat bran. Firstly, a full factorial design of experiments was implemented in order to investigate the effect of MC (70%, 75% and 80% wet basis) and temperature (25 °C, 30 °C and 35 °C) on its production. SSF was carried out in 500 mL Erlenmeyer flasks, containing 30 g solid dry substrate. Lipase activity was measured by colorimetric assay, using *p*-nitrophenyl butyrate as substrate.

In general, MC presented a significant effect ( $p < 0.05$ ) on lipase production. Temperature presented a significant effect only for *A. ibericus*. Maximum lipase production was obtained at 70% of MC for all microorganisms, showing the need to investigate lower values of moisture. Thus, MC values from 35% to 70% were investigated at 30 °C for *A. ibericus* and *A. niger*, and at 25 °C for *A. tubingensis*.

Results showed an increase in lipase production with lower MC: 3-fold for *A. ibericus* with 60% MC, 7-fold for *A. niger* with 50% MC, and 4-fold for *A. tubingensis* with 35% MC.

Finally, the effect of inoculum size from  $10^5$  to  $10^8$  spores/mL was also investigated, and it was found that this condition did not present a significant effect on lipase production. *A. ibericus* was the best lipase producer, leading to 117.33 U/gds. *A. niger* and *A. tubingensis* produced 80.86 and 8.60 U/gds, respectively.

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## Industrial Microbiology & Biotechnology

P096/F05

### RESVERATROL PRODUCTION IN BIOREACTORS: ASSESSMENT OF CELL PHYSIOLOGY STATES BY FLOW CYTOMETRY AND PLASMID SEGREGATIONAL INSTABILITY BY REAL-TIME QPCR

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Plant secondary metabolites have a high medicinal value. Resveratrol (3,5,4'-*trans*-hydroxystilbene) is a plant secondary metabolite produced under stress (phytoalexin), and mainly present in peanuts and grapevines. This polyphenolic compound, widely used since ancient times, can act as an antioxidant and antimicrobial compound, having several health benefits such as anti-aging and anticancer activities. Being a multifunctional molecule, this stilbene is a desirable product for the cosmetic, pharmaceutical and nutraceutical industries. In an attempt to develop alternative ways for improving resveratrol production, new recombinant yeast and bacteria were proposed for recombinant resveratrol production. In this study, *Saccharomyces cerevisiae* and *Escherichia coli* were used to optimize resveratrol production in bioreactors. The impact of resveratrol production on cell physiology and plasmid segregational instability was assessed through flow cytometry and real-time qPCR, respectively. For resveratrol quantification, a liquid-liquid resveratrol extraction from culture media was performed using ethyl acetate for subsequent injection on a High Performance Liquid Chromatography – Diode Array Detector (HPLC-DAD). After validation of the HPLC-DAD method, *S. cerevisiae* and *E. coli* were evaluated for resveratrol production in shake flasks under six variables: pH, temperature, medium composition, agitation, optical density (OD<sub>600</sub>) at time of induction and inducer concentration. The data obtained were used to create assays to perform in bioreactors in order to improve and optimize resveratrol production. Shake flasks screening assays revealed a 30 times higher resveratrol yields by *E. coli* (about 100 µg/mL) when compared to *S. cerevisiae*, which led to the choice of the first microorganism for scale up optimization studies. The results obtained in shake flasks were used for designing further optimization assays in bioreactors, the only considered factors were those with the highest impact on resveratrol production: temperature, pH, inducer concentration and optical density (OD<sub>600</sub>) at time of induction. These assays allowed to achieve 160 µg/mL of resveratrol, which is the highest yield obtained to date for this recombinant microorganism. The population of depolarized cells varied according to the conditions used – sometimes resulting in a 10% difference between higher and lower production assays. Segregational instability has also been observed during the bioprocess, with variations in the values of plasmid copy number (PCN), with higher production assays generally presenting the highest PCN amounts. It is possible to conclude that cellular viability and plasmid segregational instability may affect the final resveratrol production. In sum, this study outlines the optimization of resveratrol production in bioreactors using flow cytometry and RT-qPCR for bioprocess monitoring. It was demonstrated that using the appropriate tools to optimize and monitor resveratrol production process, solutions can be found for the production of large quantities of this stilbene, providing an effective alternative to chemical synthesis and avoiding the depletion of natural sources.



## Industrial Microbiology & Biotechnology

P097/F27

### CHARACTERIZATION OF THE *GAM* CLUSTER OF A *BACILLUS SUBTILIS* GUT STRAIN

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The genome sequence of an undomesticated gut-associated strain of *Bacillus subtilis*, BSP1, was recently determined [1]. BSP1 is closely related to the widely used laboratory strain *B. subtilis* 168, carrying however about 200 genes not found in the latter strain. Most of these genes were presumably acquired horizontally and might be related to adaptation of life in gut [1]. One newly identified group of genes, the *gam* cluster, comprises a putative extracellular beta-mannanase (A7A1\_2761), and most likely a specific system for the synergistic degradation and utilization of mannan polymers, such as galactoglucomannan [1].

In this work we report the genetic organization of the *gam* cluster. *In silico* and *in vivo* mRNA analyses revealed that the *gam* cluster, comprising nine genes, is organized and expressed in at least six different transcriptional units. Furthermore, cloning, overexpression and purification of the product encoded by A7A1\_2761 was achieved. The enzymatic kinetic parameters and physical properties were determined. Enzymatic activity towards different substrates revealed beta-mannanase activity. Moreover, thin-layer chromatography analysis using mannose oligomers as substrate suggests an endo-acting mode of action.

This beta-mannanase displays a modular architecture comprising a carbohydrate-binding domain (CBM), a feature rarely found in hemicellulolytic enzymes of the laboratory strain 168, supporting the hypothesis of acquisition by horizontal gene transfer. Interestingly, preliminary analyses indicate that the gut strain BSP1 possesses higher hemicellulolytic capacity compared to that of the laboratory strain 168.

This project was partially supported by PEst-OE/BIA/UI0457/2011 from Fundação para a Ciência e a Tecnologia (FCT).

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## Session 2

# Environmental Microbiology and Biotechnology



# Plenary Lecture

## KEY FEATURES OF SYNTROPHIC METHANOGENIC COMMUNITIES

Alfons Stams; Caroline Plugge; Diana Sousa

*Wageningen University*

Syntrophic associations of anaerobic bacteria and methanogenic archaea are important in methanogenic environments. Syntrophic conversion of organic compounds is driven by interspecies hydrogen and formate transfer. Hydrogen transfer is best studied, but knowledge about formate transfer is increasing. Bacteria that grow in syntrophy with methanogens can be found within the Gram-negative and Gram-positive bacteria. Phylogenetically, such bacteria are often similar to bacteria that cannot grow syntrophically. However, it is not completely understood which properties determine the ability to grow syntrophically or not.

*Syntrophobacter fumaroxidans* is a mesophilic deltaproteobacterium and *Pelotomaculum thermopropionicum* is a thermophilic bacterium grouped in the *Desulfotomaculum* cluster 1. These two bacteria can grow with propionate in syntrophy with methanogens. Physiological studies and genome analysis revealed the pathway and electron mechanisms in these two bacteria.

*Desulfobulbus propionicus* and *Desulfotomaculum kuznetsovii* are two sulfate-reducing bacteria that can grow with propionate and sulfate, but they lack the ability to grow with propionate in syntrophy with methanogens. Analysis of the available genome sequences of these two bacteria allows to identify the key differences

# Keynote Lectures

## KL02

# MICROBIAL RESOURCE RESEARCH INFRASTRUCTURE (MIRRI) FACILITATING IMPROVED PATHWAYS TO DISCOVERY

David Smith

*CABI*

Europe is laying down the foundation for a major push to harness biodiversity in its battle to overcome natural resource depletion and reduce our impact on the environment through the European Strategy Forum for Research Infrastructures (ESFRI) which establishes pan-European structures to drive innovation and provide the resources, technologies and services necessary to underpin research. The European Consortium of Microbial Resource Centres (EMbaRC) in collaboration with the Global Biological Resource Centre Network (GBRCN) and the European Culture Collection's Organisation (ECCO) played a major role in ensuring the Microbial Resource Research Infrastructure (MIRRI) is one of the thirteen research infrastructures ESFRI has launched in the biological and medical science area. MIRRI entered its preparatory phase funded by the European Commission in 2012. The ESFRI strategy recognises the weakness of fragmented individual policies and supports a coherent strategy on research infrastructures in Europe, which facilitates multilateral initiatives and provides Europe with the most up-to-date Research Infrastructures. Science frontiers are evolving and knowledge-based technologies need to be used more widely. In the microbial area, the OECD Biological Resource Centre initiative (1999 to 2006) proved important in providing frameworks for best practice and biological resource networking. Now MIRRI will integrate services and resources, and encourage innovative solutions. It will provide coherence in the application of quality standards, homogeneity in data storage and management, and workload sharing to help scientists release the potential of microorganisms. MIRRI brings together European microbial resource collections with users, policy makers, potential funders and the plethora of microbial research teams. It aims at improving access to enhanced quality microbial resources in an appropriate legal framework. It will build the European platform for microorganisms within the future Global Biological Resource Centre Network (GBRCN) and will facilitate improved pathways to discovery for the future. MIRRI will enhance existing European microbial collections linking them to non-European country partners globally. It will tackle key obstacles to research in a co-ordinated way and will improve the provision of services beyond what is currently supplied by individual microbial resource collections. MIRRI will focus efforts through a cluster model to improve resources to meet user needs. MIRRI will support the efforts of ECCO, the World Federation for Culture Collections and its World Data Centre for Microorganisms adding value and quality.

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## KL11

PATHOADAPTATION OF *ESCHERICHIA COLI* TO MACROPHAGESMigla Miskinyte, Ana Sousa, Ricardo Ramiro, Jorge Sousa, Isabel Gordo*Instituto Gulbenkian de Ciência*

The selective pressure imposed by host innate immunity is an important component of microbial adaptation from commensalism to pathogenicity. Here we use experimental evolution to study the initial steps of the adaptation of *Escherichia coli* to cells of the innate immune system, *i.e.* macrophages. We show that *E. coli* evolves remarkably fast, and acquires adaptive mutations, which lead to increased survival inside macrophages and/or ability to escape macrophage engulfment. The mechanism underlying this pathoadaptive process involves the accumulation of mutations caused by transposon insertions. These mutations lead to increasing pathogenicity *in vivo* as assayed in a mouse infection model. These findings reveal the remarkable fast pace at which bacteria can evolve to escape a central component of the host innate immunity, namely macrophages.

# Oral Presentations

## OP05

## SEQUENCED MOBILE ELEMENTS FROM SUBMARINE HYDROTHERMAL VENTS INCLUDE SIMULTANEOUSLY GENETIC DETERMINANTS FOR HEAVY METAL AND ANTIBIOTIC RESISTANCE

Christophe Espfrito Santo<sup>1</sup>; Pedro Farias<sup>2</sup>; Lars Hansen<sup>3</sup>; Soren Sorensen<sup>3</sup>; Romeu Francisco<sup>1</sup>; Paula V. Morais<sup>4</sup>

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Vent sites from the Mid-Atlantic Ridge (MAR) exhibit varied environmental conditions, resulting from depth variation of the axis and associated physical parameters, and different source rocks.

It is known that, many genes conferring antibiotic resistance are located on mobile genetic elements, some of which are easily exchanged among phylogenetically distant bacteria. Many of these mobile genetic elements encode resistance to multiple antibiotics, heavy metals and other toxic compounds. Selective pressure by one of such compounds indirectly selects for other resistances. Hence the possibility of deep-sea bacteria to harbor antibiotic/metal resistance traits via horizontal transformation can be anticipated.

The present work aimed to, in metal-rich-environments free of anthropogenic influences, assess the metal and antibiotic resistant microorganisms and their mobile genetic elements. The objective was to find out the genetic determinants responsible for metal resistance and their association to potential antibiotic resistances and other determinants involved in microbial pathogenicity. The hydrothermal Lucky Strike field in the Azores archipelago (between 11N and 38N), at the MAR, protected under the OSPAR Convention was sampled. 9 isolates with at least 6 simultaneous resistances to metals (Cu, Zn, Co, Cd, U, Cr) and to antibiotics (at least one of each class) were selected for plasmid isolation. The 9 purified plasmids had sizes ranging from 7.5 to 264 kbp were sequenced by Illumina, assembled by EDENA V3 program and, contigs with coverage higher than 8, were annotated using RAST server. The analyses owed the presence of genes coding for multiresistance pumps, specific metal and antibiotic resistance proteins and for virulence factors. Genes for conjugational transfer systems (IncF, F pili) and for other mobile elements (transposases) were also found.

Plasmids carried multiple genes for resistance-nodulation-cell division superfamily (RND) pumps (cobalt-zinc-cadmium efflux system genes, and copper-induced-efflux system (*cusCFBA*)), and genes for copper homeostasis genes (copper translocating P-type ATPase, multicopper oxidase), siderophore production and acquisition and multiple  $\beta$ -lactamase genes. Furthermore, resistance to fluoroquinolones (*gyrB*) and reactive oxygen species (superoxide dismutases, catalases and peroxidases) were also found.

Plasmids described here might prove to be useful in developing new genetic tools for the organisms inhabiting these environments. Also, they can be seen as promoters and potential sources of new genes for antibiotic and metal resistance.

OP06

ASSESSMENT OF QUERCUS SUBER SOIL MICROBIOME USING DGGE FINGERPRINTS AND BARCODED PYROSEQUENCING

Diogo Pinho<sup>1</sup>; Paula Gomes<sup>1</sup>; Cristina Barroso<sup>1</sup>; Miguel Pinheiro<sup>1</sup>; Isabel Henriques<sup>2</sup>; António Correia<sup>2</sup>; Mariana Telles<sup>3</sup>; Conceição Silva<sup>3</sup>; Conceição Egas<sup>1</sup>

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<sup>3</sup>*Associação de Produtores Florestais do Concelho de Coruche e Limitrofes*

Plants maintain a relationship with soil microorganisms that can be beneficial or harmful to plant, while soil microbiome is influenced by plant root exudates. The use of molecular techniques is enabling us to analyze the diversity and structure of microbial communities and explore the interaction of microbes with plants.

In the Mediterranean region, the combination of severe summer drought and pathogens appears to be the main cause for *Quercus suber* decline, however, the influence of the soil microbial community has never been addressed. For that, the soil microbiome of *Q. suber* was extensively characterized using the 16S rDNA and the ITS regions as DNA barcodes for identification of bacterial and fungal communities, respectively, and to correlate results with tree health. For this, DGGE fingerprinting was firstly used to ascertain differences between microbial communities, and then barcode pyrosequencing was performed for a highly detailed study of the soil composition in both health status of cork oak trees.

DGGE revealed differences between healthy and decrepit trees and some of the samples were pyrosequenced and analysed with Metabiodiverse. This bioinformatics tool is designed to identify the organisms present in a community from total DNA, contemplating all the kingdoms, Eukarya, Archaea and Bacteria, and only needs the correct curated database for the identification of the rDNA specific region. A total of 124,428 sequences were obtained, which generated, in average, 278 fungal and 972 bacterial OTUs per sample. PCA clearly supported the hypothesis that the bacterial and fungal communities were different according to the health status of *Q. suber*. *Cortinarius* and *Russula* were the most abundant fungal genus in the healthy and diseased trees, respectively, and a decrease of mycorrhiza was observed from healthy to diseased trees. Soil bacterial community composition was less variable according to tree health, but mycorrhiza-associated bacteria as *Pseudomonas*, *Bacillus*, *Streptomyces* and *Paenibacillus* were mostly found in the healthy soil microbiome, where the mycorrhizal fungi were more abundant.

This study provides a deep description of soil microbiome associated with the health status of cork oak trees, suggesting an important role of mycorrhiza in tree decline. Future studies are required to understand the functional relevance of mycorrhiza-bacteria-plant interaction with the tree health, and to correlate *Quercus* decline to biotic and abiotic factors.



## OP07

## ENTEROBACTERIA CARRYING OXA-48-LIKE GENES ISOLATED FROM NON-HUMAN SOURCES

Marta Tacão; António Correia; Artur Alves; Isabel Henriques

*Biology Department and CESAM, University of Aveiro*

Last-resort antibiotics, such as carbapenems, are drugs of choice to treat infections caused by multiresistant bacteria or when all other therapeutics fail. Since its first description in 2004, the class D carbapenemase OXA-48 and its variants among Enterobacteriaceae are a matter of great concern. Genes encoding these enzymes rapidly disseminated in clinics all over the globe and have been implicated in numerous death-causing outbreaks. We isolated twelve environmental OXA-48-like-producing strains from rivers and estuarine ecosystems. These strains were identified by sequencing the 16S rRNA gene as *Citrobacter freundii* (n=2), *Enterobacter asburiae* (n=1), *Enterobacter cancerigenus* (n=1), *Enterobacter ludwigii* (n=1), *Escherichia coli* (n=4), *Pantoea eucalypti* (n=1), *Providencia alcalifaciens* (n=1) and *Serratia marcescens* (n=1). The presence of genes encoding OXA-48 or its variants was investigated by PCR and sequencing. Besides *bla*<sub>OXA-48</sub> genes (n=4) also *bla*<sub>OXA-181</sub> (n=4) was detected. In addition a new variant was identified in 4 strains, differing in 2 or 3 aminoacids from the enzyme sequences previously described. Antimicrobial susceptibility tests revealed that the majority of the strains were also resistant to 3<sup>rd</sup> generation cephalosporins and other non-beta-lactams as quinolones and aminoglycosides. This is the first report on OXA-48-like-producing enterobacteria in Portugal. Moreover, this study reports for the first time a member of the genus *Pantoea* (*P. eucalypti*) carrying *bla*<sub>OXA-48</sub>-like genes. The diversity of OXA-48-like encoding genes and the phylogenetic range of OXA-48-producers found were totally unexpected, again confirming the importance of natural environments as places of evolution and spread of antibiotic resistance.

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## OP08

### BACTERIA ASSOCIATED WITH MARINE *ERYLUS* SPONGES: THEIR DIVERSITY AND BIOACTIVITY

Ana Patrícia Graça<sup>1,2</sup>; Joana Bondoso<sup>1,2</sup>; Flávia Viana<sup>1</sup>; Sofia Cunha<sup>1</sup>; Cátia Moreira<sup>1</sup>; Inês Correia<sup>1</sup>; Helena Gaspar<sup>3</sup>; Olga Maria Lage<sup>1,2</sup>

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The world's oceans comprise a rich still unknown diversity of microbial life which holds great, underexplored potential for drug discovery. Sponges have been reported to possess a high bioactivity which, most probably, is due to the rich microbial community that lives in association with these organisms. In the framework of the project NIDOS, heterotrophic bacteria associated with six *Erylus* sponges (three specimens of *Erylus* cf. *deficiens* Topsent, 1927 collected in the Gorringe Bank, two specimens of *Erylus discophorus* (Schmidt, 1862) in Berlengas islands and one specimen of *Erylus mamillaris* (Schmidt, 1862) in São Miguel island, Azores) were isolated in pure culture and taxonomically identified through the 16S rRNA gene sequencing. Furthermore, bioassays to assess the bioactivity of the isolated bacteria were performed as well as the search of genes involved in the synthesis of polyketide synthase (PKS) and non-ribosomal peptide synthetases (NRPS). Bacterial diversity and bioactivity results will be presented and discussed and the biotechnological potential of *Erylus* associated bacteria will be highlighted.

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## OP19

BACTERIAL SENSING OF KEY ENVIRONMENTAL PARAMETERS: STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF CHEMOTAXIS SENSOR PROTEINS FROM *G. SULFURREDUCTENS*Marta A. Silva<sup>1</sup>; Teresa Catarino<sup>2</sup>; Cláudio M. Gomes<sup>3</sup>; Carlos A. Salgueiro<sup>1</sup><sup>1</sup>Requimte-CQFB, Dep. Química, FCT-UNL, Campus Caparica, Portugal<sup>2</sup>Requimte-CQFB, Dep. Química, FCT-UNL, Campus Caparica, Portugal<sup>3</sup>ITQB-UNL, Oeiras, Portugal; 3ITQB-UNL, Oeiras, Portugal

The ability of *Geobacter* species to reductively precipitate uranium and related contaminants and to produce current densities higher than any other known organism in microbial fuel cells has led to the development of bioremediation and others biotechnological applications [1]. Bacterial chemotaxis systems sense and regulate the microbe mobility in response to environmental conditions. Such mechanisms constitute a striking example of cell motility to gain advantages for cell survival and permit the bacteria to fill important niches in a diversity of anaerobic environments. *Geobacter* species have an unusually large number of methyl-accepting chemotaxis (MCP) genes. Several MCP genes are located in proximity to other chemotaxis genes on the genome and therefore are likely to be involved in signal transduction pathways mediated by chemotaxis-like systems [2]. In the present work complementary biophysical spectroscopies and stopped-flow techniques were used to characterize the heme-sensor domains of two methyl-accepting chemotaxis proteins from *Geobacter sulfurreducens*: GSU935 and GSU582. The results showed substantial differences in the thermodynamic stability and kinetics properties of these sensors. Overall the results provide evidence that the sensing threshold and intensity of the propagated allosteric effect is linked to the stability of the PAS-fold, as this property modulates domain swapping and dimerization [3]. The different working potential ranges revealed by their different reduction potential values may allow the bacteria to trigger an adequate response in different anoxic subsurface environments. The work reported constitutes the first step in understanding of how *G. sulfurreducens* sense key environmental parameters in contaminated sites and may have implications in future rational manipulations.

Acknowledgments: PTDC/BBB-BEP/0753/2012 and SFRH/BD/61952/2009 from FCT (Portugal)

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## OP20

### NATURAL HOTSPOTS FOR MULTIPLE RESISTANCES GAIN: ARSENIC RESISTANCE IN HETEROTROPHIC AEROBIC BACTERIA FROM MARINE HYDROTHERMAL VENTS

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Organism habitats include the ocean and Earth's crust. Microorganisms are responsible for multiple antibiotic resistances which have been associated with resistance/tolerance to heavy-metals with consequences to public health. The circulation of seawater through fractured rocks at mid oceanic ridges produces important exchanges between lithosphere and hydrosphere. The hydrothermal Lucky Strike field in the Azores archipelago (North Atlantic, between 11N and 38N.), at the Mid-Atlantic Ridge (MAR), protected under the OSPAR Convention was sampled.

Ecological studies have reported that metal and antibiotic resistance is becoming a global phenomenon. Many genes conferring antibiotic resistance are located on mobile genetic elements, easily exchanged among phylogenetically distant bacteria. Many of these elements encode resistance to multiple antibiotics, heavy metals, and other compounds. Therefore, it is likely that selective pressure by one of such compounds indirectly select for other resistances, and some studies indicated that the frequency of antibiotic resistance was raised significantly in heavy metal-contaminated environments. The objective of the present work was to isolate arsenic, antimonite and antibiotic resistant strains from deep-sea hydrothermal sediments and to determine the existence of plasmids harboring antibiotic/arsenic/antimonite resistance traits.

A total of 166 strains from 18 different species were isolated in low carbon content medium under aerobic conditions, in the presence of arsenate, arsenic, antimonite or antibiotics. The arsenic resistant isolates carried ACR3 arsenic resistance gene. Moreover, the 2 antimonite resistant isolates were simultaneously resistant to arsenate but surprisingly not to arsenite. This result suggests that a specific mechanism for antimonite resistance, not yet described, could be responsible for the resistance.

Cross resistances – simultaneous resistance to metals and antibiotics – were found in 56 strains. In these strains, the presence of plasmids was determined. Purified plasmids were sequenced by Illumina, assembled using the program EDENA V3 and contigs with a high coverage (>8) were annotated using the Rapid Annotation using Subsystems Technology (RAST) server.

A number of species show simultaneous metal and antibiotic resistance. Presence of genes coding for arsenic resistance and antibiotic resistance genes in the same mobile element were found, anticipating the possibility of horizontal gene transfer and distribution of these resistances in the bacterial population.

## OP21

## CYANOBACTERIAL DIVERSITY IN MICROBIAL MATS FROM ONE OF THE LARGEST HYPERSALINE COASTAL LAGOONS COMPLEX: AN IN-DEPTH POLYPHASIC APPROACH

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Cyanobacterial mats are complex micro-scale ecosystems that can be found in a diverse range of environments around the world, and are recognized as main contributors for the formation of some modern microbialites (i.e. rock formation driven by microbial growth). In marine systems, they can be observed in flat, hydrodynamically low-energy coasts, and in salterns or other hypersaline aquatic systems. The latter are considered extreme environments, and the organisms that there inhabit need to be adapted to daily salinity fluctuations and to seasonal periods of desiccation. Globally, cyanobacteria are metabolically versatile and a very diverse group of microorganisms, which can explain their successful evolutionary adaptation to such harsh environments. In fact, many halophilic and/or halotolerant cyanobacterial species are known to occur in these habitats.

To further deepen the knowledge on photosynthetic microbial mats in tropical hypersaline environments, we have characterized the cyanobacterial diversity present in mats from three hypersaline ponds of the Araruama complex lagoon (Rio de Janeiro, Brazil). To this end, we have adopted culture-dependent and -independent methodologies, either using morphological or molecular (16S rDNA)-based techniques.

More than 30 morphospecies (i.e. morphotypes identified by microscopy) could be differentiated in the three original samples. Nine cyanobacterial strains were isolated and identified as belonging to genera *Geitlerinema*, *Leptolyngbya* and *Nodosilinea*. Molecular independent techniques included PCR-DGGE (denaturing gradient gel electrophoresis) and 454-pyrosequencing technology to perform high-throughput deep sequencing. In total, these methods generated 38 DGGE band sequences and 164 cyanobacterial operational taxonomic units (OTUs) sequences from >9000 reads, respectively. Molecular phylogenies inferred from these data were then compared with morphological ones. A good number of sequences could be associated with the morphospecies observed. Most of the 16S rRNA gene sequences demonstrated to be closely affiliated with cyanobacterial strains from ecologically similar habitats. One interesting finding of the phylogenetic analysis was that many sequences have no close relatives in the public databases, being placed as 'loner' sequences. This seems to reveal the presence of novel cyanobacterial diversity in the mats of the Araruama complex.

## OP43

### FUNGAL ACTIVITY IN UNTREATED SURFACE WATER: MYCOTOXINS PRODUCTION AND ESTROGENIC ACTIVITY

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Filamentous fungi are known to occur ubiquously in the environment therefore reports of their occurrence in different water matrices were not surprising. Water contamination with fungi has been intensively studied in the past few years, showing that most of the different species of filamentous fungi present in the soil can be transferred to water through drainage and rain offs. However, their presence in water may be associated with taste and odour problems, contamination of food and beverages, mycotoxin production, and health related effects. Some of the fungi that were reported to occur in the aquatic environment (e.g. *Aspergillus*, *Fusarium* and *Penicillium*) have the ability to naturally produce mycotoxins, toxic secondary metabolites. Recently mycotoxins and some of its metabolites, that have been shown to be several times more estrogenic than other environmental estrogens of concern, have been reported to occur at ng/L levels in the aquatic environment [1,2]. In this study different fungi species were spiked into surface water to evaluate their potential ability to produce mycotoxins in water matrix. Also, the possible estrogenic activity of metabolites produced by the fungus was monitored over time, using the yeast estrogen screen (YES) assay. The results showed that only *Aspergillus fumigatus*, *Aspergillus niger* and *Purpureocillium lilacinum* were able to produce mycotoxins (ochratoxin A and fumonisins), being aflatoxins the most produced. On the other hand, *Aspergillus niger*, *Fusarium foetens* and *Purpureocillium lilacinum* produced mycotoxins and supernatants of their growth had higher endocrine disrupting capabilities.

The production of mycotoxins in water is here described for the first time. Moreover, it was demonstrated that the existence of compounds with endocrine disrupting capabilities in the aquatic environment it is not only due to human and pharmaceutical activity but also due to the presence of fungi.

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# Poster Presentations

## Environmental Microbiology and Biotechnology

P098

### A MICROCOSM APPROACH SHOWS THAT *AEROMONAS MOLLUSCORUM* AV27 ENHANCES THE DEGRADATION OF TRIBUTYLTIN (TBT) IN AN ESTUARINE SEDIMENT

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Tributyltin (TBT) is a biocide that has been used in several industrial processes, namely as additive to antifouling paints. It is extremely toxic to a wide range of organisms from bacteria to mammals and from the molecular to the community level.

The International Maritime Organization (IMO) called for a global treaty that bans the TBT-based paints starting from 2003, and total prohibition by 2008. However, TBT continue to be produced/used, especially in countries that did not join IMO. Therefore, TBT is still a global problem, mainly due to the high levels that are trapped in the sediments. Some microorganisms are resistant and can degrade TBT into less toxic compounds (dibutyltin and monobutyltin) and thus can be used as natural remediators.

*Aeromonas molluscorum* Av27 is a TBT-degrading bacterium isolated from an estuarine system, Ria de Aveiro, Portugal.

Previous studies showed that Av27 strain is not cytotoxic, constituting an important tool to decontaminate TBT-polluted environments.

A microcosm experiment, using estuarine sediment, was followed over 150 days to determine to what extent this strain was able to degrade TBT alone or in association with the indigenous bacterial community. Subsamples were withdrawn at the beginning (time 0), after 30, 90 and 150 days, to follow the evolution of the bacterial community and also to evaluate TBT degradation along time.

The results showed that TBT degradation occurred over time, and that the indigenous community play an important role in this process. Nonetheless, TBT degradation is significantly enhanced ( $P < 0.05$ ) by the presence of Av27.

16S rRNA gene and DGGE analyses indicated alterations of the bacterial community structure along time. For instance, changes in the predominance of Proteobacteria subdivisions were observed, indicating that TBT degradation occurred over time, which confirmed the results of sediment organotin chemical analysis.

Additionally, 16S rRNA gene library analysis showed that almost 100% of the clones affiliated with unculturable-bacteria and an average of 14% affiliated with unclassified-bacteria. Proteobacteria and Bacteroidetes were the most represented phyla.



## Environmental Microbiology and Biotechnology

P099

A REPORT ON ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCI* RECOVERED FROM NASAL SAMPLES OF CAPTIVE IBERIAN LYNX (*LYNX PARDINUS*)M. Andrade Sousa<sup>1</sup>; N. Silva<sup>1</sup>; R. Madureira<sup>1</sup>; P. Gomez<sup>2</sup>; D. Benito<sup>2</sup>; R. Serra<sup>3</sup>; M.J. Perez-Aspa<sup>4</sup>; M.T. Del-Rey<sup>4</sup>; M. Caniça<sup>5</sup>; C. Torres<sup>2</sup>; G. Igrejas<sup>6</sup>; P. Poeta<sup>1</sup>

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Antibiotic resistance is now an emerging public health concern among wild animals. In the last few years more reports have been made about the prevalence of resistance determinants proving them to be a natural reservoir of resistant bacteria. An effective approach to understand the complex multidirectional exchange of resistant pathogens between humans and animals must be provided to better understand this problem. *Staphylococcus* spp. are one of the keys to this problem. Often found in the natural microbiota of the skin, nose and mouth of humans and animals, these bacteria can cause serious life-threatening diseases. There's scarce information on the prevalence of *Staphylococcus* spp. in wild animals. For this reason, one aim of this work was to determine the prevalence of these bacteria in Iberian Lynx as well as to determine the phenotype of antibiotic resistance of recovered isolates.

In this study we recovered 16 staphylococci from the nasal samples obtained from 27 captive animals from CNRLI (National Centre for Captive Breeding of the Iberian Lynx), Portugal and CCLI (Iberian Lynx's Captive Breeding Center) La Olivilla, Spain. Swabs obtained were dipped into BHI broth containing 6.5% of NaCl; then, 150µl were seeded on MSA and in ORSAB media [2 mg/L Oxacillin]. Presumptive *Staphylococcus* spp. colonies obtained in both types of media were isolated and identified by microbiological, biochemical and molecular methods (Gram staining, DNase, catalase and PCR of *mecA*, *nuc* and *sodA* genes). Susceptibility testing to 18 antimicrobial agents was performed by Kirby-Bauer's disk-diffusion test according to CLSI. The obtained *sodA* DNA amplicons were sequenced on both strands and gene homology searches were performed using BLAST analysis. Positive and negative controls were used from the bacterial collection of the University of La Rioja, Spain. From the 16 isolates we identified one coagulase-positive (6%) and 15 (94%) coagulase-negative (CNS): nine were *S. sciuri* (two of them sensitive to all antibiotics tested) and five *S. felis*, one *S. aureus* (SA) and one *S. chromogenes*, all sensitive to all antibiotics tested. Seven *Staphylococcus sciuri* were resistant to various antibiotics: mupirocin (n=1), fusidic acid (n=7), clindamycin (n=7), penicillin (n=7), cefoxitin (n=2), oxacillin (n=1) and chloranphenicol (n=1).

On one hand, the nose of wild animals proved to be a natural reservoir for SA and coagulase negative staphylococci (CNS). Indeed, we verified a diversity of staphylococci in the nasal microbiota of *Lynx pardinus* and their resistance profile was characterized. Due to the convergence between habitats, wildlife contact with other animals and humans is now more common and this involves an increased risk to human and animal health due to the exchange of resistance factors between emerging pathogens. On the other hand, this contact is needed as we are talking about *Lynx pardinus*, the most endangered felid in the world; and in this case, we cannot put the possibility of the exchange of resistance to antibiotics above the conservation of this endangered felid. In any way, the treatment of these felids by antibiotics it's not yet at risk in what took to commensal staphylococci.

## Environmental Microbiology and Biotechnology

### P100

#### ACIDOGENIC FERMENTATION OF HSSL FOR SCFA PRODUCTION

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Polyhydroxyalkanoates (PHA) derived from industrial residuals or wastes and open mixed cultures (OMC) represents a strategy to reduce the production costs. OMC produce PHA from short-chain fatty acids (SCFA), where the SCFA composition determines the PHA copolymer composition and, consequently, its material properties. For this reason, it is important to understand how operational conditions influence SCFA production during acidogenic fermentation of industrial wastes such as hardwood sulphite spent liquor (HSSL). In this work, the evaluation of the acidogenic fermentation capacity of HSSL in a continuously stirred tank reactor (CSTR) was studied, with emphasis on the influence of the organic loading rate (OLR). The imposed conditions allowed for the selection of a SCFA-producing sludge that converted sugars present in HSSL to acetic, lactic, propionic and butyric acids. The first two were produced even at high OLR (15 g COD/L.d), although propionic and butyric acid were only produced at low OLR (7.5 to 3.75 g COD/L.d). The highest production yield was obtained at the lowest OLR (3.75 g COD/L.d). The fermented HSSL was utilized in as feedstock for PHA accumulation, where the produced SCFA were utilized for PHA accumulation by an OMC. The production was similar for both synthetic and fermented media (35% vs. 32% PHA of DCW with composition 75:25 vs. 76:24 %HB:HV). The rate of PHA accumulation and the copolymer composition were not found to be significantly influenced by the complex matrix of fermented HSSL that contained 96 % non-VFA COD. Thus it was found that, for PHA production using OMC, even a complex fermented industrial feedstock can be utilized for the polymer accumulation step with a biomass that was not otherwise acclimated to this carbon source.

## Environmental Microbiology and Biotechnology

### P101

#### ANTIBIOTICS AND HEAVY METALS CO-RESISTANCE AMONG PHYLOSOSPHERE BACTERIA IN SALTMARSHES

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The phytosphere is a microbial hot-spot which supports the growth of numerous and diverse microorganisms, of which bacteria are undoubtedly the most dominant. This ecosystem has been poorly investigated and the knowledge on the associated microbial communities and their activities is still limited. The main goal of this study was to characterize bacteria associated to the phytosphere of plants inhabiting saltmarsh environments, where substantial amounts of anthropogenic contaminants are accumulated. Specific goals were to determine phylogenetic diversity and also the antibiotics and metal resistance profiles. Here we report the results obtained for bacteria associated to the phytosphere. Samples were collected from the plant *Halimione portulacoides* in saltmarshes with and without heavy metal contamination. Clonal relationships were assessed by BOX-PCR and the phylogenetic affiliation determined by 16S rDNA sequencing. Patterns of resistance to 16 antibiotics from 6 classes were determined, and the minimal inhibitory concentration of 6 heavy metals (As, Cr, Cu, Hg, Ni and Zn) was evaluated. Phylogenetic diversity comprised 13 different bacterial genera. The majority of strains isolated from the control site affiliated with *Vibrio* sp. (62.5%) but members of Enterobacteriaceae were also identified (18.8%). On contaminated sites, the majority affiliated with members of Enterobacteriaceae (34.5%) followed by *Pseudomonas* spp. (23.6%).

Multiresistance (defined as resistance to 3 or more classes of antibiotics) was higher on contaminated sites, with 43.6% vs. 28.1% of the strains from control site.

As of heavy metals tolerance, differences between sites were evident concerning copper: all strains isolated from control site were susceptible to this metal while 92.7% from the contaminated sites were resistant.

A principal component analysis of the susceptibility levels to antibiotics and heavy metals showed a clear separation between strains from contaminated and non-contaminated sites. Overall it was observed that epiphytic bacterial community associated to *Halimione portulacoides* in contaminated and non-contaminated sites varied in terms of composition but also in what concerns antibiotic multiresistance and metals tolerance. Ongoing work is addressing also the endophytic bacterial community.

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## Environmental Microbiology and Biotechnology

P102

### AQUACULTURES AS RESERVOIRS OF PLASMID-MEDIATED QUINOLONE RESISTANCE GENES

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Quinolones are critical antibiotics (AB) for the treatment of human infections. Plasmid-mediated quinolone resistance genes (PMQR) were described in aquatic settings but the input of aquacultures for their spread in nature remains scarce. We aimed to study the contribution of trout farms to the spread of bacteria carrying PMQR genes to aquatic environments downstream of the production systems.

Samples were collected from water/sediments upstream (n=13) and downstream (n=13) of farms, water/sediments from juvenile/adult fish ponds (n=15) and feed (n=6) from 2 Portuguese trout aquacultures (TR-A/TR-B; winter/summer; 2010–2012). They were plated in MacConkey with ciprofloxacin and *Aeromonas* Agar after enrichment. Genes coding resistance to fluoroquinolones [*qnr*, *qepA*, *aac(6')-Ib-cr*, *oqxAB*] and other AB were searched by PCR/sequencing. AB resistance was studied by agar diffusion/Etest (CLSI/EUCAST). Species were identified by ID32GN/16SrDNA sequencing. Clonality (MLST; *Escherichia coli*), characterization of plasmid (PL) and integron backbones (PCR, rep-PCR and/or sequencing), conjugation assays and genomic location (I-CeuI/S1 PFGE hybridization) were done.

PMQR genes were found in 9% (n=14/160) of the isolates: *Aeromonas* spp (3; upstream/downstream river water/adult ponds water/TR-A), *E. coli* (6; ST423-CC23, ST641, ST661, ST1049, ST2739; upstream/downstream river water/TR-A, juvenile/adult ponds water or sediment/TR-B), *Klebsiella* spp (2; downstream river sediment/TR-A, feed/TR-B) and *Citrobacter freundii* complex (3; sediment from adult pond/TR output/TR-B; feed/TR-A) with MIC to ciprofloxacin (0.19–1 mg/L) above ECOFF for *Enterobacteriaceae*. The *qnrS1* was found in an untypable (UN) PL of *E. coli* (2); *qnrS2* in UN PL of *Aeromonas*; *qnrS3* in PL (2 UN/1 IncN) of *E. coli* and in an IncN PL of a *C. freundii* complex. The *oqxAB* in IncN-F hybrid or UN PL of 2 MDR *Klebsiella* spp and *aac(6')-Ib-cr* in IncU PL in *Aeromonas*. Different *qnrB* alleles, including a new variant, were chromosome located in *E. coli* (1), *Klebsiella* spp (1) and *C. freundii* complex (1). Transferable PL were found only in *E. coli* carrying *qnrS1* (2) or *qnrS3* (2). Most strains carried other AB resistance genes, class 1 integrons and/or IS26.

The detection of different PMQR genes in different species and samples in both TR strongly suggests that this ecological niche might constitute a reservoir/vehicle for antibiotic resistance genes of relevance for human and animal health.

## Environmental Microbiology and Biotechnology

### P103

#### ASSOCIATIONS BETWEEN ANTIMICROBIAL RESIDUES, BACTERIAL COMMUNITIES AND ANTIBIOTIC RESISTANCE IN A HOSPITAL-URBAN WASTEWATER TREATMENT PLANT

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The discharge of untreated hospital effluents has been associated with the dissemination of antibiotic resistant bacteria into the environment. However, studies on the impact of hospital effluents on the receiving urban wastewater treatment plants (UWTP) are scarce. The main goal of this study was to compare the loads of antimicrobials, the antibiotic resistance prevalence and the bacterial community structure of a hospital effluent with those of the receiving UWTP.

These parameters were assessed through successive sampling campaigns of raw hospital effluent (HE) and raw (RWW) and treated (TWW) wastewater of the UWTP. Samples were characterized for amoxicillin and ciprofloxacin resistance prevalence, content of heavy metals and antimicrobial residues and bacterial community structure, based on 16S rRNA gene PCR-DGGE.

The bacterial communities found in HE and RWW of the UWTP were distinct, although with higher similarity between them than with the treated outflow (TWW). While the levels of fluoroquinolones, arsenic and mercury were in general higher in HE than in RWW, the opposite was observed for tetracyclines, sulfonamides and penicillin G. The prevalence of ciprofloxacin, but not of amoxicillin, resistance was significantly higher in HE than in RWW ( $p < 0.05$ ). It was possible to correlate the variations of the bacterial community in wastewater with the concentration of antimicrobials and with antibiotic resistance prevalence. Moreover, the concentration of antimicrobial residues was observed to be significantly correlated with the prevalence of antibiotic resistant bacteria.

Hospital effluent was confirmed as an important, although not the unique, source of antimicrobial residues and antibiotic resistant bacteria to the UWTP. Given the high loads of antibiotic residues and antibiotic resistant bacteria found in hospital effluents, these wastewater habitats serve as models to study and predict the impact of antibiotic residues on bacterial communities.

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## Environmental Microbiology and Biotechnology

### P104

#### **BACILLUS AMYLOLIQUEFACIENS SMA1 ISOLATED FROM AN HEAVY METAL CONTAMINATED ENVIRONMENT PRODUCES AN ANTI-GRAM-NEGATIVE COMPOUND**

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Gram-negative bacteria are a source of community and hospital-acquired infections. The presence of an outer membrane in their cell wall provides a barrier to the entrance of several antibiotics into the cell. This is one of the reasons behind the difficulty to identify new anti-Gram-negative drugs. A promising alternative to overcome this problem has been the search of novel clusters using bioinformatic tools and the exploitation of unique microbial adaptations to adverse environments. In this context, 22 bacterial strains belonging to Firmicutes phylum were isolated from two different environments highly contaminated with heavy metals: i) sediment from the open pit of Cunha Baixa abandoned uranium mine, Portugal, and ii) roots of the plant *Eryngium* sp. collected in the abandoned iron mine Ait Amar, Morocco. Their bacteriocinogenic potential was assessed against the Gram-negative indicators *Aeromonas hydrophyla* ATCC 7966, *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* 100603, *Pseudomonas aeruginosa* PAO1 and *Salmonella enteritidis* ATCC 13076. Colony-bioassay results showed that 9 *Bacillus* sp. strains were able to inhibit at least one of the following indicators: *A. hydrophyla*, *E. coli* or *S. enteritidis*. No antibacterial activity was detected against *K. pneumoniae* and *P. aeruginosa*. Among all the *Bacillus* sp. strains, the SMA1 strain (isolated from Cunha Baixa) was selected for further studies since it showed antibacterial activity against *A. hydrophyla*, *E. coli* and *S. enteritidis*. After *gyrA* gene sequencing, the SMA1 strain was classified as *B. amyloliquefaciens*. Presently, 11 *B. amyloliquefaciens* genome projects are finalized. The detection of genetic determinants for secondary metabolites production on these chromosomes was performed *in silico* using the antiSMASH 2.0 platform. Biosynthetic clusters of NRPS, PKS and terpenes were detected in all of them and one of a lantibiotic was found exclusively in one strain. The latter was also identified by PCR in the SMA1 strain and encodes the biosynthesis of mersacidin lantibiotic. Nonetheless, mersacidin is known to be active exclusively against Gram-positive bacteria. Thus, the bioactivity of SMA1 strain could be due to one of the other antibacterial compounds classes identified by genome mining. Therefore, identification studies are in course to identify the compound(s) responsible for such interesting spectrum of activity.

## Environmental Microbiology and Biotechnology

## P105

**BACILLUS INVICTUS SP. NOV., A NEW SPECIES ISOLATED FROM MEDICINAL PRODUCTS IN PORTUGAL**

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Three Gram-positive, rod-shaped endospore-forming *Bacillus* isolates Bi.FFUP1, Bi.FFUP2, and Bi.FFUP3 recovered in Portugal from medicines' contaminants were subjected to a polyphasic study. Acid production from mannose and its absence when inositol, mannitol, methyl  $\alpha$ -D-glucopyranoside, maltose, turanose and L-tryptophan is tested, discriminate these new isolates from its closest relatives, *Bacillus pumilus* and *Bacillus safensis*. Additionally, a significant different protein and carbohydrate signature was evidenced by spectroscopic techniques. PCA (principal component analysis) of this spectral data clearly delineated the novel species isolates. 16S rDNA analysis placed isolates within the genus *Bacillus* with highest similarities ( $\geq 99.7\%$ ) with *B. safensis* and *B. pumilus*. Nevertheless, only 49–50% DNA relatedness (DDH studies) was observed between Bi.FFUP1 and *B. safensis* FO-036bT. Variable DDH values were obtained when Bi.FFUP1 was compared with *B. pumilus* ATCC 14884 ( $39.75\% \pm 0.35$ ) and ATCC 7061<sup>T</sup> ( $69.40\% \pm 0.2$ ), despite the genotypic and phenotypic similarity of these two *B. pumilus* strains, highlighting the ambiguities in the taxonomic grouping based on a strict DDH cut-off. However, novel species isolates share 93.0% *gyrB* similarity with *B. pumilus* ATCC 14884 and 7061<sup>T</sup> and 91.3% with *B. safensis* FO-036b<sup>T</sup>. Further, *rpoB* similarity of 96.4% with close reference strains, together with *gyrB* and *rpoB* phylogenetic tree topology supported the delineation of a new species. On the basis of phenotypic characteristics (metabolic profile, protein and carbohydrate content) and phylogenetic analyses of *rpoB* and *gyrB* sequences, the three isolates represent a novel species of *Bacillus* genus, for which the name *Bacillus invictus* sp. nov. is proposed, with strain Bi.FFUP1 as the type strain.

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### P106

#### BACTERIA IMMOBILISED ON HYDROXYAPATITE SURFACE FOR WASTE WATER TREATMENT

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Heavy metals pollution in the environment is a big cause of concern, due to the toxicity of the metals themselves. In this work we propose an innovative method for heavy metals removal from contaminated waste water – a combination of hydroxyapatite (already used for water treatment) and microorganisms. Selected bacterial strains were immobilised on the surface of hydroxyapatite of natural origin (extracted from cod fish bones). These systems were used to tested on heavy metals, such as zinc and cadmium, from water solutions. The three strains used were *Pseudomonas fluorescens*, *Microbacterium oxydans* and *Cupravidus sp.*; they were selected as they showed high heavy metal resistance. SEM micrographies were used to study their immobilization on the hydroxyapatite surface. Solutions containing different concentrations of either Zn (II), Cd (II) or both metals at the same time were used. Results showed that the combination of hydroxyapatite and bacteria increases the metal uptake, indicating a synergistic action between the solid support and the bacteria biofilm. Particularly high removal rates were observed for higher heavy metal concentrations and for the *Pseudomonas fluorescens* strain. This work shows a promising way for environmental remediation, combining products of natural origin with microbiology.

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P107

### BIOACTIVE POTENTIAL OF MEDICINAL PLANTS FROM CERRADO, BRASIL

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Brazilian Cerrado is one of the world's biodiversity hotspots being a priority for conservation. It is threatened by logging and fire, which lead to the loss of medicinal plants still poorly known regarding their bioactive compounds. Within this context information has been growing 8% a year, although this is a biome with the largest plant diversity in the world and many medicinal plants are well known by the populations.

The goal of this work was to carry out a preliminary characterisation of crude foliar extracts of native medicinal plants in order to detect secondary metabolite groups with potential biological activity.

Samples were collected from 9 species (cajuí – *Anacardium othonianum* Rizz., inhare – *Brosimum gaudichaudii* Trécul., jatobá – *Hymenaea courbaril* L., jenipapo – *Genipa americana* L., aroeira – *Myracrodruon urundeuva* Fr. All., negramina – *Siparuna guianensis* Aublet., barbatimão – *Stryphnodendron obovatum* Benth., assa peixe – *Vernonia brasiliana* (L.) Druce, embaúba – *Cecropia pachystachya* T.). Ethanol and methanol extracts were obtained from oven dried material dried, filtered and concentrated on a rotary evaporator under reduced pressure, weighed and placed again in an oven. Yield (m/m) resulting from the ratio of the mass extract concentrated and after dry was determined. Tests were conducted for detection of the major groups of metabolites: saponins, phenols and tannins, catechins, steroids and triterpenoids, coumarins, anthraquinones and flavonoids.

Saponins, phenols, tannins, steroids, triterpenoids, and flavonoids were detected in the majority of species. The detection of phenols and flavonoids in all species except *B. gaudichaudii* is a relevant indicative of the presence of antimicrobial, antioxidant, and anti-insect activities.

The data gathered is strategic for strengthening biodiversity conservation policies of Cerrado, considering the medicinal resources still available in the native flora and their potential applications.

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## Environmental Microbiology and Biotechnology

### P108

#### BIOCIDAL MECHANISMS OF METALLIC COPPER SURFACES: A CLOSER LOOK INTO MEMBRANE DAMAGE

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Around the world billions of people touch surfaces many times a day, an important way of transmission of microbial pathogens in public and healthcare facilities. Among hospital users, hospital acquired infections (HAI) are one of the leading causes of death and morbidity among hospital users. The Centers for Disease Control and Prevention reported a total of one hundred thousand deaths only in the United States of America in the year of 2009 with aggravated costs over forty-seven billion dollars. In England, a report from 2005 estimates that each year there are approximately 5,000 people killed by HAIs and over one billion British pounds are spent in costs associated with HAI.

Copper minerals were used throughout the ages by many different civilizations for its natural medicinal and sanitizing powers. However, only recently, research has proven that applying metallic copper strongly reduces microbial surface-burden, both in laboratory settings and healthcare environments. This unique antimicrobial property of metallic copper (MC) is increasingly becoming recognized by microbiologists and hygiene specialists as a very promising novel tool for reducing hospital acquired infections.

This present work explores the molecular mechanism that leads to microbial death on MC surfaces. Previous data showed that cells are quickly inactivated by dry contact on MC. Cell contact on the MC leads to surface oxidation with release of high amounts of copper and, in addition, generation of toxic reactive oxygen species. Subsequently, this inflicts damage to the cell, more specifically to the membrane lipids which are the closest biomolecules. Eventually the severe membrane damage results in lethal structural instability. The cell's membrane potential is lost and cytoplasmic content may be released. After cell death other biomolecules are also degraded by oxidation, including degradation of cellular DNA.

Here, we explore the effect of the presence of osmoregulated periplasmic glucans (OPG) on the survival of *Escherichia coli* cells on MC. OPGs, among other functions, are membrane stabilization molecules. Cells with mutations on genes involved in the OPG biosynthesis are more sensitive to MC exposure. Furthermore, we investigated which fatty acids are more targeted by MC toxicity. Longer, ramified and the most predominant fatty acids are the most affect.

This work is the first step to deciphering which membrane constituents play a role in the killing mechanisms by MC exposure.

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## BIODEGRADATION OF PHARMACEUTICALS BY ACTIVATED SLUDGE AND PURE CULTURES

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The occurrence of pharmaceuticals in the environment has been a topic of increasing concern. Most of the pharmaceuticals are not completely mineralized in the human body and are released on the sewage systems as the pharmaceutical itself and as their “biologically active” metabolites through excretion, as well as by improper elimination and disposal. As conventional wastewater treatment plants (WWTPs) are not designed to remove these emerging pollutant, they are easily released into the environment. The effects of halogen on biological properties of molecules have had a marked impact on various fields such as pharmacology. Stability, bioavailability and interactions with the biological target can be improved, however the recalcitrance of those molecules to biotic and abiotic degradation increases. Ciprofloxacin (CPF) and diclofenac (DCF) are two widely used halogenated pharmaceuticals, commonly found in the environment in concentrations ranging from  $\text{ng L}^{-1}$  to  $\text{mg L}^{-1}$ . CPF is a common human and veterinary broad-spectrum fluoroquinolone antibiotic. DCF is a non-steroidal anti-inflammatory drug (NSAID), also used as analgesic and antithermic treatment. In the present study, biodegradation of CPF and DCF was assessed by selective enrichments with activated sludge and with pure cultures of three different strains – *Labrys portucalensis* (F11) and two *Rhodococcus* spp. (FP1 and S2) – which have previously demonstrated capacity to degrade a range of halogenated compounds. For CPF selective enrichments, degradation of 100% was achieved after ca. 6 months, although without fluoride release. These findings indicate that these selective enrichments are good candidates to find a bacterial strain able to biodegrade CPF. Regarding the pure cultures, F11 exhibited the highest degradation capacity, but no defluorination was observed. Concerning DCF selective enrichments, removal and liberation of chloride occurred after ca 4 months. Bacterial strains F11 and S2 were able to stoichiometrically dechlorinate the compound. These results indicate that these two strains are promising for DCF biodegradation.

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### CHANGES ON BACTERIAL NUCLEIC ACIDS AFTER PHOTODYNAMIC INACTIVATION BY CATIONIC PORPHYRINS

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The cellular damages caused by photodynamic treatment have already been studied but the mechanism of bacteria photodynamic inactivation (PDI) is not yet fully understood. Some authors have suggested that although DNA damage occurs, it is not the primary cause of bacterial cell photoinactivation. In the present study we will evaluate the in vivo effect of the photodynamic process on the nucleic acids of *Escherichia coli* ATCC 13706 by the two cationic porphyrins Tetra-Py<sup>+</sup>-Me and Tri-Py<sup>+</sup>-Me-PF.

The two photosensitizers (PS) were used at 5.0 µM upon white light irradiation (4.0 mW cm<sup>-2</sup>). Total nucleic acids were extracted from photosensitized bacteria after different times of irradiation and analyzed by agarose gel electrophoresis. The double-stranded DNA was quantified by spectofluorimetry. The integrity of the *E. coli* cell membrane was evaluated by determination of the release of material absorbing at 260 nm.

In order to evaluate the direct effect of PDI in the nucleic acids, *E. coli* nucleic acids were extracted, dissolved in TE buffer containing the PS and irradiated. After irradiation, the double-stranded DNA was quantified by spectofluorimetry and the samples were analyzed by electrophoresis. Fluorimetric analysis of DNA unwinding (FADU) was applied to detect DNA strand breaks in the bacterial nucleic acids after PDI. In this communication will be discussed all the experimental details and the main results obtained.

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### P111

#### CHARACTERIZATION OF *ESCHERICHIA COLI* FROM WATER AND FAECAL POLLUTION SOURCES: DATA TO ASSESS HEALTH RISKS AND TO IDENTIFY SOURCES OF CONTAMINATION

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Faecal contamination of aquatic systems is a major human health concern. The presence of faecal indicator bacteria (e.g. *Escherichia coli*) may represent, *per se*, significant risk, as they constitute reservoirs of antibiotic resistance (AR). We hypothesize that risk is significantly different depending on the source of contamination since different hosts imply different selective pressures (e.g. antibiotic exposure). This study compared the phylogenetic diversity and AR phenotypes and genotypes of *E. coli* strains from Berlenga Island from different sources. Strains were from contaminated seawater (n=166) and from two putative sources of contamination: seagull faeces (n=179) and domestic sewage (n=69).

Over 70% of the isolates belonged to phylogenetic groups A and B1, representing commensal strains. Group B2 was only present in sewage (10.1%) and gull faeces (3.9%). This group is known to comprise extraintestinal pathogenic strains. AR profiles were determined for 16 antibiotics. High percentages of resistance were detected, particularly to penicillins, aminoglycosides and tetracyclines. Surprisingly, levels of resistance were frequently higher in isolates from gull faeces and seawater. However, resistance to 3<sup>rd</sup> generation cephalosporins, imipenem and ciprofloxacin was mostly restricted to isolates from sewage. A high rate of multiresistant isolates was found, being lower for seawater and gull faeces (32%) and higher for sewage (39%). Differences in terms of AR profiles between sources were statistically significant. Isolates were screened for the presence of 15 AR genes. *bla*<sub>TEM</sub>, *tet*(A), *tet*(B), *sul2* and *sul1* genes were frequently detected in all sources. Genes conferring resistance to 3<sup>rd</sup> generation cephalosporins were detected in water (*bla*<sub>CTX-M-1</sub>) and gull faeces (*bla*<sub>CMY-2</sub>). The genetic context of these genes was identical to the one described for clinical isolates.

Our results suggest that the origin of faecal contamination affects the prevalence and diversity of AR genes and resistant bacteria in contaminated aquatic systems. The risk associated to contamination by wild animals is not neglectable as they constitute reservoirs of AR, including resistance to last-line antibiotics preferentially used in humans. AR phenotypes and genotypes can thus be used as markers to identify sources of faecal pollution in aquatic environments.

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### CHARACTERIZATION OF THE HYDROPHOBIC CHAIN OF *LACTOBACILLUS PENTOSUS* BIOSURFACTANT

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Nowadays there is high interest in the industrial use of natural detergents, named biosurfactants. Surface-active compounds have a wide range of applications e.g., these are used in the pharmaceutical, cosmetic, petroleum, environmental and food industries. Surfactants enhance the solubility of hydrophobic compounds in water. Polycyclic aromatic hydrocarbons (PAHs) are one of the most important and frequent soil and sediment contaminants. An ecofriendly strategy to remove these hydrophobic organic contaminants in soil could include the use of leaching agents such biosurfactants obtained from lactic acid bacteria. However, before adding biosurfactants to the soil it is necessary to carry out a complete characterization of the biosurfactants. Thus, in this work the fatty acid chain of biosurfactant from *L. pentosus* was characterized by was gas chromatography-mass spectrometry.

Biosurfactant from *L. pentosus* were produced and extracted following the methodology proposed by Vecino et al. (2012). Prior to the fatty acid characterization, biosurfactant was submitted to vacuum distillation, to remove the aqueous phase, and it was then dissolved in dichloromethane. The fatty acids were separated on a ZB-WAX column (length, 60 m; internal diameter, 0.25 mm and thickness 0.25 µm) and analysed by gas chromatography-mass spectrometry. The sample was dissolved in 500 µL of methyl tertiary-butyl ether and 250 µL of trimethylsulfonium hydroxide, and 1 µL of sample was injected into the chromatograph (at 250 °C, flow rate 100 mL/min). The standard used to identify the fatty acids of biosurfactant from *L. pentosus* consisted of Supelco 37 Component FAME Mix (10 mg/mL of the FAME reference standard mix in methylene chloride).

Results showed that the hydrophobic chain of the biosurfactant extracted from *L. pentosus* was composed by: linoelaidic acid, oleic or elaidic acid, palmitic acid and stearic acid in a ratio of 10:5:3:1 respectively.

The fatty acid chain of biosurfactant from *L. pentosus* is composed mainly by fatty acids containing 18 carbon atoms.

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**References:** Vecino Bello, X.; Devesa-Rey, R.; Cruz, J.M.; Moldes, A.B. Study of the synergistic effects of salinity, pH, and temperature on the surface-active properties of biosurfactants produced by *Lactobacillus pentosus*. *J. Agri. Food Chem.* 2012, *60*, 1258-1265.

## Environmental Microbiology and Biotechnology

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COLONIZATION OF *ARABIDOPSIS THALIANA* BY NEMATOCIDAL *SERRATIA* SP. M24T3 AND ITS EFFECTS ON PLANT DEVELOPMENTDiogo Neves Proença<sup>1</sup>; Stefan Schwab<sup>2</sup>; Márcia Soares Vidal<sup>2</sup>; José Ivo Baldani<sup>3</sup>; Gustavo Ribeiro Xavier<sup>3</sup>; Paula V. Morais<sup>4</sup>

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*Serratia* sp. M24T3 was found associated with the pinewood nematode *Bursaphelenchus xylophilus* from pine wilt disease (PWD) *Pinus pinaster* trees, as well as part of the pine endophytic microbial community. *Serratia* sp. M24T3 has shown to have nematocidal activity against *Bursaphelenchus xylophilus*, the causal agent of PWD, and its genome was sequenced.

The aim of this study was to evaluate the colonization process in *Arabidopsis thaliana* by *Serratia* sp. M24T3 marked with the *gfp* and *gusA* reporter genes, as well as plant growth factors produced by the strain.

*Arabidopsis thaliana* seedlings were inoculated *in vitro* with *Serratia* sp. M24T3 carrying the *gfp::gusA* plasmid pHRGFPGUS ( $10^6$  CFU.g<sup>-1</sup>) and separately with the original untransformed strain. Confocal laser scanning microscopy confirmed the presence of *gfp*-tagged M24T3 inside the root tissue, intercellular spaces as well as inside plant cells and vascular zones.

Inoculation of *Serratia* sp. M24T3, transformed and untransformed, decreased plant root elongation but increased the number of lateral roots, an effect probably related with the production of IAA by bacteria, which has been shown *in vitro* in the absence of plants. ACC deaminase gene was found in M24T3 genome suggesting that the strain can promote plant growth by lowering plant ethylene levels. Additionally, *Serratia* sp. M24T3 was able to produce siderophores and solubilizing phosphate and zinc oxide as plant growth promoting factors.

In conclusion, the plasmid pHRGFPGUS was an important tool to mark strain M24T3 and monitor the bacterial colonization of *A. thaliana*, without cause harm to plant. *Serratia* sp. M24T3 seems to be useful as a plant growth promoting bacteria and demonstrated to be able to colonize other plants beyond pine trees.

## Environmental Microbiology and Biotechnology

### P114

#### CULTURABLE ENDOPHYTIC BACTERIA FROM *HALIMIONE PORTULACOIDES*: DIVERSITY AND INFLUENCE OF Hg CONTAMINATION

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*Halimione portulacoides* is a halophyte salt marsh plant that tolerates high metal concentrations. Endophytic bacteria residing in the internal tissues of plants are known to improve plant stress tolerance and phytoremediation. However, information about the composition of this bacterial community is scarce.

Here, the phylogenetic diversity of cultivable endophytic bacteria from *H. portulacoides* was explored in plants from two sampling sites in *Ria de Aveiro*: one contaminated mainly with mercury (Hg site), and one non-contaminated (C site).

Endophytic isolates were obtained from above (AG) and belowground (BG) tissues (321 from the C site, 344 from the Hg site). Molecular typing of the isolates was performed by BOX- and ERIC-PCR fingerprinting. Representatives of each profile (n=328) were identified by 16S rDNA sequencing.

Isolates affiliated with 4 phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. The latter dominated both tissues and sampling sites (66–78%).  $\alpha$ -proteobacteria was the dominant class in BG tissues (50–58%), while  $\alpha$ - and  $\gamma$ -proteobacteria shared dominance in AG tissues (16–31% and 35–62%, respectively). From a total of 68 genera, 21 were found to be exclusive to the C site, whereas 23 were exclusive to the Hg site. The dominant genera were *Marinilactibacillus*, *Salinicola* and *Vibrio* in the C site and *Altererythrobacter* and *Labrenzia* at the Hg site.

Considering a cutoff value of 97% for same-species similarity based on 16S rDNA sequence, 260 isolates from the C site were attributed to 60 operational taxonomic units (OTUs), and 248 isolates from the Hg site were attributed to 61 OTUs. Both sites shared 31 OTUs and presented a Bray-Curtis similarity index of 0.35. Diversity analyses showed higher Shannon's diversity (3.68) and evenness (0.89) scores at the Hg site than in the C site (3.20 and 0.78, respectively).

This study revealed structural differences in endophytic bacterial communities from plants sampled in Hg and C sites and exposed an array of uncharted diversity present in internal tissues of a metal resistant halophyte. Further studies regarding metal resistance and plant growth promotion traits from these communities will be conducted.

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## Environmental Microbiology and Biotechnology

### P115

#### DIVERSITY AND ANTIBIOTIC RESISTANCE PATTERNS OF GRAM-NEGATIVE BACTERIA ISOLATED FROM WASTEWATER IN THE PRESENCE OF COPPER OR ZINC SALTS

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Some metals are nowadays worrying environmental pollutants. Although some, like copper and zinc, are essential for microorganisms, their presence in high concentrations can be toxic or exert a selective pressure in bacterial communities. Some studies propose that this selective pressure could also select for antibiotic resistant bacteria, and thus may play an important role in the maintenance and proliferation of antibiotic resistance. This is of particular concern considering that anthropogenic levels of heavy metals in the environment are currently several orders of magnitude above those of antibiotics. This study aims to investigate the effect of two metals ( $\text{Cu}^{2+}$  or  $\text{Zn}^{2+}$ ) on the survival and antibiotic resistance profiles of bacteria isolated from raw wastewater (urban and hospital).

Enterobacteria were isolated on mFC agar or on this culture medium supplemented with 1 mM  $\text{Cu}^{2+}$  ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) or  $\text{Zn}^{2+}$  ( $\text{ZnCl}_2$ ). Heterotrophic bacteria tolerant to the same metals were also enriched (3–5 successive transfers) from hospital effluent. Bacterial isolates were typed by RAPD-PCR, identified by 16S rRNA gene sequence analysis and characterized for antibiotic resistance. For *Escherichia coli*, the phylogenetic groups (A, B1, B2 and D) were also determined.

Enterobacterial counts were similar in urban and hospital wastewater. In both types of wastewater, the presence of metals in the culture medium inhibited the growth of enterobacteria, by up to 66% for  $\text{Cu}^{2+}$  and 98 % for  $\text{Zn}^{2+}$ . As expected, most of the isolates were *E. coli*, and the phylogenetic groups A and B2 were predominant. Resistance to ciprofloxacin, cephalothin and streptomycin was more prevalent among the isolates from  $\text{Zn}^{2+}$  supplemented culture medium than from metal-free medium.

Other heterotrophic bacteria isolated from metal enrichment cultures were identified as *Cupriavidus pauculus*, *Burkholderia anthina*, *Ralstonia picketti* and *Elisabethkingia anophelis*. Most (95%) of these isolates were resistant to three or more classes of antibiotics, contrary to what was observed for the enterobacteria in which only ~ 20% presented that phenotype.

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## Environmental Microbiology and Biotechnology

### P116

#### DIVERSITY AND PHYLOGENY OF DIAPORTHE SPECIES FROM DIFFERENT PLANT HOSTS

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The fungal genus *Diaporthe* includes species with a worldwide distribution occurring on a very wide range of hosts. Some species are known as plant pathogens of economically important hosts causing root and fruit rots, diebacks, cankers, leaf spots, blights, decay and wilts. Members of the genus are commonly reported as endophytes of many plant hosts and occasionally as pathogens of humans and other mammals. In addition some species are recognised as producers of relevant secondary metabolites.

In the past, recognition of *Diaporthe* species was based on morphology, culture characteristics and host association. However, with the introduction of DNA sequence data into species recognition it became clear that these criteria, especially host association, were not useful to discriminate species. Currently, much effort is being devoted to redefine species using a combination of phylogenetic, morphological and mating data.

The aim of this study was to resolve the *Diaporthe* species associated with a wide range of plant hosts, ranging from forest trees (e.g. eucalypts), to fruit trees (e.g. pear) and herbaceous plants (e.g. fennel), in Portugal.

A total of 177 isolates were obtained and subjected to BOX-PCR fingerprinting. Representatives of each group determined in this analysis were selected for further subjected to multi-locus phylogenetic analyses combining sequence data from the rDNA internal transcribed spacer (ITS) with partial sequences from protein coding genes, namely translation elongation factor 1- $\alpha$  (TEF1), beta-tubulin (TUB), histone H3 (HIS) and calmodulin (CAL).

The maximum likelihood phylogenetic analysis of the combined five loci clearly resolved all species. The isolates studied were distributed among nine clades, of which five correspond to known species: *D. novem*, *D. angelicae*, *D. infecunda*, *D. foeniculacea* (= *D. neotheicola*), *D. viticola*. The remaining four clades represent potentially new species that need to be further characterised. New reports for the country and new host-associations were identified.

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## DIVERSITY OF THE ENDOPHYTIC MICROBIAL COMMUNITY OF CORK OAK TREES FROM HEALTHY AND DECLINING AREAS OF MONTADO

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Plants namely trees, and hence the huge biomass of forests, constitute a true microbial ecosystem as there are very ancient terrestrial ecosystems, providing special ecological niches. The association between bacteria and plants is recognized, as well as the mechanisms leading to the selection of the microorganisms by them. The function of these microorganisms, mentioned as endophytic bacteria, ranges from the protection of the plant to promoting growth. There is also a large variety of plant pathogenic bacteria responsible for various diseases. Although, there is a large endophytic community in forest trees, specific pathogenic bacteria for these trees were not found.

The structural diversity of the endophytic community of *cork oak* was studied in two stands, Herdade do Freixo do Meio (FM) and Herdade da Gouveia de Baixo (GB). Each is constituted by two areas of contrasting tree health: area A with visually-healthy cork oaks, and area B with declining cork oaks and/or associated tree mortality.

The oaks were sampled at the trunk level and at the branches. A total of 680 colonies were isolated on R2A at 26°C, typed and identified by phylogenetic analysis based on 16S rRNA gene sequence.

In all areas analyzed, and regardless of the state of the stands, species of the genus *Curtobacterium* were found only in the branches where they constitute about 60% of the microbial community. In the branches were isolated 28 strains of the species *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, recognized as a pest of quarantine EPPO A2. The number of different bacterial species isolated could not be correlated with the sampling area of FM and GB or with the declined or healthy areas.

The functionality of the microbial species found is part of the ongoing research.

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### EFFECT OF SALINITY IN THE PRODUCTION OF RHAMNOLIPIDS BY *PSEUDOMONAS* STRAINS ISOLATED FROM RIA DE AVEIRO

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Biosurfactants have been gaining interest as a promising alternative over chemical surfactants mostly because of their lower toxicity, higher biodegradability, better environmental compatibility, and activity at extreme conditions. The production of biosurfactants by microorganisms is affected by the properties of the cultivation medium, namely in terms of nutrients and salinity.

In this study, the effect of the concentration of NaCl in the culture medium on the production of rhamnolipids by environmental strains of *Pseudomonas* previously isolated from the estuarine system Ria de Aveiro was studied. Changes in the structure of the biosurfactant produced, related to the salinity of the culture medium were also assessed.

Biosurfactant producing *Pseudomonas* strains were inoculated in half-concentration TSB medium supplemented with variable concentrations of NaCl in a range between 0 g L<sup>-1</sup> and 30 g L<sup>-1</sup>. Rhamnolipid production in the different experimental conditions was estimated in rhamnose equivalents, analysed by the orcinol method. In parallel, the structure of the rhamnolipids produced was also assessed by MS.

An inverse relation between salinity and biosurfactant production was observed in all tested strains but the decrease in the rhamnolipids production was particularly marked for salinity values above 20.

Considering that bacterial consortia of biosurfactant producing and pollutant degrading strains are regarded as a promising approach for the biorremediation of hydrophobic pollutants, the *Pseudomonas* strains isolated from Ria de Aveiro are rather tolerant to salinity and may have potential for application the recovery of impacted brackish-water systems.

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### ENDOPHYTIC MYCOBIOTA OF THE SALT-MARSH PLANT *HALIMIONE PORTULACOIDES*: DIVERSITY AND BIOTECHNOLOGICAL POTENTIAL

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Fungal endophytes are recognised as a natural source of useful secondary metabolites with potential applications ranging from agriculture to medicine. Moreover, there is a growing interest in this group of microorganisms as resources to improve crop production and ecosystem management.

*Halimione portulacoides* is a halophytic salt-marsh plant that is known to tolerate high levels of metal contamination. Although this plant has been widely studied virtually nothing is known regarding its endophytic mycobiota. Here we studied the diversity of endophytic fungi associated with this plant as well as their ability to produce extracellular enzymatic activities.

Isolations from root, stem and leaf segments from healthy plants collected in three sites from Ria de Aveiro yielded 111 isolates that were subjected to BOX-PCR fingerprinting. The rDNA internal transcribed spacer (ITS) region sequence was determined for representatives of each group retrieved in BOX-PCR analysis allowing the identification of 25 OTUs (operational taxonomic units). Most of the isolates (84,7%) were assigned to the Phylum Ascomycota with the majority of them (74,5%) belonging to the order Pleosporales. The remaining isolates were identified as fungi-like organisms belonging to the Oomycetes (Stramenopiles) with a clear dominance of the genus *Halophytophthora*. Fungal endophytic assemblages differed considerable between sampling sites and plant tissues, with only one OTU being present in all sites (*Fusarium incarnatum*) and another present in all tissues (an unidentified Pleosporales). Several OTUs could not be assigned to any ascomycete genus or species. These represent putative new genera and species that need further characterisation.

Most isolates showed the ability to produce extracellular hydrolytic enzymes, namely amylase, protease, lipase, pectinase, cellulase, xylanase, and urease. However, only two OTUs were able to synthesize laccases. Our findings indicate a complex and diverse endophytic mycobita associated with *H. portulacoides* tissues that represent a source of useful enzymes with biotechnological potential that should be further explored.

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### ENRICHMENT AND ISOLATION OF A BACTERIAL STRAIN ABLE TO DEGRADE SULFAMETHOXAZOLE

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Sulfonamides are a large family of synthetic compounds, which include antimicrobials, antidiabetics, diuretics, and pesticides. Hence, environmental contamination with these active ingredients is inevitable. Besides the toxic effects, some of these micropollutants may contribute to the acquisition and spread of antibiotic resistance.

The major aim of this study was the isolation of a microbial culture, with potential to be used in the treatment of sulfonamides-polluted waters.

A mixed culture able to mineralize sulfamethoxazole (SMX) under aerobic conditions was isolated from an enrichment culture of activated sludge and treated domestic wastewater. The mixed culture, comprising four different bacteria, could degrade SMX with the simultaneous stoichiometric accumulation of 3-amino-5-methylisoxazole (3A5MI), indicating that only the aniline moiety of SMX was transformed. The aniline moiety of SMX was observed to support bacterial growth, although with low biomass yields ( $0.100 \pm 0.023$  g SMX g cell dry wt<sup>-1</sup> at), when used as single source of carbon and energy (10 mM SMX) in mineral medium. In addition, it was evidenced that the aniline moiety of SMX was majorly transformed into CO<sub>2</sub>. The accumulation of 3A5MI at concentrations up to 20 mM did not inhibit SMX degradation.

Among the four members of the mixed culture, only *Achromobacter denitrificans* strain PR1 showed ability to degrade SMX in pure culture. However, in opposition to the mixed culture, strain PR1 could not degrade SMX in mineral medium and required an additional source of carbon and energy, such as succinate, or amino acids and/or vitamins and nitrogen bases to growth and remove the antibiotic. These observations suggest that strains PR2, PR3 and PR4, contribute for the activity of the mixed culture by providing strain PR1 with growth factors. Strain PR1, due to its ability to degrade SMX, may be an interesting tool to be used in wastewater treatment processes, since 3A5MI is inactive as antimicrobial agent.

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### *ENTEROCOCCUS HIRAE* INACTIVATION USING UV PHOTOLYSIS AND ADVANCED OXIDATION PROCESSES

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Chlorine is the most common disinfection agent used on the first line of microbial disinfection in food industries, water treatment and hospital surfaces.

The disadvantage of this use is the formation of disinfection by-products (DBPs), that occur by reaction of organic matter with chlorine and bromide present in the water. These compounds bioaccumulate in the tissues of animals and human and can be linked to development of cancer and reproductive problems [1,2]. Moreover, when used in certain surfaces it can damage them enabling bacterial adhesion, growth, and resistance to washing and disinfecting treatments.

Therefore, the use of ultraviolet (UV) radiation is gaining importance to inactivate contaminant microorganisms, due to its disinfection effectiveness against a wide range of pathogens. UV may be used directly or as advanced oxidation processes (AOPs) which are extremely promising techniques to inactivate microorganisms. AOPs are innovative inactivation technologies that rely on the generation of highly reactive, transitory radicals such as the hydroxyl radicals. Among the heterogeneous catalysts widely tested, titanium dioxide revealed to be one of the most promising materials for promoting a good level of disinfection.

In this study *Enterococcus hirae* was selected as a model microorganism from the Gram positive bacterial group. We used a bench-scale UV quasi-collimated beam set-up to compare the inactivation effectiveness of UV photolysis and UV photocatalysis (with different concentrations of titanium dioxide) at different exposure times. The results obtained show that direct photolysis is extremely efficient and achieves inactivation of *Enterococcus hirae*. Therefore using UV radiation seems to be a very promising safe chemical-free technique to be used in disinfection treatments in hospital ward surfaces, cleaning water and other industrial surfaces.

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### P122

#### *ENTEROCOCCUS* SPP. AND *ESCHERICHIA COLI* IDENTIFICATION BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS FINGERPRINTING

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*Enterococcus* spp. and *Escherichia coli* are commensal microorganisms from the gastrointestinal tract of humans and animals. The excessive use of antibiotics is an important factor in the natural selection of bacterial resistance in such bacterial populations. The identification method for routinely use in bacterial species identification should meet the following criteria: simplicity, reliability, high specificity and uniformity in analysis of various groups of microorganisms. Recently, Matrix-assisted laser desorption/ionization – time of flight – mass spectrometry (MALDI-TOF MS) was introduced for microorganism identification and it was demonstrated to be an accurate, rapid and cost-effective method. MALDI-TOF MS requires minimal sample preparation, achieving more than 92% correct species identification. Bacteria can be identified, for example, using the polymerase chain reaction (PCR), but even this sensitive technique does not provide sufficient information to unambiguously discriminate between species.

In this study, 120 enterococci and 120 *E. coli* isolates from wild birds of the Archipelago Azores were analysed through a streamlined protocol going from the seeding of bacteria up to the bioinformatics study of the MALDI-TOF MS data obtained. Using the bioinformatics web tool Speclust it was possible to compare 1200 mass spectra, 600 spectra of the *Enterococcus* spp. isolates and 600 spectra of the *Escherichia coli* isolates. In total, 215 distinct peaks were obtained by MALDI-TOF MS. Among these, it should be noted that the mass peak at  $m/z$   $4428 \pm 3$  was exclusively present in all the studied isolates of *Enterococcus* spp. and other two peaks ( $m/z$   $5379 \pm 3$  e  $6253 \pm 3$ ) were discovered only in the spectra of isolates from the genus *Escherichia* and not in the spectra of the isolates of enterococci. Suggesting that these peaks could represent a genus-specific biomarker. Notably, the spectra revealed unique mass peaks that could help the separation of the four species of enterococci from this study. The results from clustering also revealed a clear separation between the isolates of enterococci and *E. coli*. It's feasible that with further bioinformatics tools these spectra would discriminate peaks associated with antibiotic resistance. Furthermore, studies have shown that despite the high number of spectra obtained by this method, it is possible to detect common peaks among mass-related isolates, suggesting the possibility of identifying specific biomarkers of bacterial characteristics.



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**ENTEROCOCCUS SPP. RECOVERED FROM NASAL SAMPLES OF IBERIAN LYNX (*LYNX PARDINUS*): A GENOMIC APPROACH ON ANTIBIOTIC RESISTANCE DETERMINANTS**

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The Iberian Lynx (*Lynx pardinus*) is the most endangered felid in the world. Studies that contribute to this species conservation are important to secure the viability of the wild fauna in Iberic Peninsula. Antibiotic resistance is an emerging public health concern and some resistance determinants have been previously reported in bacteria recovered from fecal samples of wild animals, suggesting them as possible reservoirs.

Along with other study of our group on staphylococci, 17 enterococci were recovered from the nasal samples of 27 captive Iberian Lynx from CNRLI (National Centre for Captive Breeding of the Iberian Lynx), Portugal and CCLI (Iberian Lynx's Captive Breeding Center) La Olivilla, Spain. Swabs obtained were dipped into BHI broth, containing 6.5% NaCl; then, 150µl were seeded on MSA and in ORSAB media [2 mg/L Oxacillin]. Gram-positive cocci, others than staphylococci, were isolated in both media. During identification by GRAM staining, catalase test, bile-aesculin reaction, and molecular methods (*SodA* PCR and sequencing and Multiplex PCR *E. faecium/E. faecalis*) we defined the colonies from ORSAB medium as enterococci: 12 *E. faecalis*, 3 *E. faecium* and 2 *Enterococcus* spp. Susceptibility testing to 11 antimicrobial agents was performed by Kirby-Bauer's disk-diffusion test according to CLSI. Six enterococci showed susceptibility to all antibiotics tested and others presented resistance to the following antimicrobial agents: ampicillin (n=1), erythromycin (n=3), tetracycline (n=9), ciprofloxacin (n=5), trimethoprim/sulphamethoxazole (n=1), chloranphenicol (n=1) and high concentration of gentamicin (n=1), streptomycin (n=2) and kanamycin (n=3). The genes involved in these resistance profiles were *erm(B)*, *tetM*, *dfiK*, *aac(6')-aph(2'')*, *ant(6)-Ia* and *aph(3')-III*. Positive controls were used from the bacterial collection of the University of La Rioja, Spain. Enterococci's nasal carriage was never studied or reported and it's interesting to notice that commensal fecal bacteria can settle and be part of the natural bacterial microbiota of another animal body part. This could mean an exchange of resistance factors between bacteria by proximity. We think that animal behavior like eating and sniffing other animals' feces could have influenced our findings. Indeed, captive Iberian Lynx may possibly be contaminated through the food chain because the presence of resistant strains has been previously demonstrated in some food-producing animals and in their prey of preference, the wild rabbit. The proximity to humans may also contribute for the exchange of pathogens within their natural bacteria flora. One more time, wild animals are suggested as natural reservoir of bacteria resistant to multiple antibiotics. When genes that confer resistance to antibiotics settle into commensal bacteria in wild populations it's a matter of concern. The use of antibiotics it's affecting endangered "long distance targets".

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### ENZYMATIC SYSTEMS AS BIOMARKERS OF CULTURAL HERITAGE DECAY: A NEW APPROACH TO ASSESS ARTWORKS BIODETERIORATION STATUS

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Cultural Heritage decay is a serious problem with social and economic impact. Mural painting is one of the artistic expressions widely represented in Portugal, which over the time has suffered numerous forms of deterioration. Heritage decay depends on the physical properties and chemical nature of the materials, several environmental parameters and biological agents. The most harmful organisms involved in biodeterioration are fungi and bacteria, which are responsible by loss of cohesion, cracks, detachment of the paint layer and biofilms formation, fact which may induce microorganisms development, increasing the damage and promoting pigment alterations and discolorations of the paintings due to their metabolic activity [1, 2].

The main goal of this work was to identify the biodeteriogenic agents, evaluate and understand their effect in the mural paintings by metabolic activity monitoring, in order to obtain useful information for establish effective remediation strategies.

Four enzymatic systems were chosen to assess the physiological features of the predominant mural paintings colonizers and their biodeteriorative potential.  $\beta$ -glucosidase, phosphatase and arylsulphatase hydrolyse and catalyse specific reactions involved in the biogeochemical transformations of carbon, phosphorus and sulphur. On the other hand, dehydrogenase, detect viable organisms and can be considered an accurate measure of the microbial oxidative activity [3]. Real mortar microfragments were also enzymatically monitored and analysed by SEM to detect microbial contamination.

The predominant fungal isolates from mural paintings were *Rhodotorula* sp., *Penicillium* sp., *Cladosporium* sp. and *Aspergillus* sp., which seem to be the main responsible for artworks structural deterioration.

The metabolic activity of the microbial population can be correlated with the contamination levels detected and with biodeterioration status of the paintings. Therefore the enzymatic systems used constitute good biomarkers to be applied in this research field and are useful to detect biodeteriogenic agents.

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EVALUATION OF INHIBITORY EFFECTS OF *TRICHODERMA ASPERELLUM* METABOLITES ON MYCELIAL GROWTH OF *SCLEROTINIA SCLEROTIUM*

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Common bean (*Phaseolus vulgaris* L.) is an economically important food commodity in Brazil. However, it can suffer serious damage by white mould disease caused by the pathogen *Sclerotinia sclerotiorum*. Species of the genus *Trichoderma* can act as biocontrol agents against of this pathogen. This study describes the identification based on morphological, MALDI-TOF MS and molecular analysis of these 29 *Trichoderma* sp. isolates and their *in vitro* antagonistic behaviour against *S. sclerotiorum*. In order to evaluate the disease incidence greenhouse experiments were also performed using bean seedling. According to the results obtained, MALDI-TOF MS technique was appropriate for all *Trichoderma* species identification confirming the morphological and molecular-based identifications through analysis of rDNA ITS sequence data. *In vitro* inhibition experiments showed that 38% of *Trichoderma* isolates colonised the entire surface of the medium (grade 1 evaluation score) in dual cultures against *S. sclerotiorum*. Pathogen exposition to non-volatile metabolites produced by *Trichoderma* resulted in the inhibition of their mycelia growth between *c.a.* 83 and 100%. Moreover, 19 *Trichoderma* isolates enabled a total suppression of disease in bean seedlings when evaluated in greenhouse experiments. Two *Trichoderma* isolates identified as *Trichoderma asperellum* (CEN201 and CEN162) were responsible to the highest rates of growth promotion in bean plants, which ranged from 26 to 34%. The MALDI-TOF technique was appropriate for species designation for the majority of *Trichoderma* species, confirming most molecular-based identifications through analysis of rDNA ITS sequence data.

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### **EVALUATION OF MICROBIAL CONTAMINATION IN PUBLIC TRANSPORTS**

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Microorganisms are present in the environment and can colonize and survive in many places and inanimate objects, transforming them into sources of contamination. It is estimated that public transport vehicles have a high level of microbiological contamination, which may have effects on the health of users. Therefore, it is essential to assess the variability of microbiological agents in urban public transports, as well as the necessary conditions for their development. This study was based on sampling surfaces taking five smears from the surfaces in four buses of a public transport, before and after they were cleaned. In fact, users of this type of transport are exposed to bacteria, mostly Gram positive, and several genera of fungi. Through this research, it was found that even the cleaning is not always effective, and may sometimes even increase the proliferation of microorganisms. These results imply the use of more effective cleaning techniques so that minimize the risk of exposure, in order to protect workers and users of public transports.

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## EVALUATION OF PLANT–MICROBE INTERACTION IN 2-METHYLNAPHTHALENE CONTAMINATED SEDIMENTS

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Salt marsh sediments are sinks for various anthropogenic contaminants, such as petroleum hydrocarbons. Due to their ecological, commercial and economical importance, restoration approaches based on natural recovery processes are relevant. The interaction between plants and their associated indigenous degrading microorganisms (rhizo- and endosphere) have been explored as a process for removal or sequestration of pollutants. In this study, a factorial microcosm approach was used to investigate the response of the salt marsh halophyte *Halimione portulacoides* to the contamination with 2-methylnaphthalene. The effect of inoculation with indigenous endophytic hydrocarbon degrading bacteria was also assessed. During the experiments, plant photosynthetic performance was monitored by Pulse Amplitude Modulated (PAM) fluorometry, the structural diversity of sediment bacterial communities was assessed by denaturing Gradient Gel Electrophoresis (DGGE) analysis of 16S rRNA gene sequences and the clearance of the added hydrocarbon was evaluated by chemical analysis of the sediments.

The microbial community in the sediments significantly changed in response to the 2-methylnaphthalene amendment. However, inoculation of the plants did not improve their physiological condition when exposed to this hydrocarbon. Nevertheless, the inoculation had a positive impact in plant physiology in inoculated plants. The addition of 2-methylnaphthalene increased the frequency of genes encoding the large subunit of naphthalene-1,2-dioxygenase (*nahAc*) in sediment bacteria (with and without inoculation).

These results indicate that endophytic hydrocarbon degrading bacteria may have a growth promoting effect on the wild salt-marsh halophyte *Halimione portulacoides* and may be regarded as promising for microbe-assisted phytoremediation approaches.

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### EXTRACTION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) FROM SOIL BY USING BIOSURFACTANTS FROM *L. PENTOSUS* AS WASHING AGENT

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It has been demonstrated that *Lactobacillus pentosus* is able to produce biosurfactants by fermentation of hydrolyzates from trimming vine shoots, that were proposed to improve the spontaneous biodegradation of octane in soils (Moldes et al., 2013). However, no references there are in the literature about the utilization of this biosurfactant in the extraction of PAHs from soil.

Thus, in the current work biosurfactant from *L. pentosus* was evaluated as washing agent to extract different PAHs from soil.

Biosurfactant from *L. pentosus* was produced from vineyard pruning waste (Vecino et al., 2012). Following, *lactobacillus* cells were recovered by centrifugation, from the fermentation medium, washed twice in deionized water, and resuspended in phosphate-buffer (Moldes et al., 2013).

Soil samples, contaminated with a final concentration of 500 mg/Kg of PAHs, were treated with biosurfactant from *L. pentosus*.

To determine the amount of PAHs removed from the samples, the concentration of PAHs in the supernatant were analysed by spectrophotometry at 240 nm. Soil samples, without PAHs, washed with the same surfactant-ligand solution, were used as controls.

Results showed that biosurfactant from *L. pentosus*, was able to remove, in some cases, about 70 % of PAHs from soil. Thus, biosurfactant from *L. pentosus* could replace to the chemical surfactants used for the extraction of PAHs from soil like Triton X-100; Tergitol NP-10, Brij 30, SDS or Tween 80.

Biosurfactant from *L. pentosus*, obtained after fermentation of a low cost agricultural residue, can be used as washing agent to extract PAHs from soil.

Acknowledgments: We are grateful to Spanish Ministry of Economy and Competitiveness (project CTM2012-31873) and to the University of Vigo for the predoctoral contract of X. Vecino.

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### FUNGIWATCH: BENEFITS AND HURDLES ASSOCIATED WITH THE PRESENCE OF FUNGI IN WATER

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The main objective of this work was to evaluate the benefits and hurdles associated with the presence of fungi in water. An initial screening of the hundreds of isolates was therefore undertaken to select a significantly different group that was identified at species level and further evaluated in terms of their beneficial potential to degrade xenobiotics as well as their toxicity. In addition, since the effect of water treatment disinfectants in this area was unknown, ultraviolet radiation and chlorine (two extremely powerful disinfectants) were used to evaluate their disinfection effectiveness towards fungi.

After screening hundreds of isolates, 252 morphologically and microscopically different isolates were identified at genus level, 106 isolates were identified at species level, and 24 species were reported for the first time to occur in the aquatic environment<sup>1</sup>.

In terms of benefits associated with the presence of fungi in water, *Penicillium citrinum*, *Aspergillus fumigatus*, *Aspergillus terreus*, and *Trichoderma harzianum* were found to be resilient to a constant input of pesticides in the aquatic environment and to be able to degrade chlorfenvinphos when spiked individually and as mixtures in biodegradation experiments. These fungi may therefore be important in future bioremediation studies of agro–industrial effluents.

In terms of hurdles associated with the presence of fungi in water, we have shown that 41 of the 106 analyzed fungi were able to grow at high temperatures and 66% of those have their conidia measurements lower than 5 µm being therefore considered as potential pathogenic species to humans and animals<sup>1</sup>.

The effectiveness of UV direct photolysis as well as free and combined chlorine to inactivate fungi was also evaluated. Compared to other microorganisms, fungi require higher UV fluences to attain the same levels of inactivation and are expected to be more resistant to chlorine inactivation than *E. coli* and Polio 1, and less resistant than *Cryptosporidium* oocysts. With varying resistance towards disinfection, some fungi species are expected to be able to resist conventional drinking water treatment and to be present in the biofilms formed in distribution systems<sup>2–3</sup>.

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### GREEN FLUORESCENT PROTEIN APPLICATION TO VISUALIZE PROLIFERATION OF BACTERIA IN MORTAR FRAGMENTS

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Mural paintings support the growth of microorganisms commonly involved in biodeterioration, contributing to the esthetical damage such as overgrowth and discoloration, and structural damage like cracks and detachment of the painting [1]. Mural paintings are an integral part of the monuments, and their deterioration constitutes a loss of a significant part of the world's cultural heritage. Microorganisms such as bacteria can grow on the painting surfaces, providing different ecological niches that are exploited by a large variety of microbial species like fungi, algae and lichens. The microbial flora development is supported by many environmental conditions such as humidity, temperature, light and pH, but also by the nature and physical properties of the material [2].

The conventional techniques applied in this field seem not to be efficiently enough to visualise proliferation of microorganisms as bacteria, so it is necessary to develop new methodologies to visualise microbial proliferation, namely in consolidation materials applied in the field of conservation and restoration.

The aim of this work is to develop an alternative method for the detection of bacterial proliferation. This method involves a genetic alteration in bacteria to produce a green fluorescent protein.

The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* is the most commonly used reporter protein for monitoring gene expression [3]. GFP can be produced in bacteria containing the pGLO plasmid, which also contains the arabinose promoter that regulates the gene expression. The arabinose promoter controls the expression of the GFP gene in the pGLO plasmid.

In this study, the pGLO plasmid containing the gene of GFP was expressed in *Escherichia coli* JM109 HB101 K-12.

The transformation was confirmed with ultraviolet light and by fluorescence microscopy, and, the integrity of the pGLO plasmid was checked by agarose gel electrophoresis.

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HYPER ACCUMULATION OF ARSENIC IN MUTANTS OF *OCHROBACTRUM TRITICI* SILENCED FOR ARSENITE EFFLUX PUMPS (ARSB AND ACR3)Tânia Sousa<sup>1</sup>; Rita Branco<sup>1</sup>; Paula V. Morais<sup>2</sup><sup>1</sup>IMAR-CMA, 3004-517 Coimbra, Portugal<sup>2</sup>Department of Life Sciences, University of Coimbra, 3001-401 Coimbra, Portugal

Arsenic (As) is a natural metalloid widely spread in the environment due to natural reasons and anthropogenic activities. This element can occur in different oxidation states, but the most stable species are the forms of arsenite [As(III)] and arsenate [As(V)]. As-resistant microorganisms have developed a variety of mechanisms to resist to the harmful effects of arsenical compounds. One of these organisms is *Ochrobactrum tritici* SC1124<sup>T</sup>, a highly As-resistant bacterium that has two arsenic resistance operons, *ars1* and *ars2*. Among a large number of genes, these operons contain the *arsB* and *ACR3* genes that encode the arsenite efflux pumps responsible for arsenic resistance.

In this work, two single mutants (As1 and As2) and one double mutant (As3) of *O. tritici* were constructed. Whereas *O. tritici* As1 was obtained by disruption of *arsB* gene through insertion of a gentamycin resistance cassette (Gm<sup>r</sup>), the *O. tritici* As2 resulted from the insertion of a hygromycin resistance cassette (Hyg<sup>r</sup>) into *ACR3* gene. The mutant As3 was constructed using the previous strategy to disrupt both *arsB* and *ACR3* genes. Mutated and non-mutated strains were tested for arsenite resistance capacity. Both wild type strain and single mutant As2 showed a high resistance to As(III), being able to grow up to 20 mM of arsenite. The mutants As1 and As3 exhibited a low resistance capacity since they were not able to grow for concentrations higher than 5 mM and 1 mM, respectively. Mutants were also evaluated for their ability to accumulate arsenic when exposed to arsenite stress for 3 hours. In the presence of 1 mM As(III), double mutant was able to accumulate the highest intracellular arsenic concentration (up to 17 ng(As)/mg protein), while the type strain and the single mutants accumulated approximately only 2 ng(As)/mg protein. In assays with high arsenite concentration (5 mM), mutant As1 was able to accumulate the highest concentration of arsenic (up to 10 ng(As)/mg protein). These results show that *arsB* is the main gene responsible for arsenite resistance in *O. tritici* rather than *ACR3*. However, we should not ignore the fact that both genes play a crucial role in this resistance mechanism.

In conclusion, for moderate arsenite concentrations, the double mutant As3 exhibits a great ability to accumulate arsenic into the cells, which can be seen as a promising bioremediation tool for environmental arsenic detoxification.

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### IDENTIFICATION AND PHYLOGENY OF *TRICHODERMA* SPECIES BY MALDI-TOF ANALYSIS AND CHARACTERIZATION OF ANTAGONISTIC ACTIVITIES AGAINST *SCLEROTINIA SCLEROTI*

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Common bean (*Phaseolus vulgaris* L.) is an economically important food commodity in Brazil. However, it can suffer serious damage by white mould disease caused by the pathogen *Sclerotinia sclerotiorum*. Species of the genus *Trichoderma* can act as biocontrol agents against of this pathogen. This study describes the identification based on morphological, MALDI-TOF MS and molecular analysis of these 29 *Trichoderma* sp. isolates and their *in vitro* antagonistic behaviour against *S. sclerotiorum*. In order to evaluate the disease incidence greenhouse experiments were also performed using bean seedling. According to the results obtained, MALDI-TOF MS technique was appropriate for all *Trichoderma* species identification confirming the morphological and molecular-based identifications through analysis of rDNA ITS sequence data. *In vitro* inhibition experiments showed that 38% of *Trichoderma* isolates colonised the entire surface of the medium (grade 1 evaluation score) in dual cultures against *S. sclerotiorum*. Pathogen exposition to non-volatile metabolites produced by *Trichoderma* resulted in the inhibition of their mycelia growth between *c.a.* 83 and 100%. Moreover, 19 *Trichoderma* isolates enabled a total suppression of disease in bean seedlings when evaluated in greenhouse experiments. Two *Trichoderma* isolates identified as *Trichoderma asperellum* (CEN201 and CEN162) were responsible to the highest rates of growth promotion in bean plants, which ranged from 26 to 34%. The MALDI-TOF technique was appropriate for species designation for the majority of *Trichoderma* species, confirming most molecular-based identifications through analysis of rDNA ITS sequence data.

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## INTRACELLULAR POLY-P ASSESSMENT BY DAPI STAINING AND IMAGE ANALYSIS

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In wastewater treatment, enhanced biological phosphorus removal (EBPR) is considered a well-established process to remove phosphate (P). EBPR is based on the activity of polyphosphate-accumulating organisms (PAOs) able to take up and store large amounts of P as intracellular (poly-P) granules. However, monitoring poly-P in mixed cultures is usually performed by a laborious and time consuming off-line chemical analysis. Thus, there is a clear need to develop new techniques to rapidly monitor these processes, such as image analysis coupled to sample staining and microscopy inspection.

A lab-scale sequencing batch reactor (SBR) was fed with synthetic wastewater containing acetate and propionate as main carbon sources and an orthophosphate solution was added. A COD/P ratio of 10 mg COD mg P-PO<sub>4</sub><sup>-1</sup> was used to provide selective advantages to PAOs. The SBR was operated with a cycle time of 6 h: 120 min anaerobic including 5 min feed, 180 min aerobic and 60 min wasting/settling. Biomass samples were collected at the end of the aerobic stage. Bulk P concentration was determined by segmented flow analysis and total P concentration was similarly measured following acid digestion at 100°C. Intracellular poly-P concentration was determined by subtracting the bulk P from the total P. Intracellular poly-P granules were observed in epifluorescence microscopy using DAPI staining with a 25 µg mL<sup>-1</sup> DAPI solution. A long pass filter was used with an excitation bandpass of 365–370 nm and emission cut off at 421 nm. A specially developed program in Matlab was used for image analysis.

A total of 41 samples were collected. Two thirds were fed as training data to the partial least squares (PLS) model and the remaining used for validation. Both absolute (in mg poly-P / L) and relative (in mg poly-P / g MLSS) intracellular poly-P concentrations were studied. This procedure was found to predict, at some extent, the relative intracellular poly-P concentration (real poly-P = 0.971 x predicted poly-P, R<sup>2</sup> of 0.744). Regarding the absolute intracellular poly-P concentration, a total of 3 samples needed to be discarded in order to obtain a similar result (real poly-P = 1.005 x predicted poly-P, R<sup>2</sup> of 0.731).

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### ISOLATION AND CHARACTERIZATION OF PHAS-ACCUMULATING BACTERIA FROM HSSL

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Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible biopolymers. PHAs emerge as a possible solution as substitutes of petroleum based plastics, being produced under the Biorefinery concept, in which wastes and by-products of numerous industries may be used as carbon source.

This project aimed the isolation and characterization of organisms able to store PHAs from Hardwood Sulphite Spent Liquor (HSSL), a by-product of the pulp and paper industry. Isolation was performed from a Mixed Microbial Culture (MMC) selected under feast and famine conditions, using some components present in HSSL as substrates, such as acetic acid and xylose. Five pure isolates able to produce PHAs resulted from the successive streaking in solid medium containing HSSL. Two strains were identified as *Rhodococcus* spp. and three as *Pseudomonas* spp.. One isolate of each genus was selected and further studied in terms of growth and PHAs accumulation capability from three distinct carbon sources (HSSL, acetic acid and xylose). Both isolates, *Rhodococcus* spp. and *Pseudomonas* spp., were able to grow and use the three carbon sources as well as to produce PHAs. Both strains showed a higher maximum specific growth rate ( $\mu_{max}$ ) when HSSL was used as carbon source,  $0.212 \pm 0.0219 \text{ h}^{-1}$  and  $0.251 \pm 0.0526 \text{ h}^{-1}$ , respectively. A qualitative evaluation of the PHAs accumulation through Nile Blue staining showed a higher accumulation when acetic acid was used as sole carbon source. In an attempt to identify some of the species responsible for PHAs accumulation of the selected MMC, belonging to the dominant class, *Alphaproteobacteria*, a 16S rRNA gene clone library was constructed. The clonal analysis indicated the possible involvement of *Novosphingobium*, *Sphingobium*, and *Pleomorphomonas* species in the PHA storage process.

In conclusion starting from a MMC selected under feast and famine conditions for PHAs production from HSSL, strains belonging to four out of the 6 groups identified of the microbial community were isolated or identified. Five strains were successfully isolated using an isolation approach using solid medium containing HSSL. Strains belonging to the dominant class of the MMC were identified, through the construction of a 16S rRNA gene clone library, constructed on DNA extracted from the selected MMC.

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#### METHANESULFONATE-DEGRADING MARINE BACTERIA: A MOLECULAR APPROACH

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Methanesulfonate (MSA) is a very relevant product of the photo-oxidation of dimethylsulfide in the atmosphere representing possibly 50% of the atmospheric biogenic S fallout. The absence of MSA accumulation in the biosphere demonstrates that this chemically stable compound must be actively degraded in Nature. Indeed, bacterial strains capable of degrading MSA have been isolated and studied and the genes responsible for the import of MSA into the cell (*msmEFGH*) and for its oxidation to formaldehyde (*msmABCD*) have been sequenced from soil bacterium *Methylosulfonomonas methylovora* str. M2 while genes for the MSA monooxygenase (*msmABC* and *msmABD*) have been sequenced from marine bacterium *Marinosulfonomonas methylophila* str. TR3. Additionally, a previous screening of the Sargasso Sea metagenome for homologues of the *msm* genes retrieved one scaffold bearing sequences with high identity to the *msmABCD* cluster plus two scaffolds bearing genes highly identical to the *msmEFGH* operon.

These findings suggest that MSA-utilisers may be prominent members in the marine methylophobic community. However, the comparison with the total lack of genes encoding other typical C1 catabolic enzymes is striking and would seem to hint that the degradation of MSA, rather than other C1 substrates, may be a major methylophobic pathway active in ocean surface waters.

The goal of this work is to get ecological information about methanesulfonic acid-degrading bacteria from data on the distribution of the *msm* genes and to define the real relevance of this methylophobic pathway in ocean surface waters.

Through bioinformatic analysis and molecular biology techniques, such as PCR, cloning and sequencing, we aim at obtaining new *msm* gene sequences from partially explored bacterial isolates such as *Marinosulfonomonas* and metagenomic data from an enrichment previously obtained from ocean water from the coast of northern Portugal, and through the analysis of the data design new and more flexible PCR primers. The amplification and sequencing of 16S rRNA genes from the most representative amplicons will be performed: this will afford a general view of the taxonomic diversity in this community.

Up to now we have obtained new sequence data from 2 Sargasso Metagenome clones and from 2 novel marine strains. These preliminary results confirm the presence of this methylophobic pathway among microorganisms from diverse genera and ecological environments.

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### MICROBIOLOGICAL AND PHYSICAL-CHEMICAL CHARACTERIZATION OF SLUDGE GENERATED FROM WATER PURIFICATION PROCESS OF HUMAN WATER CONSUMPTION IN BRAZIL

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The water treatment plants (WTP) generate waste as residual decanters sludge due producing potable water. In Brazil, wastes generated from sewage treatment station (STS) have more importance than ones from WTP. The sludge releasing into water bodies can cause impairment to physical-chemical quality of water and injury to human health because of high frequency of pathogens. The objective of this study was characterize the sludge microbiologically from three collections of two WTP (A and B) of Londrina, Paraná, Brazil through microbiological indicators as *Enterococcus* spp, Clostridium sulphite-reducing and a pathogen *Salmonella* spp. The multiple tubes technique was used to isolated bacteria. For analyze physical-chemical parameters we used Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), turbidity, pH, apparent color, according to APHA, AWWA and WEF 2005. The results showed that in WTP-A for collection one, the most probable number (MPN) of microorganism per 100mL was  $2,1 \times 10^4$  *Enterococcus* and  $1,1 \times 10^5$  clostridium; for the collection 2, it presented absence of *Enterococcus* and  $>2,4 \times 10^5$  clostridium; and for collection 3,  $1,0 \times 10^5$  *Enterococcus* and  $2,4 \times 10^5$  clostridium. *Salmonella* spp. was not detected in any sample analyzed. The BOD was 113,9 mg of oxygen/L, COD of 6184,09 mg of oxygen/L, turbidity of 24000 NTU, pH 6,0 and apparent color of 100000 uH for collection 1. For the collection 2, BOD of 4334,59 mg of oxygen/L, COD (was not determine), turbidity 16800 NTU, pH 6,38 and apparent color of 6000 uH. In collection 3, BOD of 73,3 mg of oxygen/L, COD 2172,20 mg of oxygen/L, turbidity 4370 NTU, pH 6,90 and apparent color of 16700 uH. In WTP-B, for collection 1 we detected absence of *Enterococcus*,  $4,6 \times 10^5$  clostridium and  $3,0 \times 10^2$  *Salmonella* spp; for collection 2,  $1,5 \times 10^1$  *Enterococcus* and  $4,6 \times 10^5$  clostridium; and for collection 3  $4,3 \times 10^3$  *Enterococcus* e  $1,1 \times 10^6$  clostridium. For collection 1 the BOD was 156,5 mg of oxygen/L, COD 4372,7 mg of oxygen/L, turbidity of 24733 NTU, pH 6,01 and apparent color of 121666 uH. For collection 2, BDO (was not determine), COD 5848,6 mg of oxygen/L, turbidity of 49400 NTU, pH 6,99 and apparent color of 28000 uH. For collection 3, BDO 56,6 mg of oxygen/L, COD of 8363,9 mg of oxygen/L, turbidity of 48800 NTU, pH 6,51 and apparent color of 132500 uH. The microbiological contamination was relevant and high levels of physical-chemical parameters were detected in the decanters sludge, indicating high destructive potential for the environmental and healthy human population.

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### MONOCLONAL ANTIBODIES: PRODUCTION AND CHARACTERISATION TO RECOGNIZE PROTEIC BINDERS IN EASEL PAINTINGS

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Nowadays immunological techniques have been an important topic in heritage studies, like in easel paintings, for the identification of biopolymers such as proteins, in order to make their conservation and restoration. Enzyme-Linked ImmunoSorbent Assay (ELISA) is a very sensitive and specific technique and is capable of resolving complex mixtures of proteins, offering unique advantages over other analytical approaches mainly based on chromatography (usually combined with mass spectrometric detection). The commercial production of monoclonal antibodies (Mabs) for use in heritage studies has been developed for albumin, ovalbumin and casein binders, and holds enormous potential, however exist a lack of specific antibodies for animal glues [1].

The aim of this work is the production of Mabs for the detection of protein binders from animal glues, by hybridoma technology, and their purification and characterization.

For the production of specific antibodies, Balb-C mice were immunized with rabbit glue over six weeks. Three days after the last immunization, mice were bled and the titer was determined by ELISA. Subsequently, the spleen cells were fused with Sp2/0 Ag 14 in the presence of PEG. The Mabs were produced from clones in *in vitro* culture (RPMI 1640 +10% [v/v] fetal bovine serum) at 37°C and 5% CO<sub>2</sub> [2]. The antibodies produced were used in indirect ELISA assays allowing the detection of the target antigen (rabbit glue). The specificity of Mabs as well as their class and subclass were investigated by using Western Blotting and Ouchterlony double diffusion analysis, respectively. The purification of Mabs was carried out by immobilized metal affinity chromatography (IMAC) on epoxy-activated Sepharose 4B-IDA containing zinc ions.

This methodology shows high specificity that allows for resolving complex mixture of proteins distinguishing their biological source. This study offers advancement in this field and improvements in conservation and restoration of easel paintings and other artefacts.

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### MYCOSPORINES: YEAST BORN NATURAL SUNSCREENS

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The harmfulness of UV radiation to organisms is well known. The production of secondary metabolites as sunscreens has evolved as a mechanism to counteract the damaging effects of UV radiation, allowing the colonization of extremely insolated habitats. Mycosporines (MYCs) are low-molecular-weight water soluble molecules, absorbing UV radiation in the wavelength range of 310–365nm. They are produced and accumulated in prokaryotes (cyanobacteria), as well as in eukaryotes (microalgae, yeasts and fungi), playing an important and well-established role in UV protection. MYCs have also other physiological functions (e.g. as potent anti-oxidizers), being their production induced by photosynthetically active radiation (PAR), as well as by UV radiation, especially UVB.

This study was focused in a collection of yeasts recovered from highly insolated habitats: a uranium mine in Viseu, and an open lake resulting from acid mine drainage, located in Alentejo. All strains were tested for their resistance to UVA, UVB and UVC, and the minimal lethal doses (LD<sub>90</sub> and LD<sub>99</sub>) were calculated. The search for MYCs production was also performed by the preparation of aqueous methanolic cell extracts and analysis of the respective UV absorption spectra. Two yeast strains, each belonging to a different species (*Erythrobasidium hasegawianum* and *Rhodotorula lactosa*) and previously reported as presenting a high UV resistance and MYC production, were used as reference for UV resistance and for MYC presence.

Several yeast isolates under study showed higher resistance levels to UV radiation than the controls (LD doses = 311 to >405 mJ cm<sup>-2</sup> for UVB and 15 to 54 mJ cm<sup>-2</sup> for UVC). It was also possible to detect MYCs in one of these strains. However, probably this tolerance is not exclusively attributed to MYCs, as the presence of these compounds were not detected in the other isolates tested so far. Thus, other compounds should be evolved and further studies should to be conducted, in order to a better understanding of these tolerance mechanisms.

The study of natural sun protective compounds, such as MYCs, may represent a solution for a currently relevant issue: the production of highly efficient and non-harmful sunscreens formulations. Therefore, these type of natural, photoprotective compounds have clearly a huge biotechnological application, namely in cosmetics, and justify the effort for their study.



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## P139

OCCURRENCE OF IMP-METALLO- $\beta$ -LACTAMASES TYPES LOCATED ON CLASS ONE INTEGRONS AND OTHER ESBLs IN BACTERIAL ISOLATES FROM TUNISIAN RIVERSChedly Chouchani<sup>1</sup>; Rim Marrakchi<sup>1</sup>; Isabel Henriques<sup>2</sup>; Antonio Correia<sup>3</sup>

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Antibiotic-resistant bacteria (ARB) have been surveyed widely in water bodies, but few studies have determined the diversity of ARB in river waters. This study has been undertaken to investigate the origin of resistance among polluted-rivers bacterial isolates in Tunisia.

In this study 128 isolates resistant to  $\beta$ -lactam antibiotics were obtained from two polluted rivers in north of Tunisia. Isolates were identified using Phoenix phenotyping criteria. The occurrence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>CMY</sub>, *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> was studied by PCR amplification and sequencing and the genetic relatedness of the 16 IMP-producing *K. pneumoniae* isolates was analyzed by comparison of *Xba*I PFGE profiles.

Using Phoenix phenotyping criteria, diverse genus of bacteria was identified with different rates of prevalence with different MICs against different antibiotics. The occurrence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>CMY</sub>, *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> genes was confirmed. The DNA sequences upstream and downstream *bla*<sub>IMP</sub> genes were determined revealing that all IMP encoding genes constituted the first cassette of class one integrons, followed by *aacA* gene cassettes encoding aminoglycoside resistance. Comparison of PFGE profiles showed that only 2 of the isolates were clonal, the other 14 displaying a unique profile. The *bla*<sub>CTX-M</sub> gene was the most dominant of the ESBL genes, while the *bla*<sub>TEM</sub> gene was the second-most dominant.

The discovery of highly diverse ESBL-producing bacteria and MBL, particularly *bla*<sub>IMP</sub>, in polluted river raises alarms for potential dissemination of ARB in communities through river environments.

## Environmental Microbiology and Biotechnology

### P140

#### PHENOTYPIC AND GENETIC DIVERSITY OF RHIZOBIA ISOLATED FROM NODULES OF THE LEGUME GENERA *HEDYSARUM CORONARIUM* IN NORTH WEST REGION OF MOROCCO

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The species of *Hedysarum coronarium* L. (Tribe Hedysareae), Known by the Italian name of sulla or Spanish sainfoin, ranges within the mediterranean basin from northern Africa to southern Spain and in Italy from centrally to southern. In Morocco this phylogenetic resources are currently damaged by severe genetic erosion due to overgazing, irregular rainfall and reduction of rangeland.

Phenotypic and genotypic of 70 isolats, isolated from root nodules of *hedysarum coronarium* in north west region of Morocco, were studied using both phenotypic and genetic techniques.

The phenotypic tests (growth rate, tolerance to salinity, pH, Temperature, resistance to antibiotics and metals) revealed that most of isolats are belonging to rhizobium and genetic tests clustered all isolats into 26 different profiles.

Results of 16 S rARN sequencing revealed that th isolats were phylogenetically related to *Rhizobium Sulla strain IS 123T*.

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### PHENOTYPIC AND GENOTYPIC FEATURES OF ANTIBIOTIC RESISTANCE IN *SALMONELLA ENTERICA* FROM GULLS

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Gulls have been described as an important source of faecal contamination of coastal environments. However, the risks for human health associated to gull faeces are largely unknown. Several studies have shown that gull faeces carry potentially pathogenic bacteria like *Campylobacter* spp., *Salmonella* spp., *Listeria* spp. and verocytotoxin-producing *Escherichia coli* O157. In Europe, salmonellosis is the second most frequently reported zoonosis in humans. In the last years, resistance to antibiotics has increased significantly in *Salmonella* spp. with a marked proliferation of multiresistant strains such as *S. Typhimurium* DT104 and *S. Typhimurium* monophasic variant (4,[5],12:i:~).

The aims of this study were to investigate the diversity of *Salmonella* isolates from gull faeces and to assess their role as vectors for dissemination of antibiotic resistance. A total of 176 *Salmonella* isolates were typed by BOX-PCR. Representatives of each unique profile (n=66) were further characterized for serotype, antimicrobial susceptibility and antimicrobial resistance genetic determinants. Strains were also screened for the presence of genes encoding class 1 and 2 integron integrases and plasmids conferring drug resistance among Enterobacteriaceae.

The most frequently isolated serotypes in gull faeces were *S. Anatum* (43.9%), *S. Derby* (21.2%), *S. Typhimurium* monophasic variant (19.7%) and *S. Typhimurium* (10.6%). Prevalence of antibiotic resistance was higher for nalidixic acid (56.1%) and streptomycin (36.4%) followed by beta-lactams and sulphonamides. In addition, more than 90% of strains were resistant to at least one of the 16 antibiotics tested and approximately 6% were multiresistant. The resistance genes, *bla*<sub>TEM</sub>, *aadA1*, *qnrB* and *sul2*, and *gyrA* mutations were detected among the drug-resistant strains. Class 1 and class 2 integrons were detected in 17% and 3% of the strains, respectively. IncI1 plasmids were also identified in 17% of the *Salmonella* strains.

Our results show that Typhimurium and monophasic variant serovars were common in gull faeces and that the presence of multiresistance phenotype and mobile genetic elements were strongly associated to them. These evidences along with the fact that these serovars are among the most common agents of salmonellosis highlight the health risks associated to gull faeces.

## Environmental Microbiology and Biotechnology

### P142

#### PHOSPHOLIPID-DERIVED FATTY ACID (PLFA) ANALYSIS OF STANDARD EUROPEAN SOILS – STANDARD METHODOLOGY VERSUS MIDI ADAPTATION

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Two methods of soil phospholipid-derived fatty acid analysis are presented in this work, which are accessible for most research and analytical laboratories involved in soil sciences. The first method, here mentioned as standard procedure, simplifies the one described in the guideline ISO 29843-2 (ISO, 2011), which is derived from the method first proposed by Bligh and Dyer (1959) and later modified by White et al. (1979), Frostegard et al. (1993), and Feng et al. (2003). Soils are lyophilized and lipids are extracted using the Bligh and Dyer [1959] extraction procedure. Lipid extracts are separated by liquid chromatography using an SI-column. Phospholipids are transformed into fatty acid methyl esters (FAME) by mild alkaline hydrolysis followed by methylation. The different FAMEs are measured by Gas Chromatography (GC), identified and quantified using standards or Sherlock MIS data base. The second one uses the lipid extraction and separation of the standard procedure and the derivatisation and transmethylation of the MIDI FAME protocol (MIDI, Inc., Newark, DE, United States). These methodologies can both be used with a wide range of soils with different soil properties.

The modifications introduced to the method allowed substantial improvements. All modifications contributed to reduce the ISO/DTS 29843-2 protocol operational time. The time for PLFA extraction/separation was decreased in 1 day and 1 hour, respectively. The modifications introduced (Standard Method) did not change the balance of PLFA in comparison to the method described in the ISO/DTS29843-2. In addition, the modifications increased the total PLFA mass, by adding a lyophilization step. The alternative MIDI protocol for the Derivatization – Transmethylation step decreased the time needed for sample treatment in 32% and the volume of solvents in 62%. The total PLFA mass was in average, 40% lower when using the MIDI protocol, but the balance of PLFA was similar to the standard protocol and ISO/DTS 29843-2, and results were reproducible. Some bottlenecks are avoided using the MIDI protocol, such as the space available in a centrifuge, when using 20-30 ml tubes. This allows for the simultaneous treatment of more samples and therefore improves the throughput of the methodology.

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## PHOTODYNAMIC INACTIVATION OF BACTERIA: INFLUENCE OF EXTERNAL BACTERIAL STRUCTURES

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Photodynamic inactivation (PDI) is receiving considerable attention for its potential as a new form of bacterial inactivation. PDI combines a nontoxic photosensitizer with visible light to generate singlet oxygen and free radicals which are able to irreversibly oxidize bacterial vital constituents resulting in lethal damage. The main targets of PDI are the external bacterial structures, cytoplasmic membrane and cell wall. The aim of this work was to evaluate the influence of the external bacterial structures in the efficiency of PDI. To reach this objective a tetra-cationic porphyrin (Tetra-Py<sup>+</sup>-Me) at 5.0 µM, was tested against 8 bacteria with distinct external structures, 4 Gram-negative bacteria (*Escherichia coli*, with typical Gram-negative external structures; *Aeromonas salmonicida*, *Aeromonas hydrophila* both with a S-layer and *Rhodopirellula* sp., with a peptidoglycan-less proteinaceous cell wall) and 4 Gram-positive bacteria (*Staphylococcus aureus*, with typical Gram-positive external structures; *Truepera radiovictrix*, *Deinococcus geothermalis* and *Deinococcus radiodurans*, all with thick cell walls that give them Gram-positive stains, but including a second complex multi-layered membrane and structurally analogous to that of Gram-negative bacteria) upon white light irradiation at 4.0 mW cm<sup>-2</sup> for 270 min. The results show that the susceptibility to PDI with Tetra-Py<sup>+</sup>-Me changes considerable among bacteria with different external structures. Although all Gram-positive bacteria were inactivated to the detection limit (reduction of 8 log) after 60–180 min of irradiation, the inactivation followed distinct patterns (reductions of 4 log for *T. radiovictrix* and *D. geothermalis*, 6 log for *S. aureus* and 7 log for *D. radiodurans* after 30 min). Among the Gram-negative bacteria, *E. coli* was the only species to be inactivated to the detection limit (8 log after 180 min) under the PDI conditions used. The efficiency of inactivation of the two species of *Aeromonas* was similar (reduction of 5–6 log after 270 min). *Rhodopirellula* was the least susceptible to inactivation (reduction of 4 log after 270 min). The results support the statement that the outer cell structures are major bacterial targets for PDI. Moreover, the chemical composition of the external structures seems to have a stronger effect on PDI efficiency than complexity and number of layers of the external coating and lipids seem to be an important target of PDI, being even more important than proteins.

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### P144

#### PLASMID ASSOCIATIONS WITH INTEGRON-CARRYING BACTERIA FROM A COASTAL ENVIRONMENT

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The spread of multi-resistance genetic traits in bacteria from wild animals and natural environments mediated by conjugative plasmids may pose an important risk to both human and ecosystem health. For this reason, the characterization of conjugative plasmids outside clinical environments has gained a particular interest in the recent years.

In this study, we examined the prevalence, diversity and transferability of conjugative plasmids in 76 integron-carrying *Escherichia coli* strains obtained from seagull faeces and marine waters, on the Natural Reservoir of the Berlenga Island, Portugal. Strains were characterized in terms of phylogenetic group, antibiotic resistance patterns, replicon typing and mobilization to rifampicin-resistant *E. coli* CV601.

The majority of strains affiliated to commensal phylogroups B1 (37%), A0 (24%) and A1 (20%). Multi-resistance to three or more different classes of antibiotics was found in 83% of strains. The most frequently resistances detected were against tetracycline (87%), streptomycin (79%), ampicillin (70%), amoxicillin (70%), trimethoprim-sulfamethoxazole (70%), piperacillin (53%) and chloramphenicol (45%).

The presence of plasmids belonging to IncF, IncI1, IncN, IncY and IncK incompatibility groups was confirmed in 80% (60/76) of integron-positive strains. Moreover, in 25% (19/76) integrons were successfully mobilized through conjugation. Integrons detected harboured gene cassettes coding for resistance to aminoglycosides, trimethoprim, chloramphenicol and quaternary ammonia compounds.

Results obtained indicate the existence of a diverse plasmid pool in this coastal environment, associated with different resistance traits and incompatibility groups. In addition, the presence of identical genetic determinants in *E. coli* strains from different sources highlights the risk of spread of harmful traits through the mobilization of conjugative plasmids in natural environments.

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## PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANTS PRODUCED BY MICROORGANISMS ISOLATED FROM BRAZILIAN OILS

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Surfactants are amphiphilic molecules that comprise both hydrophilic and hydrophobic moieties, allowing the reduction the surface and interfacial tensions, as well as the formation of oil in water or water in oil emulsions. These surface-active compounds are extensively used by petroleum industries in order to reduce the capillary forces that entrapped the oil inside the reservoir. The compounds synthesized chemically, chemical surfactants, have some applicability limitations according some environmental restrictions. Contrarily, sustainable surfactants compounds can be produced naturally by microorganisms, designed by biosurfactants. The biosurfactants are a reliable alternative, since they exhibit lower toxicity, higher biodegradability, and effectiveness at extreme temperature, salinity and pH conditions. This work studies the production of different biosurfactant produced by microorganisms isolated from Brazilian oils. It was evaluated their abilities by measuring surface tensions, interfacial oil-water tensions and emulsification activities. Two *Pseudomonas* and three *Bacillus* strains demonstrated capability to grow and produce extracellular biosurfactants at 40°C. Additionally, the biosurfactants produced were characterized using different spectroscopic techniques, namely FTIR, <sup>1</sup>H NMR, ESI/MS and MS/MS, being the biosurfactants produced by *Pseudomonas* and *Bacillus* strains characterized as rhamnolipids and surfactins, respectively. The results obtained show that it is important to characterize the biosurfactants in order to understand their surface-active properties, as well as their formation of molecular aggregates: The biosurfactants chemical characterization allows the optimization of their application in bioremediation with crude oil, or in microbial enhanced oil recovery processes.

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### PRODUCTION OF EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) BY *CYANOTHECE* SP. CCY 0110 AND THEIR INTERACTION WITH HEAVY METALS

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Many cyanobacterial strains can synthesize and secrete extracellular polymeric substances (EPS) that can remain associated to the cell or be released into the environment (RPS–released polysaccharides). The particular features of cyanobacterial EPS, namely the presence of two different uronic acids, sulphate groups and high number of different monosaccharides (usually 6–10), makes them promising for biotechnological applications such as the removal of heavy metals from polluted waters. For the successful implementation of systems based on cyanobacterial EPS, it is necessary to unveil the pathways utilized for synthesis and export, identify the physiological/environmental factors that influence the synthesis and/or the characteristics of the polymers, and characterize the interactions between the cells/EPS with the metal ions. Previously, an *in silico* analysis of cyanobacterial genome sequences was performed and a putative mechanism for the last steps of EPS production/export was proposed [1]. In addition, the physiological/environmental conditions that promote RPS production by the unicellular marine N<sub>2</sub>-fixing cyanobacterium *Cyanothece* sp. CCY 0110 were evaluated. The results revealed that this strain is among the most efficient EPS producers and that the amount of RPS is mainly related to the number of cells (growth), rather than to the amount produced by each cell. Light is a key parameter, with high light intensity enhancing significantly RPS production. The polymer produced by *Cyanothece* is highly complex, composed by 9 different monosaccharides (including 2 uronic acids), peptides and sulphate groups and shows remarkable thermo stability [2]. The effects of different concentrations of Cu<sup>2+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup>, Li<sup>+</sup> and Cr<sup>3+</sup> in the growth/EPS production by *Cyanothece* were also assessed. As expected, cell growth was negatively affected by the presence of the metals, with the cells being more sensitive to copper (cell death at 0.2 ppm), followed by lead, cadmium and lithium (cell death at 70 ppm). The addition of chromium to the culture medium triggered the formation of cell aggregates, strongly affecting growth. In general, carbohydrate production followed the pattern of growth, with the RPS constituting 50 to 60% of the amount of total carbohydrates. The differential tolerance of *Cyanothece* cells to the metals is probably correlated to mechanisms of metal uptake and accumulation [3]. Therefore, the proteomes of the cells grown in the presence or absence of different metals were compared using Isobaric tag for relative and absolute quantitation (iTRAQ) technology. In addition, in order to assess the capability of *Cyanothece* cells and its isolated RPS to remove heavy metals from aqueous solutions, metal removal assays are being performed.

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## PROTEIC BINDERS USED IN EASELS PAINTINGS: CHARACTERISATION AND PURIFICATION

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The paintings are complex systems, with chemical compositions that change with time and depend on several factors, such as the interaction between pigments, binders and adhesives; the technique followed by the artist and the conservation practices. Unfortunately degradation of the original materials, co-presence of different proteins, environmental contamination and precedent addition of restoring materials make this task particularly difficult to be accomplished.

Some studies have identified traces of some animal glue, egg (albumin), milk (casein) on paintings. These natural organic compounds contain collagen, ovalbumin and casein respectively as main distinct proteins discovery. Due to the relevance of proteins as painting materials, their recognition is of great interest to characterize the artistic technique and for conservation/restoration purposes.

The aim of this work consists of characterization and purification of animal glues (rabbit, rabbit fur, sheepskin and fish) that are used as binders in easel paintings.

The organic content of animal glues was determined and proteins were fractionated and characterized by ion exchange chromatography (IEC), size exclusion chromatography (SEC) and native PAGE.

The composition of animal glues is mainly proteins, showing also the presence of high polysaccharide content and trace of lipids. SEC and IEC allowed the separation of 7 and 3 protein peaks respectively.

The final objective is the characterization of some types of binders, yet understudied, for development of identification methodologies using monoclonal antibodies (Mabs) [3]. Our research offers advancement in this field and improvements in conservation and restoration of easels paintings.

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### SCREENING OF GROWTH PROMOTIONAL TRAITS OF FIVE METAL RESISTANT PGPR – POSSIBLE USES FOR PHYTOATTENUATION OF ZN AND CD CONTAMINATED LAND

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Maize is a fast-growing and high yield crop with both energy value and phytoattenuation potential. Plant growth promoting rhizobacteria (PGPR) constitute a biological alternative to enhance plant establishment in stressing environments such as heavy-metal contaminated soils. Although PGPR are able to produce metabolites such as indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic (ACC) deaminase, siderophores, HCN and ammonia, these beneficial traits for the plant may be disrupted or decreased by metal exposure. In this study, five PGPR (*Pseudomonas fluorescens*, *Pseudomonas reactans*, *Ralstonia eutropha*, *Cryseobacterium humi* and *Agrobacterium tumefaciens*) were screened for those traits and for phosphate solubilization ability in the presence of different Cd/Zn concentrations. Furthermore, the effects of their inoculation on maize germination and seedling growth were assessed at different concentrations of Cd and Zn – 10, 30, 50 and 100 mg L<sup>-1</sup>, and 100, 250, 500 and 1000 mg L<sup>-1</sup>, respectively.

Results showed that generally with increasing metal concentration bacteria exhibited less ability to produce plant growth promoting substances, with some of the tested bacteria suffering a strong impact of metal concentration in their promotional traits. Nevertheless, generally, high production levels of ammonia, HCN and siderophores were found. ACC-deaminase and IAA levels were different between PGPR strains when exposed to the same metal level, showing dissimilar sensibilities of these mechanisms to the heavy metals. However, neither bacterial inoculation nor metal concentration had a significant effect on the germination rate of maize. Only *Pseudomonas fluorescens* was able to solubilize phosphate at all tested Cd concentrations and up to 250 mg Zn L<sup>-1</sup>. Root and shoot elongation generally decreased with increasing metal levels, but vigor index showed that, generally, PGPR inoculation had a positive influence on plant growth parameters, increasing metal tolerance.

Results suggest that PGPR can be exploited to promote maize biomass production and tolerance in moderately contaminated land. Such knowledge may provide a new insight and ground our choices concerning beneficial(s) microorganism(s) potentially suitable for microbial assisted phytoattenuation.

## Environmental Microbiology and Biotechnology

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### SEARCH OF NOVEL BIOACTIVE COMPOUNDS FROM EXTREME ENVIRONMENTS: BIOTECHNOLOGICAL POTENTIAL OF BACTERIAL STRAINS FROM PORTUGUESE ABANDONED URANIUM MINE

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Extreme environments are promising sources of novel microbial secondary metabolites produced by microorganisms inhabiting these environments. Among them are molecules with new chemical diversity and new mechanisms of action. With this in mind a screen of antimicrobial-producer bacteria was performed in sediments of an uranium mine (Quinta do Bispo – Viseu) deactivated since 1991. The samples were collected near the open pit, which contains very acidic water and where the concentrations of uranium series radionuclides and heavy metals are very high.

Bacteria were isolated in diluted (100X) and undiluted tryptic soy agar (TSA) and nutrient broth (NB) at pH 4 and 7. Selected bacteria were screened for antimicrobial activity against several Gram negative and Gram positive strains, including human pathogens, food spoilers and infectious agents with veterinary relevance.

TL11 (*Streptomyces anulatus*) and NL19 (*Pedobacter* sp.) strains are able to inhibit the growth of a wide group of bacteria.

TL11 and NL19 showed activity against *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* 9341, *Enterococcus faecium* 547261, *Bacillus cereus* Av2, *Haemophilus influenzae* 121642, *Klebsiella pneumoniae* 100603, *Listeria monocytogenes* 71, *Salmonella enteritidis* ATCC 13076 and *Staphylococcus aureus* ATCC 29213. Additionally, NL19 can also inhibit the growth of *Escherichia coli* ATCC 35281 and *Aeromonas hydrophila* ATCC 7966.

Identification studies are in course to determine the antimicrobials responsible for this activity. Furthermore, the products of NL19 and TL11 strains will be further exploited for other activities with biotechnological application (e.g. enzymatic activity, cosmetic, antipain, anti-inflammatory, etc).

## Environmental Microbiology and Biotechnology

### P150

#### SEASONAL VARIATION OF SANITARY QUALITY OF BIVALVE HARVESTING WATERS: PRELIMINARY STUDY BEFORE APPLYING PHAGE THERAPY

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The microbiological quality of bivalve molluscs depends mostly on the microbial load of the surrounding environment at growing sites. The establishment of the relation between harvesting water and bivalve microbiological quality will contribute to the accurate classification of production zones, the monitoring strategies of the bivalve molluscs growing waters and the adequate design of the depuration strategies to follow, namely phage therapy. The shellfish production zones of Ria de Aveiro are classified with the statute B and C, of the classification system (classes A through D, corresponding to healthy, non-healthy, highly non-healthy and forbidden harvesting, respectively). The main goal of this work was to evaluate the seasonal variation of bacterial indicators (*Escherichia coli* and *Salmonella* sp.) in two authorized harvesting zones of Ria de Aveiro and in two species of bivalve molluscs (*Venerupis decussata* and *Cerastoderma edule*). For this study, water and bivalve specimens were collected from Mira Channel [classified with statute B (230 – 4600 MPN *E. coli* per 100g of in flesh and intravalvular liquid)] and Ílhavo Channel [classified with statute C (4600 – 46000 MPN *E. coli* per 100g of in flesh and intravalvular liquid)] on six dates. *E. coli* was enumerated in water by the filter membrane method (adapted ISO 9308-1 and ISO16649-2). In bivalve molluscs, *E. coli* was enumerated according to ISO 16649-2. The detection of *Salmonella* in water and bivalve molluscs were done after enrichment according to ISO 6340 and ISO 6579, respectively.

The concentrations of *E. coli* in water in the Ílhavo Channel were higher than in the Mira Channel. The highest values in the two zones were obtained in December and August, and the lowest in April 2012. The presence of *Salmonella* sp. was only detected in August in the two zones. The concentrations of *E. coli* were higher in *V. decussata* than in *C. edule* in the Ílhavo Channel, however, in the Mira Channel, the concentrations of *E. coli* in the two species of bivalve molluscs were similar. The concentrations of *E. coli* in the Ílhavo Channel were higher than in the Mira Channel in the two species of bivalve molluscs. In the two zones, the concentration of *E. coli* was higher than 230 UFC/100 g in flesh and intravalvular liquid, indicating that bivalve molluscs should be depurated before commercial distribution. The presence of *Salmonella* sp. was detected in different periods in the two species of bivalve molluscs. The seasonal variation of the concentration of bacterial indicators demonstrates the need for a careful monitoring of the harvesting waters throughout the year in order to evaluate the suitability of the shellfish production zones. The occurrence of high values of bacterial indicators in summer suggest that the summer season is a critical time period where phage therapy should be applied in order to make a more effective depuration.

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**SHEWANELLA XIAMENENSIS STRAINS ISOLATED IN RIVER WATER THAT CARRY BLAOXA-48-LIKE GENES**

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The increase in carbapenem resistance is a major concern since these antibiotics are used as last-resort drugs to treat severe infections caused by multiresistant bacteria. This resistance is usually mediated by the production of beta-lactamases. The beta-lactamase OXA-48 was identified in 2001 in Turkey hospitals and this enzyme, as well as further described molecular variants, efficiently hydrolyze carbapenems. The chromosome-encoded beta-lactamases of *Shewanella* spp. have been recognized as progenitors of *bla*<sub>OXA-48-like</sub> genes. Although initially considered as geographically restricted, it has now been demonstrated that the spread of the *bla*<sub>OXA-48</sub> gene is one of the greatest concerns in terms of antibiotic resistance.

Here we report the isolation of three *S. xiamenensis* strains from river water in Portugal, carrying *bla*<sub>OXA-48-like</sub> genes. Sequencing of the *bla*<sub>OXA-48-like</sub> genes amplified by PCR revealed that these strains carried either a *bla*<sub>OXA-48</sub> or a *bla*<sub>OXA-204</sub> gene. Antimicrobial susceptibility and MICs were determined in Mueller-Hinton agar plates and interpreted according to the CLSI guidelines. All were resistant to penicillins and carbapenems but susceptible to 3<sup>rd</sup> generation cephalosporins and fluoroquinolones. MICs of ertapenem, imipenem and meropenem for OXA-204-producing strain were at least 4 times higher than those determined for the OXA-48-producing strains. Moreover, MICs for carbapenems were also higher than those previously described for *K. pneumoniae* carrying *bla*<sub>OXA-204</sub>. Sequence analysis revealed an identical genetic context for both *bla*<sub>OXA-204</sub> and *bla*<sub>OXA-48</sub> genes, bordered by the C15 gene upstream and the *lysR* gene downstream. This constitutes the first report of a *bla*<sub>OXA-204</sub> gene on *S. xiamenensis* strains. Our findings suggest that different *bla*<sub>OXA-48-like</sub> genes probably had origin in different *Shewanella* species. This also reinforces the importance of aquatic systems on the evolution and spread of antibiotic resistance.

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) through project Phytomars (PTDC/AAC-AMB/118873/2010) program COMPETE: FCOMP-01-0124-FEDER-019328

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### STRATEGIES TO ENHANCE THE REMOVAL OF FLUOROQUINOLONES

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Fluoroquinolones (FQs) are broad-spectrum antibiotics that play an important role in the treatment of serious bacterial infections. Currently, several FQs are available but ciprofloxacin (CPF), ofloxacin (OFL) and norfloxacin (NOR) are amongst the most worldwide prescribed antibiotics. Antibiotics can reach wastewater treatment plants (WWTP) from different routes. Thus removal of these contaminants during the biotreatment process is of major importance in order to avoid their release to other environmental matrices.

Granular sludge sequencing batch reactors (SBR) constitute a novel biofilm technology for wastewater treatment extremely promising for the treatment of effluents containing toxic compounds. Therefore, in this study a granular sludge SBR, established with activated sludge from a WWTP, was operated for the treatment of an aqueous stream containing FQs. No evidence of FQ biodegradation followed by HPLC with Fluorescence Detection was observed but FQs adsorbed to the aerobic granular sludge, being gradually released into the medium after withdrawal of the FQs in the inlet stream.

In a previous study, *Labrys portucalensis* F11 demonstrated to be able to degrade FQs, namely OFL, NOR and CPF, when supplied individually or as a mixture, in the presence of an easy degradable carbon source. Different removal extents were obtained for the tested concentrations (ranging from 0.8 to 30 µM), but overall the uptake capacity of strain F11 for individual FQs decreased with increasing the initial FQ concentration. When supplied with a mixture FQs, strain F11 concomitantly removed each target antibiotic but a decrease on the biodegradability of FQs was observed which could be explained by competition mechanisms.

The ability of *Labrys portucalensis* F11 to grow using the readily available carbon source while maintain its ability to degrade FQs reinforce the potential of this strain in bioaugmentation processes. As the indigenous microbial communities in biotreatment processes rarely are able to remove such contaminants, using this promising FQ-degrading strain, bioaugmentation strategies such as inoculation of the degrading strain, as a suspension or immobilized on carrier material, or using a plasmid donor strain carrying the degradative genes, could be assessed to improve FQ removal.

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## STUDIES ON ENANTIOSELECTIVE BIODEGRADATION OF FLUOXETINE

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Fluoxetine (FLX) is a chiral fluorinated pharmaceutical indicated mainly for treatment of depression and is one of the most dispensed drugs in the world. There is a clear evidence of environmental contamination with this drug. Granular sludge sequencing batch reactors (SBR) constitute a promising technology for the treatment of effluents containing micropollutants.

In this study, a SBR was operated in order to assess its performance when treating a synthetic wastewater containing racemic FLX (rac-FLX), under continuous and intermittent feeding of the compound. The concentration of FLX enantiomers was followed using an enantioselective HPLC method. A removal of 70% of the total supplied FLX was observed in the first continuous feeding period. However, the subsequent feeding periods revealed a significant decrease in the FLX removal: FLX liberation occurred during periods when no compound was supplied. This can be probably explained by desorption of FLX previously adsorbed to the granules. No intermediate metabolites or fluoride release were detected, corroborating the hypothesis that adsorption of FLX to the aerobic granules occurred. Moreover, the absence of enantioselectivity in the decrease of FLX enantiomers concentration is also an indicator of an abiotic mechanism.

In face of the incapacity of the aerobic granules to biodegrade FLX, the ability of *Labrys portucalensis* F11, a previously isolated microbial strain with the capacity to degrade a range of fluorinated aromatic compounds, to biodegrade this compound was investigated. In this study, the enantioselective biodegradation of rac-FLX and of its enantiomers was assessed. The results obtained revealed that this strain is able to degrade both enantiomers of FLX, when supplemented as a racemic mixture, as well as when supplemented as single enantiomers. Preferential degradation of the (*R*)-enantiomer was observed. This feature makes *L. portucalensis* F11 a potential candidate for devising biodegradation technologies able to deal with contamination by this pharmaceutical.

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### SURFACE ALTERATIONS DUE TO UNEXPECTED COLOUR ACQUISITION

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Historical built heritage undergoes deterioration by biological attack in association with environmental parameters, which affect architectural structures and alter aesthetic appearance of the materials [1].

Évora Cathedral (13th century) is the biggest Portuguese Cathedral and was classified by UNESCO as World Heritage, being imperative their preservation.

The present work intends to explain the pink colour in the Évora Cathedral inner walls, investigating the anthropic or natural sources of pink colour in the walls, by surface layers characterisation and its microbiological study.

Microsamples of the surface layer were analysed by scanning electron microscopy coupled with energy dispersive X-ray spectrometry (SEM-EDS),  $\mu$ -Raman spectroscopy and micro X-ray diffraction to material characterisation. For microbiological assays, samples were aseptically collected and inoculated in selective media to microbial development. Mortars microfragments were further analysed by SEM,  $\mu$ -Raman spectroscopy and ATR-FTIR [2, 3] to evaluate microbial proliferation and to characterise the chromatic and microstructural alterations of the walls.

As expected, the material characterisation showed no presence of inorganic chromophores and therefore the use of pigments in the mortars. The microbiological study allowed the identification of several bacterial strains, filamentous fungi of the genera *Penicillium* and *Cladosporium*, and, yeasts of the genera *Rhodotorula*. Particularly relevant was the fact that, the predominant microorganism isolated, *Rhodotorula* sp. yeast, exhibited a strong pink/dark orange colour that was further investigated to establish the effect of its growth on the mortars.

Spectroscopic approaches allowed evaluating the microflora proliferation, and detect oxalates and carotenoids, probably due to metabolic activity of the microorganisms and *Rhodotorula* development, respectively. Thereby, the pink colour which over the years has modified the original aspect of the Cathedral inner walls is due to biological processes rather than to Human intention.

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TARGETED SEARCH FOR *ACTINOMYCETES* FROM THE MADEIRA ARCHIPELAGOTiago Dias;<sup>1,2</sup> Florbela Pereira;<sup>1</sup> Natalie Millán;<sup>3</sup> Sofia E. Gomes;<sup>4</sup> Pedro M. Borralho;<sup>4</sup> Cecilia M. P. Rodrigues<sup>4</sup>; Paul R. Jensen<sup>3</sup>; Ilda Santos-Sanches<sup>2</sup>; Susana P. Gaudêncio<sup>1,2</sup><sup>1</sup>REQUIMTE/CQFB, Chemistry Department<sup>2</sup>CREM, Life Sciences Department, Faculdade de Ciências e Tecnologia Universidade Nova de Lisboa, 2829-516 Monte de Caparica, Portugal<sup>3</sup>Scripps Institution of Oceanography, University of California, San Diego, USA<sup>4</sup>iMed.UL, Molecular and Cell Biology of Eukaryotic Systems Unit, Faculdade de Farmácia Universidade de Lisboa, Portugal

The oceans cover 70 % of the Earth's surface and harbor most of the planet's biodiversity. Among this biodiversity, the marine bacteria are an important and unexplored resource for drug discovery. A total of 662 sediment samples were collected along Madeira archipelago and processed for the isolation of actinomycetes. The strains recovered were identified and evaluated for their ability to produce natural products with bioactive properties: (i) antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) and *Candida albicans* strains; and (ii) cytotoxic activity against the HCT-116 cell line. Around 400 actinomycete-like bacteria were isolated and a group of 82 strains was selected for species identification. Based on 16S rRNA gene sequencing, it was observed that the genera *Streptomyces*, *Micromonospora* and *Salinispora* were predominant (81%). This represents the first report of *Salinispora* spp. from the Northeast Atlantic Ocean. Crude extracts were obtained from 126 strains. Five of these crude-extracts showed antibacterial activity against both MRSA and VRE with MIC values ranging from  $7.8 \times 10^{-3}$  to  $1.3 \times 10^{-1}$   $\mu\text{g}/\mu\text{l}$  and six showed cytotoxic activity with IC50 values ranging from 4.9 and 19.7  $\mu\text{g}/\text{ml}$ . Two of those extracts showed both antimicrobial and cytotoxic activity. We are currently determining the structure of the compounds responsible for these activities. These studies demonstrate that the regions surrounding the Madeira archipelago are a rich source of marine actinomycetes with potential applications for biotechnology.

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### THE DYNAMICS OF MICROBIAL POPULATIONS DURING WINE FERMENTATION

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Grapes and wine musts harbor a very complex microbiome which has a crucial impact in wine fermentation, contributes to the stability of wine's *bouquet*, influences the wine flavour and the wine quality, and characterize the wine's identity. Unveiling the complex microbiome of grapes and wine musts, its dynamics and understanding the ecological factors that explain such biodiversity is still a challenge to oenology.

In this study, we have designed a metagenomic approach to deeply characterize the wine microbiome from 6 Portuguese wine appellations. For this, we have characterized the microbial communities associated with wine fermentations at three different stages: on the grape or initial must and at the beginning and end of spontaneous fermentation to allow for monitoring the dynamics of microbial populations.

Our data unravelled a much higher microbial biodiversity associated with wine fermentation than previously thought. Furthermore, we have observed that the microbiome of grapes differs significantly from the fermentation's microbiome and a much higher biodiversity at grapes was observed when compared with samples from spontaneous fermentation process. This variation on biodiversity reinforces the strong impact of the wine fermentation on microbial communities, which is higher on the fungal than on bacterial diversities.

Our data brings new insights on the evolution of wine fermentation, shows the first complete look into the wine related microbial communities and characterizes the natural microbiome at grapes/ initial musts and during spontaneous fermentations associated with different Portuguese appellations.

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### THE EFFECT OF PHOSPHATE-SOLUBILIZING RHIZOBACTERIA ON ZEA MAYS GROWTH ON P-DEFICIENT SOILS

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P-deficiency in soils is a limiting factor for plant growth. Several phosphate-solubilizing rhizobacteria (PSB) were used to enhance growth of *Zea mays* growing in a P-deficient soil. Strains were screened for their ability to solubilize P and to produce plant growth promoting (PGP) substances. The best-P solubilizing strains *Rhodococcus* sp. EC35, *Pseudomonas* sp. EAV and *Arthrobacter nicotinovorans* EAPAA were inoculated in maize growing in P-deficient soils without P fertilization and amended with soluble ( $\text{KH}_2\text{PO}_4$ ) and insoluble P ( $\text{Ca}_3(\text{PO}_4)_2$ ). Results showed that PSB significantly enhanced *Z. mays* biomass production in all P-treatments. Without P fertilization, bacterial inoculation increased plant dry biomass by ca. 20%, while under soluble P conditions the enhancement was higher. *Pseudomonas* sp. EAV was the strain that better performed improving root and shoot biomass by 104% and 60 %, respectively. In soils amended with insoluble P, plant biomass was also positive influenced by bacterial inoculation. Plant growth enhancement seems to be related not only to P-solubilization but also to other PGP traits, such as IAA and ACC-deaminase. This work shows that PSB may be used as bioinoculants and consequently constitute an attractive alternative to the phosphatic fertilizers amendments used to improve crop production.

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### THE IMPACT OF THE ISO CHEMICAL STRUCTURE ON THE ECOTOXICITY OF IONIC LIQUIDS

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Although ionic liquids can lessen the risk of air pollution due to their insignificant vapor pressure, they do have measurable solubility in water [1]. The water is thus the most likely medium through which ILs will be released into the environment. Their physico-chemical and biological properties can be tailored by a judicious cation/anion combination, and allows them to be tailored as *quasi* specific fluids for one particular application. As a result of this diversity of combinations, ILs are often referred to as “*designer solvents*”. In fact, there have been attempts at understanding their biological impact in terms of the alkyl chain length [2], the anion [3], the cation core [2] and the aromatic nature [4] on their (eco)toxicity. However, despite the large number of papers reporting their “*designer solvent*” character, the impact of some specific structural alterations in the ILs behavior is still scarcely studied. In this context, the impact of structural isomerism on the ILs’ ecotoxicity using four distinct cations is here properly investigated. For that purpose, several Microtox® test assays were performed [5]. The results suggest that it is possible to manipulate the biological impact by the incorporation of branched chains and that their influence on the ecotoxicity is dependent of the IL’ aromatic/non-aromatic nature [4].

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TRANSCRIPTOME ANALYSIS OF THE TBT (TRIBUTYL TIN) RESISTANT BACTERIUM *AEROMONAS MOLLUSCORUM* AV27: UNVEIL OF A POSSIBLE RESISTANCE MECHANISMRaquel Rodrigues<sup>1</sup>; Andreia Cruz<sup>1</sup>; Satoru Suzuki<sup>2</sup>; Sónia Mendo<sup>1</sup><sup>1</sup>*Departamento de Biologia & CESAM, Universidade de Aveiro, Portugal*<sup>2</sup>*Centre for Marine Environmental Studies (CMES), Ehime University, Japan*

Tributyltin (TBT) is a toxic compound that was used for several years as a component of antifouling paints. Due to its toxicity, the use of TBT was forbidden in several countries, leading to a decrease of its concentration in the environment. However, TBT pollution remains a serious problem, since this compound is still used in some countries and due to its persistence (mainly in the sediments).

*Aeromonas molluscorum* Av27 is highly resistant to TBT (up to 3 mM) and able to degrade it into the less toxic compounds dibutyltin (DBT) and monobutyltin (MBT). A transcriptome analysis was performed in Av27 cells exposed to TBT (5  $\mu$ M and 50  $\mu$ M), aiming to understand the molecular mechanism(s) that confers this trait. With that purpose, the transcriptome was obtained by pyrosequencing analysis. Validation of the transcriptome results was performed by quantitative real-time PCR.

Based on the information retrieved from the analysed transcriptomes (control and exposed) and also supported by previous studies, part of the mechanism for TBT resistance in this bacterium can be suggested. As described, once inside the cell, TBT is degraded into DBT and MBT, probably in siderophore-like structures. However, no results were retrieved for the presence of siderophores in Av27.

Following degradation, DBT and MBT may be extruded from the cell, a process mediated by efflux pumps and transporter proteins. Proteins involved in those processes were found over-expressed in Av27 strain, following exposure to TBT as, for instance, SugE, an inner membrane protein that might be responsible for the transport of DBT and MBT into the periplasmic space. Then, the outer membrane efflux protein TolC might be involved in the efflux of the TBT degradation products from the cell.

Yet, further studies are still required to fully clarify this intricate mechanism in *A. molluscorum* Av27.

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#### URANIUM MOBILIZATION BY BACTERIAL STRAINS ISOLATED FROM CONTAINERS WITH HIGH RADON ACTIVITY (300.000 BQ/M<sup>3</sup>)

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In Portugal the mining activities for radioactive ores started in 1908, and were centered on Beiras Province. This activity ceased in 2001 with closure of the main uranium milling facilities at the Urgeiriça mine. Large amounts of wastes were produced (about 13 Mton) and deposited near the old mining areas and required action to prevent dispersion of these radioactive hazardous substances to the surrounding environments. To avoid this possible contamination a rehabilitation plan, based on an *in-situ* reclamation scheme to promote the confinement of the radioactive residues was established: placement of a multi-layer cover consisting of geological and synthetic materials over the surface of the tailing deposits. In order to evaluate the long term efficiency of a clay layer on the containment of radon, a laboratory experience was conducted. In this assay an aluminum box with a square base (20 x 20cm) and 30 cm in height, was filled with a 4 cm layer of the tailing deposit and was covered with 2 cm of a clay layer that was properly sealed to avoid any leak. During one year, periodic measures were done and no radon activity was detected in the void space inside the box. After 3 years the radon activity measured in the void space of the box was very high reaching 300.000 Bq/m<sup>3</sup> and the clay layer presented cracks and a whitish biofilm. The presence of this biofilm was somewhat surprising, and a bacterial isolation in non selective R2A medium was performed. Several isolates were recovered from this biofilm and among them 4 strains showed the ability to grow in presence of 3 mM of U(VI). These strains were selected and further characterized, since there are few studies that report U tolerance up to 3 mM of U(VI) in aerobic heterotrophic microorganisms. The phylogenetic characterization based on the 16S rRNA gene sequence showed that these strains belong to the genera *Bacillus*, *Nocardioides* and *Sinomonas*. Preliminary results also showed that these strains were able to immobilize uranium in the cells, removing it from NB medium with uranium. All strains were also able to grow in water collected at Urgeiriça mine supplement with glucose, and were able to concentrate the radionuclides in the cells. The quantification of the ability to remove uranium from the environment was determined by liquid scintillation. This technique showed different uranium sequestration abilities among the strains. *Bacillus* strain 4.4 was the most efficient strain at high uranium concentrations. The presence of alkaline and acidic phosphatases in the API ZYM characterization of these strains supports the idea that phosphate can be involved in uranium immobilization of these strains.

## Environmental Microbiology and Biotechnology

## P161

## YES – YEAST ESTROGEN SCREEN FOR THE EVALUATION OF THE EFFECTIVENESS OF DRINKING WATER TREATMENTS

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Endocrine disrupting compounds (EDCs) are chemicals that interact with the endocrine system, even at very low exposure levels (ng/L). Hormones such as 17 $\beta$ -estradiol, 17 $\alpha$ -ethinylestradiol, estrone, estriol, and progesterone are major contributors of estrogenic activity in the aquatic environment. Endocrine disrupting capability is also attributed to the pesticides atrazine and alachlor. Since these EDCs pose a risk to the aquatic environment and human health, their occurrence should be monitored in drinking water.

Yeast estrogen screen (YES) is a reliable bioassay that provides the endocrine disrupting activity of water samples. Its application avoids the individual quantification of EDCs to assess water quality. YES relies on the use of a human estrogen receptor transfected *Saccharomyces cerevisiae* that was developed to identify compounds that can interact with the human estrogen receptor [1]. The receptor activation by estrogenic compounds leads to the expression of the reporter gene *Lac-Z*. This gene encodes the enzyme  $\beta$ -galactosidase, which metabolises the chromogenic substrate chlorophenol red- $\beta$ -D-galactopyranoside. The colour change observed due to this reaction is translated into estrogenic activity units – estradiol equivalents.

In this study YES is applied to evaluate the effectiveness of a drinking water treatment combining low pressure ultraviolet (UV) photolysis with nanofiltration to remove the aforementioned EDCs from a natural water. The removal of the target compounds from the treated water (often >90%) was accompanied by an extremely high decrease of estrogenic activity (>97%) for the hormones. Even though the pesticides UV by-products identified by mass spectrometry were retained by the membrane, others were generated at levels that did not enable detection. Following the formation/disappearance of such compounds, some of which presenting higher estrogenic activity than the target compounds, was then possible through the YES assay. Removals from the retentate higher than 79% were also obtained for all EDCs along with a decrease in estrogenic activity up to 95.5%. Data obtained show that high quality water was produced by the proposed treatment processes.

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## Environmental Microbiology and Biotechnology

P162/F20

### STUDY OF CHEMICAL OXYGEN DEMAND AND AMMONIA REMOVAL EFFICIENCIES BY IMAGE ANALYSIS AND MULTIVARIATE STATISTICS TOOLS

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Activated sludge systems are frequently used in wastewater treatment for chemical oxygen demand (COD) and ammonia removal. However, several problems can affect the operation of these systems leading to abnormal conditions such as filamentous bulking, viscous bulking and pinpoint flocs, among others. These occurrences, which may lead to the decrease of COD and ammonia removal efficiencies, are linked to biomass morphological and physiological changes and can be studied by microscopic evaluation. However, traditional microscopic inspection by a human operator, and correspondent manual assessment, is a subjective and labor intensive procedure. Automated image processing and analysis presents considerable convenience in such cases.

For this study, a lab-scale activated sludge reactor was operated for 100 days and monitored through microscopic staining and image analysis. The operational parameters were modified inducing the above mentioned abnormal conditions, apart from the normal operation. Biomass morphology was obtained by bright field microscopy combined with grayscale image processing. Biomass physiology was also studied by employing epifluorescence combined with color image processing. The LIVE/DEAD® BacLight™ Bacterial Viability Kit was employed to determine the biomass viability, and the LIVE BacLight™ Bacterial Gram Stain Kit for the biomass Gram status. Two ad-hoc Matlab specially developed programs were employed.

COD and ammonia removal efficiencies were studied by clustering the data points in two large clusters: “95% or above” and “below 95%” for the COD, and “90% or above” and “below 90%” for ammonia. These clusters were selected based on the behavior of these two parameters throughout the experiment time. The results showed that the COD removal efficiency was well predicted by the best 10 physiological parameters with an overall accuracy of 94.1%, for the ensemble of the tested conditions. Relatively high accuracies of 90.6% and 91.2% were also obtained for the ammonia removal efficiency regarding the best 9 physiological and morphological parameters, respectively. Thus, for the ammonia removal efficiency both types of parameters are equally useful, leading to 95.3% accuracy when the best 3 physiological and 6 morphological parameters were used.

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## Environmental Microbiology and Biotechnology

P163/F06

### MICROBIOLOGICAL CONTAMINATION BY USERS OF THE WAITING ROOMS OF THE HOSPITAL EMERGENCY DEPARTMENTS

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The waiting rooms of the hospital emergency departments are, every day, used by thousands of individuals – health workers, patients and their accompanying – susceptible to Nosocomial Infections, a Public Health problem. The relevance of studies to detection of microbiological agents present in such spaces becomes apparent, increasing knowledge of the microorganisms habitat of that and thus, controlling microbial contamination present, allowing maintenance of the Indoor Air Quality (IAQ) at the environment from the hospital. The objective of the present study aimed to evaluation of microbiological parameters regarding IAQ in two waiting rooms emergency department of a district hospital – waiting room after screening and waiting room of companions – located in the north central region Portugal. This involved the collection of air samples, using the equipment Air Sampler – 90 ActiveCount LightHouse and monitoring of temperature, humidity and the number of individuals present in the two rooms during the performance of crops, with subsequent quantification and identification of colony forming units (CFU) of bacteria and fungi. One of the most relevant parameters in this study was the influence of the hygiene in the variation of the quantity of microorganisms' isolated colonies. The results showed that there is microbiological contamination at defined sites and the cleaning interferes with the amount of CFU of microorganisms. The identified microorganisms were bacteria of the genus *Staphylococcus* and fungi of the genera *Absidia*, *Cladosporium*, *Geotrichum*, *Rhizopus*, *Aspergillus*, *Fusarium*, and even yeasts. So, despite the need of respecting the national legislation it's also essential to take correct measurements, concerning the methodology of cleaning the places, training and information for individuals designed for functions of cleansing of the spaces.

## Environmental Microbiology and Biotechnology

P164/F21

### NEW FINDINGS ON THE (ECO)TOXICITY OF CHOLINIUM-BASED IONIC LIQUIDS

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The ecotoxicity of ionic liquids (ILs) has been the subject of several studies. However, little attention has been paid to some IL families that are believed to be “non-toxic”. Cholinium-based ILs are an example of such a trend; the interest of industrial and academic communities into this IL family has been growing due to their *green* properties combined with their huge potential to be used in a wide range of industrial applications. Based on the fact that the cholinium is a complex B vitamin widely used as a food additive, the cholinium-based ILs are generally recognized as “harmless” and thus, accepted as “biocompatible” and “non-toxic”, being their ecotoxicological profile still underexplored. This work aims at contributing to the ecotoxicological database regarding cholinium-based salts and ILs, thus extending the surprisingly restricted knowledge about this family of compounds. The results depicted here indicate that all the 10 cholinium compounds investigated are not harmless towards the marine bacteria *Vibrio fischeri*. In general, the anion structure and the type of biological test adopted should be taken into account when assessing the (eco)toxicity of this IL family. Considering our findings, generalizations regarding the environmental hazard represented by the compounds, not based in tangible (eco)toxicity data, should be avoided.

## Environmental Microbiology and Biotechnology

P165/F08

## COMBINING ECTOMYCORRHIZAL FUNGI AND BACTERIA: A POWERFUL TOOL TO IMPROVE TREE PERFORMANCE

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Ectomycorrhizal fungi are ubiquitous to forest soils and the symbiosis between these microorganisms and plants roots is known to be essential for tree establishment and development, especially in areas of poor soil. Studies have also shown that some rhizosphere bacteria may act as plant stimulating agents and growth promoters. Additionally, bacteria and fungi strongly interact and therefore the analysis of the triangle plant–fungi–bacteria is a vital approach when aiming at the use of microbial inocula to enhance plant performance. To obtain a synergistic combination between microorganisms, a thorough selection is required since bacteria may strongly inhibit fungal growth, and vice versa, incurring in the risk of failed inoculum. In the present work we investigated the use of a dual inoculation system, consisting of a bacterial strain from the genus *Mesorhizobium* in combination with selected ectomycorrhizal fungi. *In vitro* tests were performed to assess the dynamic of co-culturing both microorganisms. The inoculum was applied at nursery stage and saplings were then transplanted into the field. Approximately 5000 seedlings were used in this experiment comprising the following species: *Quercus suber*, *Quercus robur*, *Quercus rubra*, *Pinus pinaster* and *Pinus pinea*. Seedlings were grown for 9 months in a commercial forest greenhouse and then transplanted to 4 locations in Portugal: Mindelo, Santo Tirso, Vila do Conde and Proença-a-Nova. Samples were taken at the end of nursery phase and 8 months after transplantation. Biometric and mycorrhizal parameters were analyzed and the persistence of the inoculated strains was evaluated. The presence of selected phytohormones was also determined. Results showed that the combined use of fungi–bacteria inoculum can be more effective than the use of each individual microbial partner. The use of ectomycorrhizal fungi and bacteria has great potential in forestry as a biotechnological tool to produce high performance plants at nursery stage and in the field.

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## Microbial Ecology

### P166

#### *ASPERGILLUS VERSICOLOR* AND *TRICHODERMA VIRENS* AS CONTAMINATION AGENTS IN A MUSEALIZED ROMAN FLOOR MOSAIC FROM THE 1ST CENTURY

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During the Museum Regional de Arqueologia D. Diogo de Sousa new building construction, a mosaic floor from a Roman house, dating probably from the 1st century, was found. Due to its rarity, dimension and quality, it was integrated in the underground floor of preserves not only the “in situ” mosaic but also the vestiges of a home from the Roman Era. The mosaic panels depict geometrical decorations of granitic and limestone tesserae with opposing colours.

However, the “in-situ” condition of the mosaic, with its soil base and recurrent climate dependent moisture fluctuations, created the conditions for a biocontamination to appear in more recent years.

The mosaic itself was covered by a thick, white and cream microbial biofilm, which includes a large area of the mosaic and also some adjacent home-related sections. Occasionally, some radial growth spots were detected, which seem to partially eliminate the original biofilm.

Samples were taken from specific, relevant areas, both from the mosaic and the house, from different surfaces and materials, as well as from the biofilm and the radial spots with wet (water) sterile swabs. Tesserae with and without contamination were also collected.

Observation with SEM allowed the identification of characteristic hyphae and other fungi structures. Samples were cultured in specific fungi and algae liquid media. A small number of fungal isolates was obtained and identified through ITS amplification as *Aspergillus versicolor* and *Trichoderma virens*. The results are in agreement with the observations made since *Trichoderma* sp. are fungal antagonists often used as biocontrol agents.

## Microbial Ecology

### P167

#### CAN INCUBATION CONDITIONS AFFECT BACTERIOPLANKTON BIOMASS PRODUCTIVITY ESTIMATES?

Inês Baptista<sup>1</sup>; Ana Luísa Santos<sup>1</sup>; Carla Pereira<sup>2</sup>; Newton C. M. Gomes<sup>3</sup>; Ângela Cunha<sup>3</sup>;

Não foi encontrada nenhuma entrada do índice de ilustrações.

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Heterotrophic bacterioplankton plays a crucial role in the aquatic carbon cycle, producing bacterial biomass that will be consumed by higher trophic levels of the food web. Thus, accurate estimates of bacterial biomass productivity (BBP) are crucial for microbial ecology studies and carbon flow models. The determination of BBP can be achieved by following the incorporation of radioactive leucine in bacterial proteins. Nevertheless, this technique has some limitations such as the dark incubation of the samples.

In this study, the influence of different incubation conditions on BBP estimates was studied in two different zones of the estuarine system Ria de Aveiro. Furthermore, the changes in community structure associated to different incubation conditions were monitored by denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene.

BBP results were significantly different between field and laboratory conditions. Although a defined pattern of variation was detected in the marine zone, with higher values of BBP obtained in laboratory dark conditions, this trend was absent in the brackish zone. In field conditions, variability observed in BBP results can be related to different physical and chemical characteristics of the water and to weather conditions, especially sunlight intensity. Moreover, bacterial community composition was considerably altered by the addition of leucine and during incubation in different experimental conditions..

We conclude that BBP should be determined at in situ light conditions. If not possible, the conventional dark incubation must be used in order to avoid problems such as reproduction of levels of ambient light and leucine incorporation by photoheterotrophs, cyanobacteria and phytoplankton.

## Microbial Ecology

### P168

#### HOW WILL LEAF DECOMPOSITION IN A FORESTED WETLAND BE AFFECTED BY CLIMATE CHANGE?

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Freshwater wetlands are unique habitats which sustain substantial biodiversity. Their biota is adapted to the spatial and temporal heterogeneity of those interface-systems. Most of the river plains are subjected to intense disturbances, mainly human-induced: modifications of river flow, changes in land use, decrease in groundwater level. Leaf litter breakdown rates can be a simple, powerful and low-cost tool to assess ecosystem integrity. During decomposition, organic matter undergoes a sequence of changes namely leaching, decaying and immobilization of nutrients within the decomposing litter. This chain of events is driven by environmental factors: moisture, temperature, biological colonisation, and litter quality. Changes in temperature and water level in wetlands could affect the decomposition dynamics and breakdown rates of organic matter. Our work presents the results of the decomposition of two leaf litters (alder and willow) in a forested wetland, dry in summer and wet the remaining year, located in central Portugal, in winter and summer. For that, litterbags were used to compare decomposition of alder and willow leaf litters in winter and summer. Throughout decay (days 2, 4, 8, 16, 32 and 64), bags were collected to measure dry weight loss, total-nitrogen, total-phosphorus and total organic carbon. Also, microbial colonization (colony forming units per foliar dry weight), functional microbial parameters such as community level physiological profiling (CLPP) and the presence of key-enzymes of C, P and N cycles were accessed during decomposition.

This study helps us to understand the effect of winter/summer, freely extrapolate to cold/warm and moisture/dough, on leaf decomposition upon a temperate forested wetland.

## Microbial Ecology

P169

### SIGNS OF MICROBE DOMESTICATION IN MEDITERRANEAN LINEAGES OF *SACCHAROMYCES CEREVISIAE*

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Domestication corresponds to the bred of an organism by man in such a manner that it becomes genetically distinct from its wild ancestors, in ways that make it more useful to humans to control its reproduction. The yeast *Saccharomyces cerevisiae* is employed since ancient times in products such as bread, beer and wine and it is possible that some lineages become domesticated through the selection of desirable phenotypic traits relevant for each of these products. The molecular characterization of *S. cerevisiae* strains from different human-driven fermentations revealed genetically distinct lineages, thus supporting the view that domestication occurred multiple times and, depending on the final product, has selected lineages with distinct characteristics. Despite the lack of detailed knowledge on how, where and when the multiple domestications of *S. cerevisiae* occurred, recent advances in population genomics are allowing the elucidation of the genes and the processes that underlie the phenotypic changes associated with domestication. In order to understand the evolution of domesticated phenotypes we sequenced the complete genome of representatives of the Mediterranean wild population of *S. cerevisiae* associated with oak trees and compared them with putatively domesticated lineages associated with wine fermentation. Next, phenotypic traits likely to have been selected during the domestication of wine lineages like sensitivity to copper sulphate, sulphite resistance and osmo-tolerance / freeze-thaw survival were analyzed in wild and domesticated lineages allowing us to put forward a model of wine yeast domestication.

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## Microbial Ecology

### P170

#### WARNING: TICKS ALERT!!! FIND OUT WHICH TICKS SURROUND US AND THEIR RELATIONSHIP WITH LYME BORRELIOSIS

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Bioecology of ticks, their pathogen load, the composition and density of their populations, as well as the climate, habitat structure, and human activities can influence the survival of the different tick species, determining their geographic distribution and importance as vectors. This has implications in public health, for emerging infectious pathogens that are being established in new endemic foci. In Portugal, there are suitable hosts and favorable climatic conditions that contribute to the distribution and maintenance of ticks and tick-borne diseases in nature.

In Europe 65,000 human Lyme Borreliosis (LB) annual cases are estimated, being the most prevalent arthropod-borne disease in the temperate regions of the northern hemisphere. However, regarding the high prevalence of under-reported cases, and its endemic character in USA, the world annual number of LB cases is estimated to be 255,000. In Portugal, several human LB cases have been recognized/reported since 1989, confirming the circulation of pathogenic strains in local tick populations.

The aim of this study was to evaluate the infection rate of *Borrelia burgdorferi* sensu lato (*B.b.s.l.*) in ticks collected from animals and vegetation, in nine sites of Portugal, contributing to update tick's fauna and LB epidemiology in the country.

Ticks were removed from animals, and collected by dragging/flagging from the vegetation in nine sites of Portugal. These ticks were identified to species level, and screened for *B.b.s.l.* DNA by two different nested-PCR protocols, targeting the Intergenic Spacer Region between 5S and 23S rRNA and the *flagellin* genes.

From May 2012 to June 2013, 4,487 ticks were collected (1,769 females, 1,283 males, 817 nymphs and 618 larvae's) belonging to *Ixodes* spp., *Rhipicephalus* spp., *Haemaphysalis* spp., *Hyalomma* spp. and *Dermacentor* spp., with different capture efforts. *I. ricinus*, the main LB vector in Europe, was present in six of the nine sites. From the 704 studied ticks, so far, *Borrelia* DNA was found in 4.8%, and *Borrelia garinii* was the most prevalent species.

*B.b.s.l.* DNA was found not only in *I. ricinus*, but also in other tick species, being nymphs the most infected immature stage. Although the vector competence of these other tick species hasn't been proven so far, they can be considered as potential vectors with specific distributions and sylvatic cycles, contributing to the maintenance of Lyme agents in Portugal.



## Microbial Ecology

### P171/F07

#### CROP ROTATION AND CROPPING PHASE IN RICE PADDIES AND THEIR RELATIONSHIP WITH VARIATIONS OF THE SOIL MICROBIAL COMMUNITIES

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The agricultural productivity and soil fertility are intrinsically associated with the soil microbial communities. The type of management and crops can influence the microbial communities in agriculture soils. Studies about microbial community structure and activity and the assessment of possible correlations with external factors may contribute to improve the knowledge on the role of microbiota in soils. Crop rotation consists on the sequential cultivation of at least two types of crop (e.g. legume-cereal) in the same area. In the present study, we analysed paddy soils subjected to a rotation of alfalfa (two years) and rice (two years). During the second half of the rotation cycle, *i.e.*, 1<sup>st</sup> and 2<sup>nd</sup> year of rice cropping, samples were collected before seeding, at the maximum tillering phase and after harvesting, and were characterized for physicochemical, biochemical and microbiological traits. Variations possibly due to the (i) phase of crop rotation and (ii) phase of annual rice cycle on the bacterial community structure and functional activity of these soils were assessed using multivariate analyses.

The results showed that the phase of crop rotation coincided with variations in the composition and structure of the soil bacterial community. In the 1<sup>st</sup> year of rice cultivation, the abundance of aerobic heterotrophs was positively correlated with lineages affiliated to *Rhizobiales*, *Rhodospirillales*, *Sphingomonadales*, *Flavobacteriales*, *Sphingobacteriales*, among others. In the 2<sup>nd</sup> year, the abundance of bacteria affiliated to *Bacteroidales*, *Anaerolineae* and *Chlorobi* was positively correlated with the soil carbon content. Variations on the microbial catabolic activity were in agreement with the variations in the community structure. Also the phase of the rice cropping cycle was related with variations in the microbial activity. Proteolytic activity and aerobic and anaerobic heterotrophs were predominant before rice seeding. After rice harvesting, the increase of cultivable diazotrophic microorganisms suggests a higher N<sub>2</sub> fixation. The results obtained suggest that polyphasic studies may shed some light on “cause-effect” relationships, which may be useful to improve agriculture practices.

## Microbial Ecology

P172/F19

### BACTERIONEUSTON ECOLOGY OF THE ESTUARINE SYSTEM RIA DE AVEIRO: AN OVERVIEW

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Bacterioneuston is the community of microorganisms that develops at the surface microlayer (SML), the uppermost layer of the water column of fresh water and marine ecosystems. Although very thin (1 mm), the SML is a critical compartment to global scale processes such as carbon cycling, production of climate-active aerosols, and accumulation and dispersion of pollutants. As a microbial habitat, the SML is regarded as an extreme environment because of the enhanced exposure to physical (solar radiation, surface tension) and chemical (heavy metals and hydrophobic pollutants such as hydrocarbons and pesticides) stress factors. On the other hand, this compartment is also enriched in organic nutrients that can sustain dense microbial populations. Bacterioneuston communities are often denser than bacterioplankton developing just below which has been interpreted as an evidence of the adaptation of microbes to life in the SML.

In estuaries, the stability of the SML is challenged by physical forcing mostly associated to winds and tidal currents. In Ria de Aveiro, conditions for the establishment of a defined SML occur mainly at the inner brackish water sections of the estuary, where current velocities are lower. However, the factor of enrichment of bacterioneuston communities in relation to bacterioplankton can vary considerably during the tidal and diel cycles.

Bacterioneuston communities of Ria de Aveiro are characterized by a high proportion of attached cells, high tolerance to chemical surfactants and a high frequency of biosurfactant producing strains. These features may be related to the adaptation of this community to the surface tension at the air-water interface. Bacterioneuston is also less sensitive to UV radiation and recovers more efficiently than bacterioplankton from UV-induced damage. In relation to bacterioplankton, bacterioneuston displays higher rates of extracellular enzymatic activity but lower rates of monomer incorporation, denoting an adaptation to the use of polymeric substrates as carbon sources, and also an enhanced capacity for the degradation of hydrophobic organic pollutants such as polycyclic aromatic hydrocarbons.

The results obtained so far confirm bacterioneuston as a community distinct from bacterioplankton in terms of lifestyle and function and make Ria de Aveiro a reference model system in the context of bacterioneuston ecology.

## Session 3

Health Microbiology and  
Biotechnology

# Plenary Lecture

## *PSEUDOMONAS AERUGINOSA* POPULATION DYNAMICS DURING CHRONIC LUNG INFECTIONS

Craig Winstanley

*Institute of Infection and Global Health, University of Liverpool, Liverpool, UK.*

Chronic lung infections due to *Pseudomonas aeruginosa* remain the main cause of the morbidity and mortality associated with cystic fibrosis (CF). Although many CF patients acquire their *P. aeruginosa* from environmental sources, it is clear that some strains can transmit between patients. Since its emergence in the mid 1990s, we have studied the transmissible Liverpool Epidemic Strain (LES), a CF-adapted clone that is widespread amongst CF patients in the UK and has been reported in North America. Detailed characterisation of the strain has revealed novel phenotypic and genotypic characteristics, including multiple prophages implicated in the competitiveness of the strain during infection. We have analysed both phages and populations of the LES from the sputa of multiple chronically infected adult CF patients. We detected high levels of free phages in all sputum samples. Following the establishment of a chronic infection in the lungs of CF patients, populations of *P. aeruginosa* LES adapt and diversify due to mutation. Hence, multiple single strain isolates from the same patient sample can exhibit diversity in phenotypes such as quorum sensing, mucoidy and antibiotic resistance. Using phenotypic and genome sequencing we have compared LES populations between patients and followed population changes over time, including during periods of exacerbation. Our data suggest that populations are highly variable between patients and dynamic within patients over time periods of several months. In parallel, we have used an artificial sputum medium (biofilm) model system to study potential factors driving diversification and adaptation, such as biofilm lifestyle, antibiotics, bacteriophages and other microorganisms. The potential causes and implications of diversification, and the use of genomics to track transmissible strains will be discussed.

# Keynote Lectures

### KL03

## METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*: A PROBLEM INSIDE AND OUTSIDE PORTUGUESE HOSPITALS

**Marta Aires-de-Sousa**

Escola Superior de Saúde da Cruz Vermelha Portuguesa, Lisbon, Portugal

*Staphylococcus aureus* is a major human pathogen capable of causing a wide range of infections, from relatively mild skin infections to life-threatening conditions such as septicemia, endocarditis and pneumonia. The fact that *S. aureus* can adapt rapidly to the selective pressure of antibiotics caused the emergence and spread of multiresistant strains namely of methicillin-resistant *S. aureus* (MRSA).

Currently, MRSA is a major cause of nosocomial infections worldwide. In Europe, Portugal has been reporting the highest MRSA rates for the last decade and is presently one of the two single countries showing a prevalence above 50%. Although methicillin resistance has long been exclusively a hospital infection problem, MRSA emerged in the last decade in the community as well causing severe morbidity and mortality in otherwise healthy persons. Recently, MRSA has been reported for the first time in public buses in Portugal, which might pose a new challenge for infection control teams.

The lecture will provide insights on the MRSA evolution inside and outside Portuguese hospitals.

## KL12

# LISTERIOSIS IN PORTUGAL: FROM RETROSPECTIVE STUDIES TO THE DETECTION OF THE FIRST CONFIRMED HUMAN OUTBREAK

Paula Teixeira

*Univ Católica, ESB, Porto*

*Listeria monocytogenes* is a facultative, intracellular bacterial pathogen of humans and a variety of animal species. In humans, *L. monocytogenes* infections are typically food-borne and cause an invasive and often fatal disease in pregnant women, newborns, infants, elderly, and immunocompromised individuals, with a mortality rate between 20 to 30%. Despite worldwide efforts by research organizations and the food industry to reduce the incidence of listeriosis, this pathogen remains a critical threat to human health and the food supply. In fact the incidence of listeriosis has been increasing in recent years in several European countries, including Portugal and listeriosis was the most frequent cause of hospitalisation and death due to the consumption of contaminated food in Europe in 2008–2011. In Portugal, listeriosis is not a notifiable infection and has, thus, been underestimated.

In the absence of an active microbiological surveillance of listeriosis at a national level, in 2003 a collaborative study with the main hospitals in Portugal and CBQF was established aiming to collect data on listeriosis in the country and to characterize the isolates.

The first data on the incidence of listeriosis, based on retrospective information provided by the hospitals, was published in 2006. The incidence of listeriosis was at least 1.4 cases per million inhabitants for the year 2003.

In 2010 another publication reported an increase in the prevalence of the infection between 2003 and 2007; at least 2.3 cases per million inhabitants for the year 2007.

A sudden increase in the number of cases was observed in January 2010: 12 cases, 6 of which were registered in Lisboa and Vale do Tejo region (LVTR). Twenty-nine more cases were registered until the end of June (17 in LVTR). Isolates were typed by PFGE and 19 matched the same pulsotype. The National Health (DGS) and Food Authorities (ASAE) were alerted of this occurrence. Further investigations in collaboration with DGS and ASAE identified fresh cheese produced by a company located in the Alentejo as the likely food source of this outbreak.

This work was developed by Gonçalo Almeida, Rui Magalhães, Isabel Santos, Vânia Ferreira, Joana Silva and Paula Teixeira, from CBQF, Maria Manuel Mendes, Pedro Nabais, Maria da Graça Mariano Fernandes and Maria Isabel Mâncio from ASAE and Maria Manuela Sousa from ARSLVT.



# Oral Presentations

## OP10

### LEPTOSPIROSIS IN ANGOLA: FIRST ISOLATES OF *LEPTOSPIRA* SPP FROM RODENTS AND SEROLOGICAL SURVEY AMONG HUMAN SUSPECTED OF MALARIA

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Leptospirosis is the most worldwide distributed zoonosis. The disease is caused by spirochetes of genus *Leptospira*. These bacteria are transmitted by contaminated urine, mainly from rodents, the principal reservoirs. The unspecific symptoms of leptospirosis can be confused with other febrile diseases like malaria, which is endemic in tropical countries. In Angola, the laboratory diagnosis of leptospirosis is not done so, the cases are generally considered as malaria, the most reported disease.

In order to obtain a differential diagnosis of febrile illness, this study aims to identify and characterize *Leptospira* spp isolated from reservoirs and to know the presence of leptospiral antibodies among febrile patients.

Work is ongoing in Luanda and Huambo, and the laboratory procedures take place at Reference Laboratory in Lisbon (IHMT). The rodents were trapped near the dwellings. The kidneys were extracted, and the lysates inoculated in the EMJH culture medium. Polymerase Chain Reaction (PCR) was also performed with *rrs* (16S) and *hap1* to the genus and pathogenic species, respectively. Leptospiral DNA was sequenced. To selected human patients (n = 650), a clinical and epidemiological questionnaire was applied, and blood samples collected. Sera were analysed for serologic study by Microscopic Agglutination Test (MAT) with apositivity threshold of 1:100.

A total of 77 rodents captured was identified as *Rattus rattus*, *R. norvegicus* and *Mus musculus*, from these, 37 kidney samples were cultured and obtained four(11%) *Leptospira* spp isolates, which will be submitted to serological and molecular characterization using the standard protocols. In addition, the molecular results of kidney lysates showed leptospiral DNA in 10 (13%) out of 77 rodents. This material was sequenced showing a 99% homology for *L. interrogans* and *L. borgpetersenii* genospecies. The questionnaire data revealed that all the 650 human patients were living under some exposure factor risk for *Leptospira* infection, and the serological study revealed 50 (8%) positive samples for antibodies against *L. interrogans*, and 38 (6%) samples with borderline reactivity. Ballum, Panama, Louisiana and Icterohaemorrhagiae were the most prevalent serogroups.

In conclusion, these findings suggest that wild rodents play an important source of multiple pathogenic leptospire, increasing the infection risk to the human population. Now, the differential diagnosis on febrile illness is crucial in the study areas.

## OP11

MASSIVE DISSEMINATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN BLOODSTREAM INFECTION IN PORTUGAL: EMRSA-15 ESTABLISHMENT AND DIVERSIFICATIONNuno Alexandre Faria<sup>1</sup>; Maria Miragaia<sup>1</sup>; Hermínia de Lencastre<sup>2</sup><sup>1</sup>*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa;*<sup>2</sup>*Laboratory of Microbiology and Infectious Diseases, The Rockefeller University, NY, USA*

Portugal has the highest rate of nosocomial methicillin resistant *Staphylococcus aureus* (MRSA) in Europe. Methicillin susceptible *S. aureus* (MSSA) have been also recognized as important pathogens in nosocomial infections, but their epidemiology and its relatedness with MRSA are not well understood. A national surveillance performed in 2006 showed that EMRSA-15 (ST22-IVh) was the dominant MRSA clone, accounting for 51% of MRSA infections and included four different *spa* types, while the second most prevalent clone was ST105-II-t002 (20%). The MSSA population was more diverse, including four major CC (CC30, CC5, CC45 and CC8), which represented more than 60% of the population.

In this study we aimed to perform an update of the most clinically relevant MRSA and MSSA strains circulating in Portuguese hospitals in 2011 and assess the relatedness of these two contemporary populations.

A total of 194 *S. aureus* isolates, 95 MRSA and 99 MSSA, were collected from bloodstream infections between January and June 2011 in 12 hospitals. All isolates were tested for *mecA* and *pvl* (Panton-Valentine leukocidin) presence by PCR and genetic background was assessed by *spa* typing and MLST. MRSA isolates were characterized by SCC*mec* typing. Patient status information was recorded 14 days after bacterial isolation.

Two major MRSA clones were identified, EMRSA-15 (ST22-IVh) (74%) and ST105-II (NY/Japan related) (19%). EMRSA-15 was found to be associated to 16 different *spa* types, while ST105-II was mainly associated to *spa* type t002 (94%).

The MSSA population showed a higher genetic diversity, comprising a total of 16 different clonal complexes (CC), 17 sequence types and 53 *spa* types. In spite of the diversity observed, four major CCs (CC30, CC72, CC5, and CC15) accounted for more than 50% of the MSSA population analyzed and clonal type ST72-MSSA-t148 was the most frequently found (8%).

PVL was restricted to the MSSA population (n=3). All MRSA genetic backgrounds were represented in the MSSA population. Mortality associated to MRSA (23%) was higher than that observed for MSSA (18%).

EMRSA-15 continues to be the major MRSA clone in Portuguese hospitals and its prevalence increased sharply to 74%. The expansion of EMRSA-15 clone was accompanied by an increase in *spa* type diversity, which suggests that it is still evolving. MSSA presents a dynamic population structure overtime including changes in clonal type composition and frequency. MRSA and MSSA populations were found to be related.

## OP12

### HOSPITAL'S ENVIRONMENTAL MICROBIAL COMMUNITIES: A SURVEY IN TWO HOSPITALS WITH DIFFERENT DIMENSIONS

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Hospitals are environments where both, infected and non-infected people, group. How microbial communities persist and change in indoor environments is of immense interest to public health. Nosocomial infections are a constant concern in hospitals worldwide since it leads to more expensive treatments and higher morbidity and mortality rates. Some bacterial species are more often related to nosocomial infections as is the case of *Pseudomonas aeruginosa* and *Klebsiella pneumonia* that are relatively common in several environmental niches. Hospital-associated pathogens are commonly found on physician's and nursing staff's clothing, cell phones, stethoscopes, computer keyboards, telemetry leads, electronic thermometers, blood-pressure cuffs, and gels for ultrasound probes. Despite the lack of direct evidence to prove that environmental contaminants are responsible for Hospital Acquire Infections (HAIs), there is increasing evidence suggesting that the environment may act as a reservoir for at least some of the pathogens causing HAIs. The aim of the present work was to evaluate the diversity of microorganisms that persisted on different equipments in hospitals A (1375 beds) and B (632 beds) from Central Portugal, able to grow in selective media for *Pseudomonas* spp. and for *Klebsiella* spp.

Each hospital was evaluated for a three month period, in different hospital wings. Surface samples were collected, both in wet and dry hospital equipments. The samples were inoculated in the selective media

In all wings from both hospitals, higher levels of contamination were mostly associated to wet surfaces and wet equipments. Strains from *Klebsiella* species were found sporadically. Most of the strains able to grow in the selective media used to recover *Klebsiella* belonged to the genera *Staphylococcus* (hospitals A and B) and *Kocuria* (hospital B). Ten different species of *Pseudomonas* were isolated, and most of the strains belonged to the *P. plecoglossicida*, varying from 40% to 25,7% in the total number of *Pseudomonas* recovered. Strains from *P. aeruginosa* were isolated in biofilmes from taps, in both hospitals.

This work showed that many different bacterial species can persist on hospital surfaces. The level of bacterial contamination was related with the presence of humidity on the surface. When comparing the two hospitals, the contamination level of the materials was higher in hospital A but the bacterial species recovered, on the selective media used, were the same showing a common environmental microbial community in the hospitals.

## OP27

## PLASMID DNA BASED CARRIERS FOR IMPROVED CANCER THERAPY

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The tumor suppressor gene p53 plays a crucial role in keeping the genome integrity and it is involved in several cellular pathways.<sup>1</sup> At the molecular level, mutation of the p53 gene is found in greater than 50% of human tumours and restoration of p53 functions in tumour cells could result in tumour inhibition, normal cell division and adequately responses to DNA damaging.<sup>2</sup> Conventional therapeutics, as surgery, radiotherapy and chemotherapy are widely used to treat cancers in the clinic. However, in some cases they are unsuccessful and chemotherapy is always accompanied by inevitable side effects and by the loss of sensitivity of cancer cells to anti-cancer drugs, addressing the problem of multidrug resistance (MDR). To evolve in the cancer cure, a new strategy seems to be the combination of chemical and gene therapy improving the treatment efficacy due to their synergistic effect. We present a new p53 encoding plasmid DNA (pDNA) microgel that is porous, biocompatible, and photodegradable,<sup>3-5</sup> thus suitable for the loading and release of pDNA and doxorubicin, an intrinsically fluorescent anticancer drug widely used in cancer treatment. Mathematical models were applied to fully characterize the pDNA and drug release profiles, after light irradiation and at dark conditions; significant differences were found between the two conditions. As demonstrated by gel electrophoresis, the released plasmid DNA was intact. Moreover, the delivery of the encapsulated pDNA shows the capability of cell internalization and transfection *in vitro* resulting in the expression of the p53 protein. Additionally, a fluorimetry analysis, using YOYO as a dye, demonstrated sustained pDNA release with microgels. The effect of this system on cell viability inhibition was evaluated in cancer Hela cells. The treatment with a pDNA/doxorubicin loaded microgel improves cell apoptosis. Compared with pDNA microgel or free drug, the co-delivery system has a stronger potential to suppress the development of cancer cells. Our findings clearly show the importance of pDNA encapsulation into microgels for cell uptake, transfection and bioactivity. The present study demonstrated that pDNA microgels can be considered as good candidates for potential anticancer drug and gene co-delivery system in order to achieve the synergetic effect of chemical and gene therapies improving the success of medical cancer treatment.

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## OP36

### BYPASSING THE NEED FOR REPORTER GENES

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Reporter genes, like LacZ, GFP or Luciferase, are routinely used in every molecular and cellular biology laboratory since it allows the study of heterologous gene expression and general cellular mechanisms. Even though these procedures are well characterized and broadly implemented, using reporter genes have serious problems, as it involves very time-consuming procedures and it can affect heterologous gene expression, protein stability and even the cellular behavior. The development of alternative techniques that can bypass the need of either reporter genes or specific probes for the target protein, is thus highly desired (1).

In the present work FT-MIR spectroscopy was evaluated as a substitute for reporter genes, as it is very sensitive to changes in the cell's metabolism and structural organization, it requires minimal sample preparation, no reagents are necessary and it can be operated in a high-throughput mode, using 96 to 386-microwells plates (2,3). A HEK cell line transfected with the pVAX-GFP, using Lipofectamine, was used as model. The 0-17.5% of transfection efficiency was evaluated by microscopic observation of the GFP fluorescence and by FTIR spectroscopy, using a 96-wells plate, and after dehydrating the cellular samples. By the analysis of the first derivative of the FTIR spectra clear differences between transfected and non-transfected cells were observed, mostly in the spectral regions attributed to lipids and proteins. By the determination of a few spectral bands' ratios, interesting differences between transfected and non-transfected cells were also observed, such as an increase in the RNA/DNA ratio in transfected cells, indicating a higher transcriptional activity due to the presence of the heterologous gene. Principal Component Analysis also revealed marked spectral differences when a critical level of transfection was reached. Finally, Partial Least Squares models were developed to quantify the transfection rate of the cells, yielding very good results, with a correlation coefficient of 0.93 and a RMSECV of 1.6%, representing 8.3% of the transfection rates range.

Besides bypassing the need for a reporter gene or other specific probes, FTIR spectroscopy may also allow the evaluation of the impact of the level of heterologous gene expression on the cellular metabolism in a rapid, cheaper and highly sensitive way.

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## OP37

## HIGH PREVALENCE OF NOSOCOMIAL MRSA CARRIAGE IN LUANDA, ANGOLA

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major human pathogen worldwide. Portugal shows the highest MRSA prevalence in Europe but data from African countries with close demographic relationship, namely Angola, are scarce. In the present study, we determine risk factors for MRSA nasal carriage among patients and health care workers (HCW) in Luanda, characterize the MRSA population structure and identify eventual reservoirs and transmission routes within hospitals.

Nasal swabs were recovered from 496 individuals in June 2012 (297 inpatients and 199 HCW) in two public hospitals and one private clinic in Luanda. All isolates were tested for antimicrobial susceptibility, presence of the *mecA* gene and virulence determinants, including the Panton Valentine leukocidine (PVL). Moreover, isolates were characterized by PFGE, *spa* typing, MLST and SCC*mec* typing for MRSA. Socio-demographic data were analysed by binary regression to identify carriage risk factors.

A total of 115 individuals (23.2%) were *S. aureus* nasal carriers out of which 68 (59%) were colonized with MRSA. The global prevalence of MRSA was similar between HCW and patients ( $p > 0.05$ ). The majority of the MRSA isolates ( $n=57$ , 74%) belonged to a single clonal lineage, PFGE A, *spa* t002/t105/t311/t6071/t1215/t11657, ST5-IVa, related to the epidemic Pediatric clone, firstly described in Portugal. The remaining isolates were distributed into four minor clones and one singleton. The methicillin susceptible isolates (MSSA), mainly (52%) belonged to three major clones reported in Europe: PFGE B, t050/t861/t1346/t1574/t2626/t12218, ST508 ( $n=11$ ); PFGE D, t939/t11656, ST45 ( $n=7$ ) and PFGE E, t1202/t9118, ST30 ( $n=7$ ). MSSA isolates presented a high variability of virulence factors, including PVL (7.9%). Taking antibiotics ( $p=0.005$ ) and living in households with less than 6 inhabitants ( $p=0.016$ ) seem to be protective for *S. aureus* nasal carriage. Attending a major public hospital in Luanda, HAB, is a risk factor for MRSA carriage ( $p=0.03$ ) supported by the fact that MRSA cross-transmission was found in public hospitals only.

The MRSA situation in Luanda contrasts with the remaining Portuguese-African speaking countries: the proportion of MRSA is in agreement with the rates currently reported in Portugal and the major clone corresponds to a worldwide epidemic lineage scarcely reported in Africa. Additional infection control measures in this metropolis are mandatory for a global MRSA control.

## OP38

## UNVEILING 5-FLUCYTOSINE RESISTANCE MECHANISMS IN YEAST, USING CHEMOGENOMICS AND MEMBRANE PROTEOMICS APPROACHES

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5-Flucytosine (5-FC) is currently used as an antifungal drug in combination therapy, but fungal pathogens are rapidly able to develop resistance against this drug, compromising therapeutic action. The understanding of the underlying resistance mechanisms is crucial to deal with this problem in a targeted fashion.

In this work, the *S. cerevisiae* deletion mutant collection, the so-called disruptome, was screened for increased resistance or susceptibility to the antifungal drug 5-FC. Through this chemogenomics analysis, 183 genes were found to confer resistance to this antifungal agent, while 21 were found to contribute to yeast susceptibility against it. Among these genes, several related to cell envelop composition and structure were further studied providing evidence for new players in this context, either contributing to cell wall resistance or to changes at the level of flucytosine accumulation. Furthermore, the aquaglyceroporin encoding gene *FPS1* was also identified as a determinant of yeast resistance to 5-FC. The impact of this finding in *S. cerevisiae* was extended to the homologous *CgFPS1* and *CgFPS2* genes, from the pathogenic yeast *Candida glabrata*, which were found to contribute to a decreased accumulation of radiolabelled 5-FC in *C. glabrata* cells. Additionally, studies in *C. glabrata* have highlighted the role of multidrug transporters of the Drug:H<sup>+</sup> Antiporter family in the process of flucytosine resistance [1,2]. Particularly, *CgAqr1* was found to confer resistance to this antifungal agent, contributing to decrease its intracellular accumulation [2]. Given the importance of membrane constituents in 5-FC resistance, the *C. glabrata* response to this stress agent, at the membrane proteome level was more recently assessed, using iTRAQ technology. Among the proteins whose expression was seen to vary, the role of multidrug transporters and proteins involved in lipid metabolism are being further inspected.

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## OP39

## A NEW PROCESS FOR THE RECOVERY AND CONCENTRATION OF BISPHENOL A FROM HUMAN FLUIDS

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Bisphenol A (BPA) is a key monomer in the production of epoxy resins and the most common form of polycarbonate plastics. Products using BPA-based plastics have been in commercial use since the 1950s. Nevertheless, the leaching of this compound, particularly from food storing containers, became a matter of concern in the past few years after its recognition as an endocrine disruptor. BPA is a ubiquitous component in aquatic and soil environments and it has been identified in human biological fluids. These findings pointed out the need to establish regulations regarding the production and market placement of BPA. However, when a population control is required, the low levels of BPA in biological fluids make it difficult to detect *via* conventional analytical techniques.

Aqueous biphasic systems (ABS) are currently recognized as efficient pre-treatment techniques for concentrating metabolites from aqueous phases. In this context, novel ABS composed of hydrophilic ionic liquids combined with  $K_3PO_4$  were tested for extracting and concentrating BPA from aqueous samples. Both model aqueous systems and complex matrices, such as human urine-type samples, were investigated.

Extraction efficiencies of BPA higher than 98.5 % were attained for all the investigated systems and, in general, the presence of a more complex matrix, such as urine, favors the partitioning of BPA for the ionic-liquid-rich phase. Moreover, by a proper manipulation of the phase forming components and their concentration it was possible to concentrate BPA up to 100-fold. The gathered data show the remarkable ability of ionic-liquid-based ABS to extract BPA from human fluid samples in a single-step procedure.<sup>1</sup> In summary, small kits containing the optimized ionic liquids and  $K_3PO_4$  in fixed amounts, to which the human biological fluids could be simply added, can be conceptualized as a new and commercial complement to analytical/clinical strategies where the identification/quantification of BPA is required.

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## OP44

### LOW PREVALENCE OF STREPTOCOCCUS PNEUMONIAE CARRIAGE AMONG THE ELDERLY IN PORTUGAL

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*Streptococcus pneumoniae* (or pneumococcus) is not only a major human pathogen but also a commensal of the human nasopharynx. As a pathogen, pneumococcus is a frequent cause of otitis media, pneumonia, bacteremia and meningitis, especially at the extremes of age. While, there are several studies addressing bacterial colonization in young children, very little is known about the elderly. This pioneer study conducted in Portugal aimed to evaluate pneumococcal carriage in adults aged over 60 years old. Between April 2010–December 2012, nasopharyngeal and oropharyngeal samples were collected from adults older than 60 years old, living in Oeiras (n=1945), an urban area, or in Montemor-o-Novo (n=1416), a rural area. Pneumococci were identified by routine procedures (susceptibility to optochin, solubility in sodium deoxycholate, and PCR detection of *cpsA* and *lytA* genes). The isolates were characterized by serotyping (multiplex PCR and/or by the Quellung reaction); genotyping (MLST); and antibiotype (agar disk diffusion or E-test according to the CLSI guidelines). For the latter, susceptibility to penicillin, chloramphenicol, erythromycin, clindamycin, tetracycline, cotrimoxazole and ciprofloxacin, was determined. Associations between pneumococcal carriage, sociodemographic and clinical characteristics were analyzed by multiple logistic regression. Of the 3,361 adults enrolled, 2.3% were pneumococcal carriers. There were significantly more carriers in the rural area than in the urban area (3.4% vs 1.4%, respectively,  $p < 0.001$ ). After multiple logistic regression, living in the rural area, being at a nursing home and smoking increased significantly the risk of being colonized by 2.0-fold (95%CI:1.2–3.5), 2.0-fold (95%CI:1.1–3.6) and 4.4-fold (95%CI:1.9–9.2), respectively. Among the 77 strains, 26 serotypes and 40 sequence types were identified. The most prevalent serotypes were 19A, 6C, 22F, 23A and 35F. Most of the isolates had STs previously described in the MLST database. Around 40% of the isolates were resistant to at least one antimicrobial agent. Nine isolates (11.7%) were non-susceptible to penicillin, and twelve were multidrug resistant. To our best knowledge, this is the first study on pneumococcal colonization in adults aged over 60 years in Portugal. The prevalence of pneumococcal carriage is low and serotype and genotype diversity is high. Living in the rural area, being at a nursing home and being a smoker are risk factors for pneumococcal colonization.

# Poster Presentations

## Clinical Microbiology

### P173

#### ANTIBIOTIC PROFILE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM HEALTH CARE PERSONNEL WORKING IN HEALTH CENTERS

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*Staphylococcus aureus* is known as a pathogen responsible for skin infections and invasive diseases such as meningitis, meningitides or pulmonary infection and with significant levels of carriage among in the community. Resistance to antibiotics is a problem and methicillin-resistant *S. aureus* (MRSA) have been increasingly isolated all over the world. The objective of this work was to evaluate nasal and hands carriage MRSA as well as hands carriage by among the health care staff in health care centers. From various health care centers near Porto, 322 swab samples from the nasal cavity and hands of health professionals, including volunteer doctors, nurses and auxiliaries, were collected.

Swabs were spread onto Baird–Parker Agar and characteristic colonies were further isolated in Mannitol Salt Agar. Characteristic colonies were selected and confirmed as for *S. aureus*. The susceptibility to oxacillin, penicillin and ampicillin and to antibiotics of other classes rather than betalactams, namely ciprofloxacin, gentamicin, rifampin, vancomycin, tetracycline, erythromycin, nitrofurantoin and chloramphenicol was also investigated. The presence of methicillin resistance gene (*mecA*) was also evaluated by multiplex PCR (detecting 16S rRNA, *nuc* and *mecA* genes). Enterotoxin genes *sea* – *sej* and TSST were also evaluated by multiplex PCR.

Fifty-four *S. aureus* were isolated or from nasal cavity or from hands or from both sites in the same individual representing 16.8% *S. aureus* recovered. Twenty-seven individuals carried *S. aureus* in nasal cavity (8.4%) while eight carried *S. aureus* both in nose and hands (2.5%). Eleven different individuals carried *S. aureus* only in the hands (3.4%). The prevalence of MRSA in 322 samples was only 6%, considering that and 35.2% of the *S. aureus* isolates were MRSA. The most prevalent resistance among *S. aureus* was to beta-lactams and erythromycin. Resistance to while to vancomycin and to gentamicin *S. aureus* did not show any resistance was not found. The most prevalent profile of enterotoxin genes was *sec* *bov* *seg* *sei*.

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### ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA PARAPSILOSIS SENSU LATO* STRAINS ASSOCIATED TO INVASIVE HUMAN INFECTIONS

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**Background.** Fungal infections, mainly caused by members of the genus *Candida*, have been increasing significantly in the last decades. *Candida albicans* remains the most prevalent cause of infection, although the number of infections induced by other *Candida* species is increasing. *Candida parapsilosis* is the second most prevalent *Candida* species in Portugal. However, the traditionally so-called *C. parapsilosis* currently represents a complex of species, entitled *C. parapsilosis sensu lato*, which includes *Candida parapsilosis sensu stricto* (the most commonly found), *Candida orthopsilosis* and *Candida metapsilosis*. However, to date, little is known about the epidemiology of these two recently described species and about the antifungal resistance mechanisms of *C. parapsilosis sensu lato*.

**Objectives.** The main purpose of this work was to identify clinical isolates of *C. parapsilosis sensu lato* associated to invasive human infections, using molecular methods, and to assess their resistance profile to azole antifungal drugs.

**Methods.** The identification of *Candida parapsilosis sensu lato* was accomplished by a species-specific multiplex PCR and RFLP of the ITS region of rDNA. The discrimination between the three species of the complex was achieved by the analysis of restriction polymorphisms in the secondary alcohol dehydrogenase gene (*SADH*). The antifungal resistance profile of isolates was initially screened by a Kirby-Bauer disk diffusion method, followed by the confirmatory and definitive microdilution method.

**Results and Remarks.** We were able to identify 98 clinical isolates as *C. parapsilosis sensu lato*. Of these, eight isolates were further identified as *C. orthopsilosis* (8%), four as *C. metapsilosis* (4%) and 86 as *C. parapsilosis sensu stricto* (88%). Of all isolates, 82 revealed to be susceptible to fluconazole (84%), nine were susceptible-dose dependent (9%) and seven were resistant (7%). All isolates were susceptible to voriconazol, with the exception of four strains (4%) that were found to be resistant to this antifungal. Nine isolates were susceptible-dose dependent (9%) to itraconazol and all the remaining isolates were susceptible. From these results, we can infer that the identification of the three above mentioned species is important not only to assess their epidemiology but, most importantly, to appraise their potential antifungal resistance. Further research will be necessary including a larger number of isolates.

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### P175

#### ASSESSMENT OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* FAECAL SHEDDING IN ASYMPTOMATIC PORTUGUESE CATTLE

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*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease that affects a broad range of hosts, predominantly ruminants [1]. MAP infection has also become a highly contentious issue regarding the cause of human inflammatory bowel disease (IBD) known as Crohn's disease [2]. With a worldwide distribution, paratuberculosis is a chronic and progressive intestinal granulomatous infection manifested by progressive and fatal weight loss, significant decrease of milk production, infertility, oedema and diarrhea eventually leading to death [1]. After infection, animals can remain asymptomatic for two to five years but during this period the shedding of MAP in faeces may lead to the spread of the disease to other animals [3]. Paratuberculosis is considered an underdiagnosed disease in Portugal and the prevalence of paratuberculosis in Portuguese cattle is not well known. The culture of the agent from biological samples remains the gold standard diagnostic method for paratuberculosis. However, this is laborious and lengthy due to the extremely fastidious growth of MAP in artificial media that can take up to 6 months [3]. The objective of this work was to assess the shedding of MAP in the faeces of apparently healthy Portuguese cattle using molecular and culture-based assays. Twenty four fecal samples from asymptomatic Portuguese cattle from a region of the north of Portugal were analysed. The fecal samples were cultured in specific media for the isolation of the agent. The total DNA of the fecal samples was extracted using a commercial kit and the detection of MAP was achieved by PCR-based methods. The isolates were confirmed to be acid-fast bacilli by Auramine-Rhodamine staining, identified as MAP by IS900-PCR and nested F57-PCR and further characterized by a MIRU-VNTR approach. From the 24 fecal samples, 22 were PCR positive and MAP was isolated from 12 samples. All 12 isolates shared an identical MIRU-VNTR profile. Our preliminary data suggests that MAP infection may be more widespread in Portugal than initially expected. Even with the absence of clinical signs the presence of the agent in faeces suggests that the animals are shedding the agent perpetuating the cycle of infection. This is the first study reporting the identification and isolation of MAP from Portuguese cattle.

[1] OIE 2008. OIE Terrestrial Manual (OIE), p. 276.

[2] Juste and Perez. 2011. Vet Clin Food Anim. 27: 127-128.

[3] Timms *et al.* 2011. J. Microbiol. Methods 85: 1-8.

## Clinical Microbiology

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### CHITOSAN AS AN ALTERNATIVE TREATMENT FOR ORAL CANDIDIASIS

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Oral candidiasis is particularly evident not only in cancer patients receiving chemotherapy, but also in elderly people with xerostomy. It is a fungal infection caused by *Candida* species such as *Candida albicans*. In general, *Candida* is an opportunistic pathogen, causing infections in immunocompromised people and in some cases when natural microbiota is altered. One of major virulence capacity of *C. albicans* is its ability to adapt to a variety of different habitats and the consequent formation of surface-attached microbial communities known as biofilms. Along with the current limited repertoire of clinically available antifungals, the drawbacks associated with them such as development of resistance in the pathogen, limited clinical efficacy and poor bioavailability, demand for the development of new antifungal agents. Chitosan, a natural derivative of chitin, is a polysaccharide that was proved to possess a broad spectrum of antimicrobial activity that encompasses fungi, yeast and bacteria. Possesses high biocompatibility and anti-inflammatory capacity and while recent studies have revealed a significant antibiofilm activity upon several microorganism, including *C. albicans*, little is known when regarding the impact of chitosan upon the adhesive process or mature biofilms. With that in mind, the purpose of this work was to evaluate, *in vitro*, the capability of chitosan to inhibit *C. albicans* growth and biofilm formation.

The results obtained showed that chitosan is capable of inhibiting *C. albicans* growth (HMW – 1mg/ml; LMW – 3 mg/ml). At sub-MIC concentrations (HMW –0.5 and 0.25 mg/ml; LMW – 1.75 and 0.75 mg/ml) they showed biofilm formation inhibition (percentages above 90%) and reduction of mature biofilms formation by *ca.* 65%. Additionally, chitosan was also capable of inhibiting *C. albicans* adhesion (*ca.* 95%).

These results display the potential of this molecule to be used in the oral cavity and in particular, in the treatment of oral candidiasis.

## Clinical Microbiology

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### COMPARISON OF VAGINAL MICROBIOTA FINGERPRINTS FROM HEALTHY AND BACTERIAL VAGINOSIS-POSITIVE PORTUGUESE WOMEN

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Bacterial Vaginosis (BV) is a common disease in women of reproductive age and is characterized by the substitution of *Lactobacillus* species, which are predominant in the normal vaginal microbiota, by rapidly proliferating anaerobic bacteria, particularly *Gardnerellavaginalis*. The aim of this study was to study microbial communities' structure in the vaginal microbiota of healthy and BV-positive Portuguese women. To this end, DNA obtained from vaginal samples of 22 BV-negative and 19 BV-positive women was analyzed using a PCR-DGGE approach. Total bacterial communities were amplified using general 16S rRNA gene primers. Group-specific primers were also used targeting *Lactobacillus* and *Bifidobacterium* genera and *G.vaginalis*. DGGE profiles were compared using the BioNumerics™ software package (Applied Maths, Belgium). Similarity between DGGE profiles was determined by calculating similarity indices of the densitometric curves of the compared profiles, using the Dice product-moment correlation. Different DGGE profiles could be obtained for BV-positive and BV-negative samples and this was verified for all primers sets utilized, suggesting that alteration of microbial community structure of BV-positive and -negative samples could be detected by PCR-DGGE. DGGE profiles obtained from samples of BV-positive women were more diverse than the ones from healthy women (as determined by a higher number of DGGE bands). The analysis of the standard electrophoretic bands for bacteria reveals an intrinsic diversity even within the two groups studied: similarities in bacterial DGGE profiles vary between 14–78% and 47–100% in BV-positive and BV-negative samples, respectively. Among the 19 BV-positive women studied 18 were colonized with *G.vaginalis*. *G.vaginalis* was not detected in any of the healthy women samples. The analysis of *Lactobacillus* communities revealed a higher diversity in BV-negative women than in BV-positive ones, which confirms the association of *Lactobacillus* in healthy vaginal microbial communities. A more thorough comparison between BV-negative and BV-positive, including the retrieval of sequencing data from these samples, is necessary for getting more insight on BV influence on vaginal microbiota.



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### P178

#### DIRECT DETECTION IN ANIMAL TISSUES OF MYCOBACTERIA BELONGING TO THE MYCOBACTERIUM TUBERCULOSIS COMPLEX USING A SEMI-NESTED PROBE-BASED REAL-TIME PCR

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Bovine tuberculosis has been tackled for decades by costly eradication programs in most developed countries. Definitive diagnosis is usually achieved by bacteriological culture of tissue samples, from suspected animals, for detection of *Mycobacterium tuberculosis* complex (MTC) members, namely *Mycobacterium bovis*. This analysis may take up to 6–12 weeks, during which the herd is under sanitary quarantine. The development of a rapid, sensitive and specific method that could detect the agent directly in tissues is, therefore, of utmost importance. In this work, a user-friendly DNA extraction protocol, adapted to tissues, was combined with an IS6110-targeted semi-nested duplex real-time PCR assay to improve the direct detection of MTC bacteria in animal specimens, reducing the time to achieve a diagnosis. The duplex use of a novel  $\beta$ -actin gene targeted probe, with complementary targets in most mammals, allowed the assessment of amplification inhibitors in the tissue samples. The assay was evaluated in a group of 128 fresh tissue specimens collected from bovines, wild boars, deer and foxes. *Mycobacterium bovis* has previously been cultured from 57 of these samples. Globally, the full test performance corresponds to a diagnostic sensitivity and specificity of 98.2% (CIP95% 89.4–99.9%) and 88.7% (CIP95% 78.5–94.7%), respectively. An observed kappa coefficient was estimated in 0.859 (CIP95% 0.771–0.948) for the overall agreement between the semi-nested real-time PCR assay and the bacteriological culture. Considering only bovine samples (n = 69), the diagnostic sensitivity and specificity were estimated in 100% (CIP95% 84.0–100%) and 97.7% (CIP95% 86.2–99.9%), respectively. Eight negative culture samples exhibiting TB suspected lesions were positive to the semi-nested real-time PCR, highlighting the increased potential of this molecular approach to detect MTC-infected animal tissues. This novel IS6110-targeted assay allows the fast detection of tuberculous mycobacteria in animal specimens with very high sensitivity and specificity, being amenable and cost effective for use in the routine veterinary diagnostic laboratory, with further automation possibilities.

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## Clinical Microbiology

**P179**

### EFFECT OF AZOLES IN *CANDIDA GLABRATA* BIOFILMS AND ITS RELATION WITH ERG GENES EXPRESSION

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Fungal infections has significantly increased, enhancing morbidity and mortality. The use of broad-spectrum antibiotics, catheters or immunosuppression diseases, are predisposing factors for its development. Once believed as non-pathogenic, *Candida glabrata* rapidly was perceived to be responsible for infections. Despite lacking a number of virulence factors allied to the majority of *Candida* pathogenicity, *C. glabrata* possesses high ability to colonize medical devices and human epithelium, resulting generally in biofilms formation ability. Its intrinsically low susceptibility to azoles, such as triazoles (*e.g.* fluconazole (Flu), voriconazole (Vcz)) and its biofilms tolerance is another problem. This study evaluated the effects of Flu and Vcz in the control of a reference, vaginal and urine *C. glabrata* biofilms and its relation with the expression of genes encoding for Ergosterol: *ERG3*, *ERG6* and *ERG11*. Minimum Inhibitory Concentration was determined for planktonic cells and biofilms were formed during 24h and treated (for 24h) with different concentrations of both antifungal agents. The effects of Flu and Vcz were analyzed by Colony Forming Units determination and by total biomass quantification using Crystal Violet staining. Biofilms were also analyzed by scanning electron microscopy. Total proteins and carbohydrates were quantified from biofilms' matrices and ergosterol present in the matrices was also quantified by HPLC. qRT-PCR was used to study the gene expression of selected *ERG* genes. Our results show that, unlike Flu, Vcz had a very good *Candida* biofilm eradication capacity. No fluctuations between the two azoles were noticed in terms of proteins and carbohydrates, both presenting a blocked production in the first and an over-metabolism in the second. Ergosterol was detected in the matrices. Overexpression of *ERG* genes, in the presence of the both drug compounds was noticed. This work reveals the extraordinary capacity of *C. glabrata* to change with the purpose of overcome the adversities of the environment. The ergosterol present in matrices and the overexpression of *Erg* genes could be an explanation for higher *C. glabrata* biofilms tolerance, hampering the action of drugs against the cells, and when passing to the progeny, is, undoubtedly, a great advantage to the development of resistance to antifungals.

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## Clinical Microbiology

### P180

#### EFFECT OF SIMULATED GASTROINTESTINAL CONDITIONS ON BIOFILM FORMATION BY *SALMONELLA* 1,4,[5],12:i:-

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*Salmonella* Typhimurium 1,4,[5],12:i:- is presently one of the major serovars responsible for human salmonellosis worldwide. *Salmonella* pathogenesis requires colonization and invasion of the gastrointestinal tract, depending on several virulence mechanisms, such as biofilm formation. In fact, biofilm may be responsible for chronic and relapsing infections. Since biofilm expression is influenced by several factors, like pH, temperature, media composition and dynamic conditions, we aimed to evaluate biofilm formation by *S.* 1,4,[5],12:i:- in conditions mimicking the intestinal human tract.

Biofilm formation by 133 Portuguese isolates of *S.* Typhimurium 1,4,[5],12:i:- from clinical (n=125), environmental (n=5) and veterinary (n=3) sources was evaluated determining optical density (OD) values by a modified microtiter biofilm assay. Prior to the biofilm assay, isolates were incubated in a solution simulating the gastric fluid, pH 3, for 180 min with agitation (175 rev min<sup>-1</sup>), to mimic the passage through the stomach. Isolates ability to produce biofilm was then determined in simulated intestinal fluid (SIF), pH 8, with and without agitation, at three different time points (24h, 48h and 72h), and compared with the conventional microtiter method, which uses Muller Hinton Broth (MHB). All microtiter assays were carried out in triplicate, repeated in three different occasions and the results were averaged. Statistical analyses (repeated measures ANOVA and ANOVA) were performed using the SPSS 20.0 software. *p* values ≤ 0.05 were considered statistically significant.

There were significant differences between results from the three protocols. OD mean values of biofilm expression in SIF with agitation decreased significantly with incubation time (*p* ≤ 0.001), and were significantly lower in comparison with static conditions (*p* ≤ 0.001), at all the time points studies. OD means values of biofilm expression in SIF under static conditions and agitation were lower than in MHB.

This study suggests that biofilm expression may decrease during *in vivo* infection, considering the conditions present in the intestinal tract. Therefore, this virulence factor may not be relevant in the context of gastrointestinal infection, and biofilm formation in *Salmonella* 1,4,[5],12:i:-, like in other *Salmonella* serovars, maybe be a survival strategy outside the host.

## Clinical Microbiology

### P181

#### EFFICACY OF DIFFERENT DISINFECTANTS AND WATER WASH ON ALGINATE AND ADDITION SILICONE IMPRESSIONS: A COMPARATIVE EVALUATION

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**Introduction:** The control of cross-infection is an imperative issue when dealing with dental impression materials in Dentistry. The aims of the present study consisted in evaluating the effect of water washing and of 1% and 5.25% sodium hypochlorite, 2% glutaraldehyde and the commercial disinfectant MD520 on the reduction of the microbial load of alginate and addition-reaction silicone, after mouth contact.

**Materials and methods:** Fourteen students voluntarily participated in the study. For each patient, one alginate impression and one addition-reaction silicone impression were performed in different days at the mandibular arch, under aseptic conditions and using sterilized materials. The first and second right and left molars were the selected impression area to study. These selected teeth were divided in 3 parts and each sample was constituted by a pull of one third of each semi-arch in order to minimize the difference of microbial colonization between sides. Each pull was submitted to one of the following treatments: left untreated, without any disinfection methodology; washed with tap water during 30 s; disinfection by immersion in 1% of sodium hypochlorite during 10 min; disinfection by immersion in 5.25% of sodium hypochlorite during 10 min; disinfection by immersion in 2% of glutaraldehyde; disinfection by immersion in MD520 (MD520, Durr, BietigheimBissingen, Germany) during 5 min. Following the exposure to the treatment regimes, each pull was placed in 3ml of 0.9% NaCl and vortexed 5 seconds for 3 times. The total aerobic microorganisms were determined by smearing in Brain Heart Infusion (BHI) agar and incubated at 37°C for 3 days. The colonies were counted and the results expressed as colony-forming units per milliliter (CFU/ml).

**Results:** After mouth contact, the microbial load of alginate 1.5E+05 CFU/ml was significantly higher than silicone 5.47E+03CFU/ml ( $p=0.005$ ). The tap water washing reduced the microbial load by 1.60% in alginate and by 17.4% in silicone. The immersion disinfection procedures decreased the microbial load of either alginate or silicone by more than 99.99% irrespective of the disinfectant used.

**Conclusion:** Dental impression materials can act as a transmission vehicle for oral microorganisms. Dental impression water wash alone is insufficient. The immersion of dental impressions using either one of the disinfectants is effective in reducing significantly the microbial load, so the procedure should be mandatory.

## Clinical Microbiology

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EPIDEMIOLOGY AND ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA PARAPSILOSIS* COMPLEX: A PORTUGUESE MULTICENTRE PROSPECTIVE SURVEYIsabel Faria-Ramos<sup>1</sup>; Isabel M. Miranda<sup>1</sup>; Elisabete Ricardo<sup>1</sup>; Ana Teresa Silva<sup>1</sup>; Emilia Canton<sup>2</sup>; Acácio G. Rodrigues<sup>3</sup>; Cidália Pina-Vaz<sup>4</sup><sup>1</sup>Department of Microbiology, Faculty of Medicine University of Porto;<sup>2</sup>Unidad de Microbiología Experimental, Centro de Investigación, Hospital La Fe, Valencia;<sup>3</sup>Department of Microbiology, Faculty of Medicine University of Porto and Burn Unit, Centro Hospitalar de São João, Porto;<sup>4</sup>Department of Microbiology, Faculty of Medicine University of Porto and Microbiology Department, Centro Hospitalar de São João, Porto

A 12-month prospective multicenter study including 10 Portuguese hospitals was carried out to evaluate the epidemiology of *Candida parapsilosis* complex candidemia in Portugal. Susceptibility profile to amphotericin B, 5-flucytosine, fluconazole, voriconazole, posaconazole, anidulafungin, caspofungin, and micafungin was assessed by *in vitro* microdilution as recommended by the new Clinical and Laboratory Standards Institute (CLSI) M27-S4 protocol. A total of 55 *C. parapsilosis* complex isolates were identified by molecular methods: *C. parapsilosis* (89.1%), *Candida orthopsilosis* (7.3%), and *Candida metapsilosis* (3.6%). Most cases were isolated from elderly patients, mostly women (53%), who were more frequently found in intensive care units (38%), surgery (33%), and internal medicine (24%) departments. Only 21% of *C. parapsilosis* cases became fatal. According to CLSI clinical breakpoints, all *C. orthopsilosis* and *C. metapsilosis* isolates were susceptible to the eight antifungal agents tested. *C. parapsilosis* isolates displayed resistance to fluconazole (3%), posaconazole (2%), caspofungin and anidulafungin (48.4%), and micafungin (27.4%). Clinical data from patients was scarce enabling analysis of predisposing and risk factors associated with *C. parapsilosis* infection. Nevertheless, it is herein presented the first multicentre study conducted in Portugal, giving important indications about *C. parapsilosis* national distribution and susceptibility profile.

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## Clinical Microbiology

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### EVALUATION OF ANTI-BACTERIAL AGENTS USING A HIGH-THROUGHPUT FTIR SPECTROSCOPY

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Most current antibiotics were discovered during the 1940–60s – the ‘golden years’ of antibiotic discovery – or are derivatives. Pharmaceuticals invested in developing novel anti-bacterial drugs, however, half a century has passed since a new antibiotics class was introduced to clinical practice. Consequently bacteria are still responsible for most infection-related mortalities, but of special concern to public health is the increase of drug resistant strains.

Early drug discovery platforms were based on the systematic screening of natural products, and more recently, on rational drug design and high-throughput screening. These platforms, however, extract low information on the drugs molecular mechanism of action. Consequently, it is relevant to develop new high-throughput drugs screening methods, which enable a rapid, cheap and highly sensitive way to estimate simultaneously the drugs antimicrobial properties and its impact on the bacteria cell biochemistry and metabolism. FTIR-spectroscopy could theoretical fulfil these requirements, as it represents specific molecular vibrations of the cell, characteristic of its biochemical composition, in addition, current spectroscopy equipment allows a high-throughput setup. Indeed this technique as been used for identification of microorganisms, cancer cells, transfection and test the efficiency of retroviral therapy, given that a compound or cell at a defined metabolic state presents a specific infra-red spectra, analogous to a molecular fingerprint.

Using *Escherichia coli* as a model organism this study explored FTIR-spectroscopy as a highly sensitive, high throughput technique capable of monitoring the effect of various drugs at a molecular scale. In order to investigate the potential of this technology an antibiotic of the quinolone family and a photoinactivation drug were tested. In both cases it was possible to detect by FTIR-spectroscopy, the drugs effect on the cellular composition either directly on the spectra or on score plots of principal component analysis. This technique was sensitive enough to detect the impact of drug concentrations even at 10% of the minimum inhibitory concentration, using short incubation periods and microliter quantities of the incubation mixture of the bacteria with the drug. Results suggest that the impact of the different drugs on the metabolism of bacteria can be distinguished, and some information on the molecular components of the cell can be deduced. Therefore the present work strongly supports that FTIR-spectroscopy could serve has a technological basis for a novel drug discovery platform, and as an economical and fast evaluation technique of the efficiency of a drugs treatment.

## Clinical Microbiology

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EVALUATION OF THE SPECIFICITY OF A LYTA REAL TIME PCR METHOD FOR THE IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE*Ricardo Carvalho<sup>1</sup>; Débora A. Tavares<sup>1</sup>; Carmen Ardanuy<sup>2</sup>; Josefina Liñares<sup>2</sup>; Hermínia de Lencastre<sup>3</sup>; Raquel Sá-Leão<sup>1</sup>

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*Streptococcus pneumoniae* (pneumococcus) is a human pathobiont that colonizes the human nasopharynx. Pneumococci are responsible for diseases such as otitis, pneumonia, sepsis, and meningitis. Due to similarities with closely related species sharing the same niche, it may be a challenge to correctly distinguish pneumococcus (from these species) when only using non-culture based methods. The latter can be highly sensitive although potentially to the expenses of lower sensitivity. In recent years, a diagnostic molecular method targeting the major autolysin of pneumococcus (*lytA*) by a real time (RT-) PCR assay has been proposed to identify pneumococcus. Since then, this method has been widely used worldwide. However, the presence of *lytA* gene variants have been described in closely related species such as *S. pseudopneumoniae* and *S. mitis*. The aim of our work was to evaluate the specificity of the *lytA* RT-PCR method. We tested a total of 131 previously characterized strains: 61 *S. pseudopneumoniae*, 38 *S. mitis*, two undefined, and 30 type strains from other streptococcal species. The collection included both carriage and disease isolates. The *lytA* RT-PCR assay followed the same previously described methodology [1]. Strains with a Ct value below 35 were considered *lytA* positive and were retested in two different days. All strains were negative although three (two *S. pseudopneumoniae* and one *S. sinensis*) had borderline Ct values of 34. Further assays with an expanded culture collection are ongoing. In conclusion, up until now, our results support the specificity of the *lytA* RT-PCR method proposed for the identification of pneumococcus. Nonetheless, confirmation by complementary methods may be needed for strains with Ct values close to 35.

[1]Carvalho, et al., 2007, J Clin Microbiol 45(8): 2460–2466.

## Clinical Microbiology

## P185

## FREQUENCY AND ANTIMICROBIAL RESISTANCE PATTERNS OF BACTERIA IMPLICATED IN COMMUNITY URINARY TRACT INFECTIONS: A TEN-YEAR SURVEILLANCE STUDY

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Urinary tract infection (UTI) is one of the most common infectious diseases at the community level. In order to assess the adequacy of the empirical therapy, the prevalence and the resistance pattern of the main bacteria responsible for UTI in the community (in Aveiro, Portugal) was evaluated throughout a ten-year period. In this retrospective study, all urine samples from patients of the District of Aveiro, in ambulatory regime, collected at the Clinical Analysis Laboratory Avelab during the period 2000–2009 were analysed. Samples with more than  $10^5$  CFU/mL bacteria were considered positive and, for these samples, the bacteria were identified and the profile of antibiotic susceptibility was characterized. From the 155597 samples analysed, 18797 (12.1%) were positive for bacterial infection. UTI was more frequent in women (78.5%) and its incidence varied with age, affecting more the elderly patients (38.6%). Although *Escherichia coli* was, as usual, the most common pathogen implicated in UTI, it were observed differences related to the other bacteria more implicated in UTI relatively to previous studies. The bacteria implicated in the UTI varied with the sex of the patient, being *Pseudomonas aeruginosa* a more important cause of infection in men than in women. The incidence of the main bacteria changed over the study period (*P. aeruginosa*, *Klebsiella spp* and *Providencia spp* increased and *Enterobacter spp* decreased). Although *E. coli* was responsible for more than an half of UTI, its resistance to antibiotics was low when compared with other pathogens implicated in UTI, showing also the lowest percentage of multidrug resistant (MDR) isolates (17%). Bacteria isolated from females were less resistant than those isolated from males and this difference increased with the patient age. In conclusion, the differences in sex and age must be taken in account at the moment of empirical prescription of antimicrobials. From the recommended antimicrobials by the European Association of Urology guidelines, the first line drugs (pivmecillinam and nitrofurantoin) and the alternative antibiotic amoxicillin–clavulanic acid (AMX–CLA) are appropriate to treat community–acquired UTI, but the fluoroquinolones should not be suitable to treat male infections and the trimethoprim–sulfamethoxazole (SXT) shall not be used in the treatment of UTI at this level.



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INFLUENCE OF GLUCOSE ON *CANDIDA PARAPSILOSIS* VIRULENCE FACTORSLeonel Pereira<sup>1</sup>; Sónia Silva<sup>1</sup>; Bruno Ribeiro<sup>1</sup>; Célia Rodrigues<sup>1</sup>; Mariana Henriques<sup>1</sup>; Joana Azeredo<sup>1</sup><sup>1</sup>IBB–Institute for Biotechnology and Bioengineering

*Candida parapsilosis* is a pathogenic fungus responsible for a high number of oral candidosis, predominantly due to the growth in a biofilm form on indwelling medical devices. One of the major contributions for *C. parapsilosis* virulence is its versatility to adapt to a variety of different environmental factors. The development of *Candida* biofilms has important clinical repercussions because of their resistance to antifungal therapy and the protection against host immune defences. *Candida* biofilms are characterized by easily glucose adaptation. However, it remains unclear how glucose affect *C. parapsilosis* biofilms formation and virulence.

Thus, this work aimed to study the influence of glucose in biofilm formation ability and matrix composition of *C. parapsilosis* and in the expression of virulence genes such as *BCR1*, *FKS1* and *OLE1*. For that a full characterisation of *in vitro* biofilm formation with different concentrations of glucose (0.2, 2 and 10 %) was examined. Biofilms were analysed by CFUs determination and total biomass quantification. Biofilm matrix was examined in terms of total proteins and carbohydrates. Scanner electron microscopy (SEM) was used to observe the structure of *Candida* biofilms. Furthermore, RT-qPCR was performed to examine the expression levels of *BCR1*, *FKS1* and *OLE1* genes present in biofilm cells grown in the presence of the different glucose concentrations.

The results demonstrated that glucose influences biofilm formation ability, with an increase in total biomass and number of CFUs, with an increase in a glucose: however in a strain dependent manner. Moreover, the increment of glucose causes an increase on the content of proteins and polysaccharides on *C. parapsilosis* biofilm matrix. Moreover, *BCR1*, *FKS1* and *OLE1* gene expression increased with the glucose increment. In addition, SEM images revealed the presence of pseudohyphae for higher levels of glucose (2 and 10 %). So, the results suggested that glucose enhanced pseudohyphae formation by the induced expression of *OLE1*, which influences biofilm structure. The overexpression of *BCR1* and *FKS1* confirm the influence of glucose in biofilm formation ability and in the carbohydrate synthesis and secretion (present in the matrix), respectively.

Summarizing, *C. parapsilosis* biofilms presented a great capacity to tolerate and grow in the presence of high levels of glucose that seems to be directly implicated in *C. parapsilosis* virulence.

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### INSIGHTS ON THE EXO-METABOLOME FROM TWO PATHOGENIC FUNGI: *CANDIDA ALBICANS* AND *ASPERGILLUS NIGER*

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Pathogenic fungi *Candida albicans* and *Aspergillus niger* are etiological agents that cause serious diseases in immunocompromised and nonimmunocompromised patients following surgical interventions and also in critically ill patients. Since diagnosis at the early stages of infection is very difficult, it is imperative to develop new approaches for rapid and reliable detection and subsequent identification of fungi. Fungi cultures are still the main diagnostic tool causing a possible delay in starting appropriate therapy. Metabolomics arises as a powerful feature in fungal systematics and growth estimation through the screening of the metabolites produced. This work aims to elucidate the exo-metabolome from *A. niger* and *C. albicans* through headspace solid-phase microextraction (HS-SPME), combined with a high-throughput chromatographic technique (comprehensive two dimensional - GC × GC - ToFMS). *A. niger* and *C. albicans* cultures were grown in Yeast Extract Dextrose Chloramphenicol broth, and aliquots of the media were collected at different growth times, and analyzed by HS-SPME/ GC × GC - ToFMS [1].

For *C. albicans*, up to 60 compounds were reported, belonging to terpenoids (C<sub>10</sub> and C<sub>15</sub>), alcohols, ketones, aldehydes, hydrocarbons, esters, pyrazines, sulfur derived compounds and norisoprenoids. Terpenoids and alcohols prevailed in terms of GC peak area accounting for up to 72% of the different identified compounds at the diverse growth times. For *A. niger*, up to 119 compounds were identified belonging to the same chemical families that were reported on *C. albicans* except for norisoprenoids. Chloro-derived compounds and furans were also identified, prevailing ketones and aldehydes.

Different profiles were noticed along growth of the cultures of the two fungi with changes in the expression of the identified compounds. These results elucidate of the usefulness of metabolomics for the understanding of the fungi growth and activity as the produced metabolites are an expression of the physiological status the cells. Additionally, a specific footprint was achieved for each microorganism at the earlier stages of growth, showing the potentiality to use volatile metabolites for rapid detection and identification of different fungal genera.

#### Acknowledgement:

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[1] Salvador, Â. C.; Baptista, I.; Barros, A. S.; Gomes, N. C. M.; Cunha, A.; Almeida, A.; Rocha, S. M. Can volatile organic metabolites be used to simultaneously assess microbial and mite contamination level in cereal grains and coffee beans? PloS one 2013, 8, e59338.

## Clinical Microbiology

### P188

#### IS THERE A RELATION BETWEEN PERITONITIS CAUSING AGENTS AND THE ORAL MICROBIOTA OF PD PATIENTS?

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**Introduction:** Peritoneal dialysis (PD) is a widely used therapy of renal replacement and one of its major complications is the infection-related morbidity. In our center there is a persistent low number of peritonitis every year (1 peritonite/29 months), despite the existing hygiene standard procedures, being *Streptococcus* and *Staphylococcus* (11%) responsible for most cases of gram-positive peritonitis and *Pseudomonas* (16%) the most common of gram-negative. Fungal peritonitis is rare but its treatment is usually very difficult. So, our aim was to characterize oral microbiota of PD patients and correlate it to PD-related infections.

**Material and methods:** Microbiological analysis of saliva from 35 PD patients of Hospital S. João comprised the isolation and identification of *Staphylococcus*, *Pseudomonas* and *Candida* using: Mannitol Salt culture medium and multi-test system API 32 STAPH; Cetramide selective agar 10 mL/L glycerol and microscopic visualization after Gram staining and oxidase test; and CHROMagar Candida™ and genomic sequencing, respectively.

**Results:** PD patients evaluated (average age 47 years) had low educational level, poor oral hygiene and high index of decayed, missing or filled teeth. The mean duration of PD was 13 months, and 37% had a previous peritonitis episode. The *Staphylococcus* most commonly associated with these infections were *S. aureus*, *S. warneri* and *S. epidermidis*. These species were the most commonly found in saliva and all patients had oral colonization by *Staphylococcus*. We found *Pseudomonas* in only one of the patients evaluated (2.86%) and 11% of PD patients presented fungal saliva colonization (*C. albicans* e *C. carpophila*).

**Conclusion:** The *Staphylococcus* most commonly found in the oral cavity of PD patients corresponds to the species associated with peritonitis; *Pseudomonas* was present with a very low prevalence. The yeast *Candida*, especially *C. albicans*, is a common colonizer though with low prevalence of the oral cavity of PD patients. Although *Staphylococcus* were ubiquitous in saliva, most of colonized patients did not show infectious complications, revealing the importance of promoting the use of facial mask during the PD exchange. Overall, we could not find a relation between the identification of oral microorganisms of each patient and the causative agents of peritonitis. This could be due to the fact that the evaluation of oral microbiota was not simultaneous to the peritonitis or could suggest other routes of infection.

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### PLASMID MEDIATED QUINOLONE RESISTANCE IN ENTEROBACTERIACEAE FROM NORTH MOROCCO

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**Abstract:** Background: Multidrug resistance (MDR) in Enterobacteriaceae including resistance to quinolones is rising worldwide. This resistance is achieved by a variety of elements including chromosomal mutations of target genes (topoisomerases), pump efflux, target-protecting proteins and quinolone-modifying enzymes. Some of these elements have been found to be carried by a large variety of plasmids which allows horizontal dissemination of resistance genes among different bacterial species.

**Objectives:** This study was conducted to assess the antibiotic resistance profile and to investigate for plasmid genes mediated-quinolones resistance in clinical Enterobacteriaceae collected in north Morocco.

**Methods:** We analyzed a sub-site of 111 isolates collected in north Morocco dominated by *E. coli* (62%) and *K. pneumonia* (25%). Antibiotic susceptibility was determined by disc diffusion agar method. 41 MDR strains were screened by PCR for *Aac* (6') and *qnr* genes. Variants were determined by sequencing and plasmid support of the genes was confirmed by conjugation.

**Conclusions:** Our results showed more than 53% ESBL strains carried *qnr* (50%) and *Aac* (6') (45%) determinants while only 16% of non-ESBL strains harbored both determinants. About 22% *qnrB* and 19% *qnrS* have been detected while *qnrA* was absent in this study. Furthermore, *qnrB* and *qnrS*, were detected simultaneously in more than 14%. Different variants could be determined by sequencing: *qnrB3* in *E. coli*; *qnrB6* in *E. aerogene*; *qnrB1* and *qnrB42* in *K. pneumonia* and finally *qnrB10*, *qnrB12*, and *qnrB41* in *C. freudii*. On the other hand, all ours strains were belong to *aac(6')-Ib-Cr* variant.

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### P190

#### POLYMORPHISM IN GIDB GENE AS A GENETIC MARKER FOR THE *MYCOBACTERIUM TUBERCULOSIS* Q1 CLUSTER AND IMPLICATIONS FOR THE STREPTOMYCIN RESISTANCE LEVEL

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Development of streptomycin-resistance in *Mycobacterium tuberculosis* is usually associated with mutations in *rpsL* and *rrs* genes, although up to 50% of clinical streptomycin-resistant isolates may present no mutation in either of these genes. The situation in Lisbon Health Region is similar, although mutations in *rrs* gene are only rarely detected. In the present report we investigate the role of *gidB* gene mutations in streptomycin resistance.

We have analyzed 52 streptomycin-resistant and 30 streptomycin-susceptible *Mycobacterium tuberculosis* clinical isolates by sequencing and endonuclease analysis of the *gidB* and *rpsL* genes. All clinical isolates were genotyped by 12-*loci* MIRU-VNTR. Semiquantitative drug susceptibility testing was also performed to a select set of isolates to assess the resistance levels towards streptomycin.

The *gidB* gene of 18 streptomycin-resistant isolates was sequenced and four missense mutations were found: F12L (1/18), L16R (18/18), A80P (4/18) and S100F (18/18). The remaining isolates were screened by endonuclease analysis for mutations A80P in *gidB* and K43R in *rpsL* gene. Overall, mutation A80P in *gidB* gene was found in 7 streptomycin-resistant isolates and 12 streptomycin-susceptible multidrug resistant isolates. Also noteworthy, comparison of the distribution of *gidB*, *rpsL* and *rrs* mutations revealed that *gidB* A80P mutation was only present in isolates without *rpsL* and *rrs* mutations. Moreover, this specific mutation was found among all isolates belonging to genetic cluster Q1.

Streptomycin quantitative drug susceptibility testing showed that isolates carrying the GidB A80P mutation were streptomycin intermediate-level resistant and that standard drug susceptibility testing yielded inconsistent results probably due to borderline resistance. Bioinformatic analysis on the degree of conservation showed that the GidB A80P mutation is predicted to affect protein function.

We conclude that *gidB* mutations may explain the high number of streptomycin-resistant strains with no mutation in *rpsL* or *rrs*. These mutations might occasionally confer undetected streptomycin low-level resistance in regular drug susceptibility testing. Also, GidB A80P mutations may serve as surrogate markers for Q1 cluster isolates that are associated with multidrug/extensively drug-resistant tuberculosis.

## Clinical Microbiology

### P191

#### PREVALENCE OF ORAL PROTOZOA IN PORTUGUESE PATIENTS UNDERGOING PERITONEAL DIALYSIS

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**Objectives:** The aim of the present study was to evaluate the presence of protozoa in saliva of chronic kidney disease patients (CKD) undergoing peritoneal dialysis (PD) and their healthy family members as a control group.

**Materials and methods:** The clinical and demographic data was obtained from 41 PD patients and 18 non-CKD family members controls. A non-invasive intra-oral examination was performed in order to evaluate the decayed, missing and filled teeth (DMFT) index as well as oral hygiene index. Saliva was collected before oral examination in order to examine protozoa colonization as well as evaluate salivary pH and flow rate. The smears were prepared with staining techniques: Lugol, Giemsa stain and modified Ziehl-Neelsen method for subsequent microscopic evaluation of protozoa oral colonization.

**Results:** The average age of PD patients was 45.4 14.6 and non-CKD family members 37.5 16.8. These patients presented a mean time on PD program of 12.7 15.9 months and the creatinine clearance was 10.4 ml/min. The education level was low in both control and study groups. Most of PD patients present bad oral hygiene and had a very high DMFT index, although the prevalence of decayed teeth was low in PD patients in comparison to controls ( $p < 0,05$ ). Regarding salivary flow rate, PD patients presented values below the normal range. No protozoa were found after the analysis of 118 smears from 59 participants. However, epithelial cells and microorganisms from the normal oral microbiota were observed.

**Conclusion:** The absence of oral protozoa colonization in patients and their healthy familiar controls, suggests that in Portuguese population the oral protozoa colonization may be low. This result can be geographic specific, although the absence of other studies in our country does not allow the generalization. Furthermore, the limited number of patients analysed as well as the limitations of the applied methodology could have compromised the results of this study. Future studies are required to ensure the low oral protozoa colonization in the Portuguese population, especially in this particular group of chronic kidney disease patients in peritoneal dialysis treatment.

## Clinical Microbiology

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### PROTEOMIC ANALYSIS OF A MRSA ST398 CLINICAL STRAIN

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For many years *Staphylococcus aureus* has been recognized as an important pathogen and responsible for human diseases. The difficulty in treatment and constant new appearance of multiple antibiotic resistance has made this organism an important focus of study. Proteomics is presently a powerful tool to analyze the differences in gene expression in bacterial strains. Furthermore, proteomics is an important bridge between the genomic data and functional biology, gathering information about post-transcriptional modifications and changes in protein expression. The combination of molecular biology with genomics and proteomics tools allow a precise evaluation and characterization of the diversity of origin of different selective pressures and correlations between them.

To investigate this microorganism with several important mechanisms of resistance and pathogenicity three different proteomes of a methicillin-resistant clinical strains *S. aureus* (MRSA) were determined by techniques of two-dimensional electrophoresis (2-DE) and liquid chromatography-mass spectrometry (LC-MS). Using 2-DE for cellular proteome 105 spots were excised, in which, of correlation with bioinformatics databases allowed the identification of 227 proteins. Considering the exposed cell surface proteome and the exoproteome, 236 and 99 proteins were identified respectively. Proteins related to basic cell functions were found, but also proteins related to virulence and pathogenicity, like catalase and *isdA*, main responsible for *S. aureus* infection, and proteins related to antibiotic resistance membrane proteins PBP. Among the two classes with more proteins identified named glycolysis (16.3%) in the intracellular proteome and translation (15% and 16%) in the other exposed cell membrane and exoproteome.

The results of this study also allowed comparison between the proteome and their proteins MRSA ST398 with other genetic strains of *S. aureus* to identify specific proteins from strain ST398, both intracellular and extracellular level and at the level of the exposed surface of the bacterial cell. With the comparison between the three proteomes allowed to crosslink proteins present between the interior and exterior of the cell.

These information reflect an active metabolism and highlighted the importance of proteomics tools to develop and knowledge the protein expression of MRSA.

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### Q FEVER: UNVEILING COXIELLA BURNETII POPULATION STRUCTURE IN ANIMAL RESERVOIRS IN PORTUGAL

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Q fever is a worldwide zoonosis caused by *Coxiella burnetii*, an obligate intracellular, Gram-negative bacterium. Infection in man occurs, mainly, by contact with small ruminants, which get infected by ticks. Abortions, premature birth or delivery of weak offspring are the main signals in animals, while in man it can manifest as either acute, flu-like, disease or chronic, endocarditis, illness.

Detection of sources of infection and routes of transmission are essential for the control of *C. burnetii* spread among animals and transmission from animals to humans. In Portugal, little is known about *C. burnetii* reservoirs and infection in animals. Therefore, a study was performed to detect and characterize *C. burnetii* DNA in tissue samples from domestic and wild animals.

A nested-touchdown PCR, targeting the repetitive transposon-like element (Trans-PCR) of *C. burnetii* insertion sequence IS1111, was performed on 229 DNAs from tissues and cloacal swabs, from domestic and wild animals, including vultures. Nineteen of them tested positive for *C. burnetii* (14 domestic and 5 wild animals), revealing a prevalence of infection of 8.3% within this sample panel. None of the cloacal swabs from vultures were positive.

MLVA was used to genotype the 19 *C. burnetii* DNA samples, using a set of six VNTR loci (Ms23, Ms24, Ms27, Ms28, Ms33 and Ms34). Seven different completed profiles (M1 to M7) and nine partial profiles were observed. The discriminatory power of MLVA was 0.94 for our sample setting, and the diversity indexes (*D*) of the individual markers ranged between 0.73 and 0.95, with loci Ms33 as the most discriminatory one.

UPGMA clustering of the MLVA data grouped the *C. burnetii* samples into eight different clusters, being cluster IV the one that included more than one MLVA type. Clustering of the MLVA genotypes using the minimum spanning tree method (MST) grouped cattle and goat samples from this study in one branch, while two sheep samples, from the same animal, were grouped completely apart in another branch. Using only completed profiles, this analysis corroborates the hierarchical UPGMA data, confirming cluster IV as the most representative of our sample setting.

None of our samples clustered with animal or human data reported previously in Portugal (Santos et al., 2012), or in other countries, showing a high diversity and exclusivity of *C. burnetii* genotypes within our panel of samples.

#### ACKNOWLEDGEMENTS

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#### RECENT TRENDS IN COLONIZING GROUP B STREPTOCOCCUS EPIDEMIOLOGY IN PORTUGAL (2005–2012): EMERGENCE OF A NEW EPIDEMIC TYPE IV/CC17 CLONE

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This study presents the serotype distribution and the antibiotic resistance profile of 953 colonizing group B *Streptococcus* (GBS) recovered from women of child bearing age between 2005 and 2012. Globally, serotypes Ia, II, III, and V were the most common (~80%). However, temporal trend analysis evidenced changes in GBS distribution, in particular in recent years, where formerly less prevalent serotypes seem to be replacing the prior most prevalent serotypes. In fact, the prevalence of serotype IV isolates increased from 1.4% in 2006 to 19.6% in 2012, representing a 14-fold increase. Also, a considerable proportion of recent serotype IV isolates evidenced resistance to erythromycin and clindamycin. The identification of nine serotype IV isolates presenting a novel association with the CC17 lineage, involving a putative capsular switch, may accentuate their virulence potential and ecologic success. Molecular analysis of this subgroup of isolates revealed the presence of *rib*, *IS861* and *GBSi1* within *scpB-lmb* region, reflecting high clonality and a putative common origin. A close surveillance of the emergent type IV/CC17 isolates is crucial considering the potential impact over GBS treatment guidelines and capsular vaccine development.

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#### RELEVANCE OF INOCULUM SIZE ON BIOFILM FORMATION BY DIABETIC FOOT BACTERIAL ISOLATES

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Diabetes mellitus is a serious health problem in rapid expansion worldwide. One of its more frequent complications is the development of diabetic foot infections (DFIs), which result from a complex interaction of several risk factors. Difficulties in DFIs treatment are often observed, and may be due to the presence of bacterial biofilms. These are a recognized virulence trait that facilitates bacterial persistence in the host, immune evasion and survival in the presence of high antibiotic concentrations. Nevertheless, there is little information available regarding the dynamics of biofilm formation by diabetic foot bacterial isolates.

The aim of this study was to evaluate the significance of inoculum size on biofilm formation by diabetic foot isolates. Three biofilm-producing clinical isolates obtained from DFIs, *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07 and *Acinetobacter baumannii* 1305/05, were used in this assay. 100 µl of bacterial suspensions in Tryptic Soy Broth with concentrations ranging from 10<sup>4</sup> to 10<sup>9</sup> CFU/mL were placed in 96-well microtiter plates and allowed to form biofilm during 24 hours at 37°C. After incubation, planktonic cells were removed. The remaining biofilm was washed twice, scraped, resuspended in 0.9% NaCl and sonicated. Afterwards, serial dilutions were made and plated onto Plate Count Agar. After 24 hours incubation at 37°C, CFUs were determined. Assays were repeated three times. Statistical analyses (Normality Test and Spearman correlation coefficient) were performed using the SPSS 20.0 software (IBM Corporation, New York, USA). A p-value ≤ 0.05 was considered statistically significant.

Diabetic foot isolates showed a high ability to form biofilm at 24 hours. Biofilm production was higher in *A. baumannii*, followed by *P. aeruginosa* and *S. aureus*. Although it was expected that the degree of biofilm formation was influenced by the inoculum size, a correlation between initial inoculum and the quantity of biofilm formed was only statistically significant for *S. aureus*.

In conclusion, results show that it is necessary to optimize *in vitro* protocols used for the evaluation of biofilms, since results may be influenced by several factors, such as inoculum size.

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#### SEVERE FIBRINONECROTIC ENTERITIS CAUSED BY *PSEUDOMONAS AERUGINOSA* IN A CAPTIVE MONITOR LIZARD (*VARANUS NILOTICUS*)

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*Pseudomonas aeruginosa* is an important pathogen which has been implicated in a number of serious conditions in humans and animals. Reports related with antimicrobial therapy of virulent *P. aeruginosa* infections are available in humans and many animal species; however studies concerning captive animals, especially reptiles, are scarce.

In this study, a six-year old monitor lizard, *Varanus niloticus*, from the Lisbon Zoo, which did not show previous signs of disease, died acutely. After necropsy, histopathological analysis revealed severe fibrinonecrotic enteritis with multiple bacterial aggregates. Samples of lungs, liver, spleen, kidneys and intestine were analyzed by conventional microbiological procedures.

It was possible to isolate *P. aeruginosa*, identified using biochemical galleries (API20E system, bioMérieux, France) and PCR amplification. Antimicrobial susceptibility testing was performed using the disc diffusion method, according to the Clinical and Laboratory Standards Institute guidelines, for the following compounds: aminoglycosides (gentamicin 10 µg, amikacin 10 µg), carbapenems (imipenem 10 µg, meropenem 10 µg), third generation cephalosporins (ceftazidime 30 µg, cefotaxime 30 µg), penicillin (ampicillin 2 µg), extended spectrum penicillin (piperacillin/tazobactam 75 µg) and penicillin combinations (amoxicillin/clavulanic acid 30 µg), monobactams (Aztreonam, 30 µg), fluoroquinolones (ciprofloxacin 5 µg, ofloxacin 5 µg) and sulphonamides (sulphamethoxazole-trimethoprim 25 µg). Virulence characterization was performed for the presence of hemolysins, DNases, gelatinases and biofilm by plate assays.

Isolate showed resistance to amoxicillin/clavulanic acid, ampicillin, cephotaxime and sulphamethoxazole/trimethoprim. The isolate was also able to express all the virulence traits tested.

As far as we know, this is the first report describing a severe intestinal infection in a zoo captive *Varanus niloticus* by a *P. aeruginosa* strain with multiple virulence traits, including biofilm. Although this resistance profile is usually found in *Pseudomonas*, the increased ability to persist in the environment due to biofilm formation, can easily impair the disinfection of the zoo environment, creating favorable conditions for virulent strains transmission. This study also highlights the importance of surveillance for antimicrobial resistance and virulence traits in captive animals, which may represent a challenge to practitioners.

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### STAPHYLOCOCCUS EPIDERMIDIS FROM THE EARLY ANTIBIOTIC ERA (1965) CARRIED A MECA FORM THAT DID NOT CONFER RESISTANCE TO METHICILLIN

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The first MRSA (methicillin-resistant *S. aureus*) was isolated in 1961, just one year after the introduction of methicillin into clinical practice. However, the gene encoding methicillin resistance (*mecA*) is believed to have originated from a *mecA* homologue found ubiquitously in *Staphylococcus sciuri*, and whose function was not associated to antibiotic resistance. Still, it is not known how this *mecA* homologue was incorporated into the mobile element called staphylococcal chromosomal cassette *mec* (SCC*mec*) conferring methicillin resistance in many staphylococci and how has evolved. Current data suggests that several coagulase-negative staphylococci species were involved in this process.

To address the possible role of *S. epidermidis* in the assembly of SCC*mec*, we analyzed a collection of 22 methicillin-susceptible *S. epidermidis* (MSSE) strains collected close to the beginning of the clinical use of methicillin. Isolates were screened for the presence of *mecA* and SCC*mec* structural elements (*mecA*, *ccr*, *mecI*, *mecR1*, *IS1272-mecR1* and *IS431-mecR1*) by PCR and dot-blot. Cefoxitin susceptibility was analyzed for all strains by E-test and population analysis profiles. Molecular characterization of the strains was achieved by PFGE and MLST.

All the strains were cefoxitin-susceptible, with MICs ranging from 0.38 to 1.5 mg/ml. A high genetic diversity was observed, with 12 PFGE types and 13 sequence types identified. In contrast to what was expected from the low levels of beta-lactam resistance, seven out of the 22 isolates carried *mecA*, and five of the seven harbored also *ccr* genes. Furthermore, one strain carried *mecR1* and another strain contained the *IS431-ΔmecR1* region. The presence of such elements did not seem to correlate with the resistance level; therefore, *mecA* and its close vicinity were sequenced in one strain (DKN22). In DKN22, *mecA* and its promoter region showed 99–100% nucleotide identity with *mecA* from the prototype MRSE strain, RP62A; however, in this MSSE isolate, *mecA* coding frame was disrupted by *IS431*.

The results suggest that in the early antibiotic era some *S. epidermidis* isolates carried a *mecA* element that did not confer resistance to methicillin. The disruption of *mecA* gene by *IS431* could be a convenient strategy to accommodate *mecA* in the chromosome of *S. epidermidis*, avoiding the cost of its constitutive expression while benefiting from prompt expression upon *IS431* excision, when necessary.

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### P198

#### SUSCEPTIBILITY TO BIOFILM FORMATION BY CLINICAL ISOLATES FROM PATIENTS WITH URINARY TRACT INFECTION: DEVELOPMENT OF A MULTIFACTORIAL PREDICTIVE MODEL

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Implantable medical devices help enhancing therapeutic results, saving human lives and improving life quality of patients. However, these devices can be readily colonized by bacteria and fungi, since the presence of a foreign body will reduce the number of microorganisms necessary to produce an infection (Guggenbichler et al., 2011).

Accordingly, the aim of this study was to evaluate a group of biofilm producing bacteria isolated from patients with urinary tract infections, identifying the main factors contributing to biofilm formation. It was also intended to evaluate the association of these factors with biofilm formation, in order to understand their contribution as predictors for biofilm onset, allowing an earlier and more effective selection of the required antibiotic treatment. Biofilm detection was done by the tube adherence method. The investigation of the biofilm production was performed measuring its adherence to borosilicate test tubes following a previous methodology (Christensen et al., 1985) with minor changes. Among the 156 isolates, 58 (37.2%) were biofilm producers. The patient's genre ( $p=0.022$ ), together with bacterial species ( $p < 0.001$ ), were the factors with highest influence for biofilm production. In fact, some of the isolated bacteria were biofilm producer in all cases. The assayed factors (age, gender, hospital unit, bacteria and catheterization) were used to build a predictive model in order to anticipate biofilm occurrence immediately after bacterial identification. In this way, it is possible to select a more effective antibiotic (among the susceptibility options suggested by the antibiogram) against biofilm producing bacteria, avoiding the need to change antibiotics due to acquired resistance during the treatment.

Regarding resistance profile among bacterial isolates, the  $\beta$ -lactamic antibiotics presented the highest cases/percentages: ampicillin (32/55.2%), cephalothin (30/ 51.7%), amoxicillin/clavulanic acid (22/37.9%), although the carbapenemic group still represent a good therapeutic option (2/3.4%). Quinolones (nucleic acid synthesis inhibitors) also showed high resistance percentages. Furthermore, biofilm production clearly increases bacterial resistance. Actually, almost half of biofilm producing bacteria showed resistance against at least three different species.

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### THE PROTOZOAN *TETRAHYMENA PYRIFORMIS* AS A CELLULAR ADHESION MODEL FOR *STAPHYLOCOCCUS AUREUS*

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*Staphylococcus aureus* is one of main pathogen agents responsible the same time for nosocomial and community-acquired infections. The pathogenicity of this bacterium is ensured by its different adhesion factors. The collagen and the intracellular polysaccharide adhesin are among the most important virulence factors of staphylococcus. It has been recently shown that the majority of the *S. aureus* strains carry the ica operon, responsible for biofilm production, however the coexpression of the icaA and the icaD is necessary for complete biomfilm synthesis.

Our study aims to detect by PCR, the presence of *cna* and *icaD* genes, in a collection of 15 clinical strains of *S. aureus* of different origins, then to estimate the strains ability to form a biofilm by the red Congo (CRA) method.

Also for the first time, we used the protozoan *Tetrahymena pyriformis* as a cellular model to test the adhesion ability of *S. aureus*. Finally we checked the adhesion's inhibition by some plants extracts. The molecular detection of adhesion's genes revealed that 80 % of strains are positive *cna*, and 73 % are positive *icaD*. The qualitative production showed that 66,6 % of strains are producing biofilm. Finally the adhesion test revealed that 20 % of strains are strongly adhering to *Tetrahymena pyriformis* and that the extract *C. cirrhosa* has an anti adhering effect of *S. aureus* to the protozoan *T. pyriformis*.

## Clinical Microbiology

### P200/F13

#### DIALYSIS EFFLUENT PROTOZOA COLONIZATION IN PORTUGUESE PATIENTS UNDERGOING PERITONEAL DIALYSIS

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**Introduction:** The prevalence of chronic kidney disease (CKD) is increasing worldwide. Peritoneal dialysis (PD) is a home-based and widely used therapy of renal replacement for patients with end-stage renal disease (ESRD). Despite the improvements in this renal replacement therapy, peritonitis is still one of the most important causes of technique failure in peritoneal dialysis. However, up to 20% of peritonitis are of undetermined cause. In these patients with persistent culture-negative peritonitis, consideration should be given to unusual microorganisms, such as protozoa. So, the aim of the present study was to evaluate the presence of protozoa in PD effluent from Portuguese CKD patients undergoing PD.

**Methods:** Clinical and demographic information was collected from 41 PD patients. Samples of peritoneal dialysis effluent were also collected in an aseptic environment. A total of 50 ml was centrifuged at 1500 rpm/10 minutes. Afterwards, for each sample, 50µl of PD effluent pellet were used to prepare 6 smears for microscopy evaluation. For protozoa identification it was performed direct wet smear with Lugol's solution and smears staining with Giemsa and modified Ziehl-Neelsen's. PD patients presenting protozoa in PD effluent were further characterized regarding age, gender, educational level, profession, CKD etiology, diabetes and environment/social condition such as children cohabiting, domestic poultry, consumption water source, raw vegetable (especially lettuce) consumption and rivers or lakes swimming frequency.

**Results:** We found protozoa on dialysis effluent samples collected from 5 (12%) different PD patients. In 2 PD patients we found *Blastocystis hominis* and, in other 3 PD patients, we found *Entamoeba* sp, *Giardia* sp and *Endolimax nana*. All the protozoa-positive PD patients presented low education level, different CDK etiologies and were not diabetics. Interestingly, 40% were female, have contact with domestic poultry, consume water from their private well and normally eat raw fresh vegetables. Although similar information was obtained between genders, no correlations between specific protozoa microorganism and environmental/social conditions were found.

**Conclusion:** In the present study, 12% of PD patients presented asymptomatic colonization of dialysis effluent with *Blastocystis hominis*, *Entamoeba* sp, *Giardia* sp or *Endolimax nana*, highlighting the need for a more systematic screening of protozoa in Portuguese PD population. The clinical impact of these sub-clinical infections should be further investigated. It is also noteworthy that there are no previous studies in this field in Portugal.

## Clinical Microbiology

P201/F14

### BACTERIAL RESISTANCE IN THE CONTEXT OF ORAL HEALTH

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The introduction of antibiotics revolutionized health care in the twentieth century, however, their intensive use promoted the selection of resistant phenotypes that have translated into a current public health problem worldwide. The fact that, between 7–11 %, of the total antibiotic prescription is done by dentists, some of most problematic resistant microorganisms are members of the oral microbiome and the oral environment is ideal for genetic exchange makes dentists an essential target in rational antibiotic prescription.

Although the existing studies present great geographical and methodological diversity it's a fact that there is a cause-effect relationship between the consumption of antibiotics and the development of bacterial resistance and it is estimated that in the last two decades the level of oral bacterial strains resistant to antibiotics has doubled. The aim of this work is to describe what is known about antimicrobial resistance in oral bacteria and to discuss some measures as to how dentists's rational antibiotic prescription can be stimulated.

Resistance to major antibiotics used in the treatment of oral/dental infections are presented by some of the main ethiological species. Among the species resistant to amoxicillin *Streptococcus viridans*, *Peptostreptococcus spp*, *Prevotella spp (intermedia)*, *Prevotella denticola*, *Porphyromonas spp (gingivalis)*, *Fusobacterium spp (nucleatum)* and *Veillonella spp* are included. *Streptococcus viridans*, *Streptococcus oralis*, *Streptococcus mitis (salivarius)*, *Streptococcus spp*, *Peptostreptococcus spp*, *Prevotella spp (intermediate)*, *Porphyromonas spp (gingivalis)*, *Fusobacterium spp (nucleatum)* have been described as resistant to penicillins. A significant portion of these species is also described as being resistant to clindamycin and macrolides, including erythromycin or azithromycin, frequently used by dentists. As for Metronidazole, often used in combination with other(s) antibiotic(s), the emergence of bacterial resistance has been slow, however it is described for various species, and it is particularly worrying when *Actinobacillus actinomycetemcomitans* is considered due to its role in periodontal disease. Tetracyclines, although increasingly less prescribed in the treatment of these infections, are broad spectrum antibiotics and widely used in other areas, requiring attention and surveillance of the specific resistances. Additionally, the tetracyclines may be used in association with penicillins or macrolides and may contribute to spreading of resistance determinants.

In this context, it is urgent improve dentists' awareness on rational antibiotic prescription.



## Clinical Microbiology

### P202/F35

#### THE ROLE OF *GARDNERELLA VAGINALIS* IN MIXED SPECIES BIOFILMS OCCURRENCE IN BACTERIAL VAGINOSIS AND ITS PREVALENCE IN PORTUGAL

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Bacterial vaginosis (BV) is the most common gynaecological clinical condition in reproductive -age women in reproductive age, and it has been associated with an increased risk of development of preterm labour, spontaneous abortion, and several sexually transmitted diseases such as HIV. Despite being of worldwide importance, no studies, to date, were performed in Portugal. Furthermore, BV has been a controversial topic in medical microbiology, and despite the wealth of information on this topic, the etiological agent has not yet been definitively identified. The first advances on BV pointed *Gardnerella vaginalis* as the infectious causative agent of BV but soon after it was found that *G. vaginalis* was also present in healthy women. Additionally, *G. vaginalis* was not able to cause BV consistently. Furthermore, other microorganisms started to be associated with BV, and this resulted in a shift in the paradigm to that of a multispecies infection. However, epidemiological data revealed inconsistencies with this latter theory. A couple of years ago the first descriptions of multispecies biofilm communities were described in BV. Interestingly, *G. vaginalis* was present in most cases and accounted for the majority of the biofilm biomass. Further studies demonstrated that biofilm-forming *G. vaginalis* presented higher tolerance to external stresses.

With this state of the art, I've hypothesized that strains of *G. vaginalis* that were able to form biofilms could be the causative agent of BV and proposed to conduct the first epidemiological survey of this condition in Portugal. To test my hypothesis, my research group collected near 300 vaginal samples and then isolated more than 30 bacterial species from BV patients, and also several strains of *G. vaginalis* from healthy women, and tested biofilm forming ability, initial adhesion to human vaginal cells, cytotoxicity activity, antimicrobial resistance and gene expression of known virulent genes. We also developed a novel PNA based multiplex methodology that was able to improve molecular diagnostic of BV and was then used to quantify the initial adhesion interactions between different bacterial species.

From the results gathered in the past 3 years, my research group was able to demonstrate that near 20% of Portuguese women had past or present BV episodes, and near 40% were colonized with *G. vaginalis*. Furthermore, BV associated *G. vaginalis* outcompeted all the other BV associated bacterial species in the initial adhesion to the epithelial cells and cytotoxicity assays. Furthermore, when comparing BV-associated *G. vaginalis* strains to strains isolated from healthy women, we found that all 7 strains from BV were more virulent than the 7 strains colonizing healthy women. Interestingly, no significant differences in expression of known virulence genes were detected, suggesting that the higher virulence of the BV-associated *G. vaginalis* was due to a yet unknown virulence determinant. Also, no significant differences were found in antimicrobial resistance profiles between the two groups. We then tested virulent *G. vaginalis* against other known BV-associated anaerobe pathogens, namely *Mobiluncus mulieris*, *Atopobium vaginae*, *Prevotella bivia* and *Fusobacteria nucleatum* in mixed biofilm formation quantification. Interestingly, while the other tested anaerobes did not reveal a higher initial adhesion, they did enhance biofilm formation by *G. vaginalis*.

Overall, our data revealed that BV is a prevalent condition in Portugal, and suggests that virulent variants of *G. vaginalis* have the potential to be the etiological agent of BV, while acknowledging that other anaerobes do enhance *G. vaginalis* virulence.

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## Epidemiology

### P203

#### *ACINETOBACTER CALCOACETICUS-ACINETOBACTER BAUMANNII* COMPLEX SPECIES DEPICTED BY MALDI-TOF MS AND MULTIVARIATE DATA ANALYSIS

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers the possibility of accurate, rapid and inexpensive identification of bacteria at species level. Moreover, discrimination of closely related species of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb) complex is difficult, leading to frequent misidentifications. In this study the potential of MALDI-TOF MS coupled with multivariate data analysis to discriminate the Acb complex species (*A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. calcoaceticus*, gen. sp. "Close to 13TU" and gen. sp. "Between 1&3") was assessed.

Eighty-three clinical isolates of the Acb complex (28 *A. baumannii*, 20 *A. nosocomialis*, 20 *A. pittii*, 9 *A. calcoaceticus*, 4 gen.sp. "Close to 13TU" and 2 gen.sp. "Between 1 and 3") from diverse origins and isolation dates (1950-2012) previously characterized by AFLP, ARDRA, 16S rDNA, *rpoB* and MLST were studied. MALDI-TOF MS spectra (Microflex LT benchtop, Bruker Daltonik, Germany) were acquired from cell extracts (CE) in linear positive mode (20-14000 Da) from 4 spots in 2 different days (4x2) per strain. CE were prepared according to the protocol suggested by the manufacturer. Spectra were mean centred and analysed with partial least square discriminant analysis (PLSDA) and the results were presented in the form of a dendrogram.

Acb species identification through MALDI Biotyper database leads to some misidentifications for species included or not in the database (except for *A. baumannii* and *A. calcoaceticus*). However, a detailed spectra analysis of the six species revealed a significantly different protein pattern among them. The PLSDA of the whole MS spectra allowed the discrimination of the 6 species. Despite some degree of overlapping observed between the clusters containing *A. baumannii*, *A. nosocomialis* and *A. calcoaceticus* this combined MALDI-TOF MS and multivariate data analysis approach led to higher values of sensitivity and specificity than the direct MALDI Biotyper database species identification. Moreover, this approach also allowed the discrimination of species not included in the MALDI Biotyper database.

The accuracy of this rapid and low cost method to differentiate the Acb complex species is extremely relevant for infectious disease treatment and infection control improvements.

## Epidemiology

## P204

## ARE METALS DRIVING THE EMERGENCE OF SALMONELLA NON-TYPHOID MULTIDRUG RESISTANT CLONES?

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Antibiotic resistant (AB<sup>R</sup>) *Salmonella* is a public health threat and might be selected by diverse environmental stressors (e.g. metals) used in animal production setting. We aimed to assess occurrence of known metal<sup>R</sup> genes among non-typhoid *Salmonella* belonging to clinically relevant clonal lineages and to characterize the genetic elements linked to their mobilization along with AB<sup>R</sup> genes.

*Salmonella* (n=92) of 16 serotypes, including emergent *S.* 4,[5],12:i:- (n=32) and *S.*Rissen (n=30), and 30 isolates representative of AB<sup>R</sup> clones from human/non-human sources from Portugal (2000-2011) were studied. Genes associated with AB<sup>R</sup>/integrons (Int), Cu<sup>R</sup>(*pcoD*), Ag<sup>R</sup>(*silA*), Hg<sup>R</sup>(*merA*), As<sup>R</sup>(*arsB*) or Te<sup>R</sup>(*terF*) were searched by PCR/sequencing. Conjugation assays, genomic location (I-Ceul/S1-PFGE/hybridization) and plasmid (PL) analysis (rep-PCR/sequencing) were done.

Ag<sup>R</sup>(72%), Cu<sup>R</sup>(57%), Hg<sup>R</sup>(47%), Te<sup>R</sup> (3%) or As<sup>R</sup>(1%) genes were found in different serotypes. All *S.*Rissen (ST469; 60% - *bla*TEM-*aadA*-*sul1*/*sul3*-*tetA*-*dfrA12*) carried *pcoD*+*silA* in chromosome (Ch) as *S.*Typhimurium monophasic variant from European clone (n=17; ST34), which also have co-located AB<sup>R</sup> genes (*bla*TEM-*strA*-*strB*-*sul2*-*tetB*) and the majority *merA*. In contrast, *S.*Typhimurium monophasic variant of the Spanish (n=10; ST19) and Portuguese (n=5; ST19) MDR clone carried *merA* and/or *silA* on large non-transferable IncA/C PL (110-170 Kb) or IncR PL (110-140) respectively and MDR *S.*Typhimurium (n=11; 4 clones; ST19/ST313) the *silA* (n=1; DT104 clone) on IncN PL (135Kb) with atypical type I-*sul3* integron, and *merA* (n=3) with *int1*-*oxa30*-*aadA1* (n=2) or *aadA1* (n=1) on transferable IncFII PL (140Kb). In *S.* Enteritidis (ST11) only *merA* (n=2/4) was detected on transferable IncP PL (80Kb) along with *int1*-*dfrA1*-*aadA1*. In isolates of other MDR clones (n=15; 12 serotypes), *merA* (n=13) and/or *silA*+*pcoD* (n=6) were co-located with different Int on large plasmids (>100Kb; IncHI1/IncP/IncI1/NT). The *arsB* (n=1) and/or *terF* (n=3) were located on transferable IncHI2 (240Kb) with *bla*<sub>CTX-M-9</sub> and in IncP (265kb) and untypable (250kb) PL, with *int1*-*aadA1* or *dfrA1*-*aadA1*/*pcoD*/*silA*/*merA*.

Metal<sup>R</sup> genes were often co-located with Int/AB<sup>R</sup> genes in diverse PL types carried by clinically relevant serotypes/clones, demonstrating the high genome plasticity of *Salmonella* to acquire different adaptive traits. Continued use of Cu/Ag or Hg environmental pollution might favour the selection of AB<sup>R</sup> *Salmonella* by clonal spread and/or PL horizontal transfer.

## Epidemiology

### P205

#### CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATES RECOVERED FROM HUMAN CLINICAL CASES OCCURRING IN PORTUGAL BETWEEN 2008 AND 2012

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*Listeria monocytogenes* is a ubiquitous organism in the environment and a rare cause of human disease. Infection (listeriosis), an uncommon but quite severe foodborne illness, leads to invasive disease during vulnerable stages of life. The elderly, immunocompromised people, and pregnant women and their foetuses, are at higher risk for *Listeria* bacteremia and meningitis, which can be fatal. The food consumption behaviours of these groups can put them at risk of listeriosis.

In this study, 204 isolates of *L. monocytogenes* recovered from Portuguese human cases of listeriosis, occurring between 2008 and 2012, have been characterized by: (i) biotyping (cadmium and arsenic sensitivity); (ii) geno-serotyping by multiplex-PCR; and (iii) the minimal inhibitory concentration (MIC, µg/ml) of twelve antibiotics (chloramphenicol, ampicillin, penicillin, gentamicin, ciprofloxacin, rifampicin, nitrofurantoin, vancomycin, tetracycline, erythromycin, streptomycin, and trimethoprim/sulfamethoxazole), by the agar dilution method, according to Clinical and Laboratory Standards Institute (CLSI, 2007).

Isolates were classified into three geno-serogroups: IVb (76.9%), IIb (16.2%), and IIa (6.9%). Four biotypes were differentiated: arsenic-sensitive and cadmium-sensitive (67.2%); arsenic-resistant and cadmium-sensitive (14.7%); arsenic-sensitive and cadmium-resistant (10.8%); and arsenic-resistant and cadmium-resistant (7.3%). Resistances to nitrofurantoin ( $n=87$ ), to ciprofloxacin ( $n=52$ ), and to rifampicin ( $n=28$ ) were observed. Erythromycin, ampicillin and penicillin were the most active antimicrobial agents against *L. monocytogenes*, with MIC<sub>90</sub> (i.e. MIC for 90% of isolates tested) of 0.5 µg/ml, 0.5 µg/ml and 1 µg/ml, respectively.

The implementation of national surveillance studies monitoring the incidence of listeriosis and antimicrobial resistance of strains of this clinically important pathogen would be most valuable, allowing identification of sporadic and outbreak cases, to detect general trends in antibiotic susceptibilities, and in parallel with food studies, to potentially identify food sources of clinical strain.

## Epidemiology

### P206

#### CURRENT DISSEMINATION OF CTX-M-PRODUCING *ESCHERICHIA COLI* IN PORTUGUESE HOSPITALS IS ASSOCIATED WITH WIDESPREAD CLONES FROM DIFFERENT PHYLOGROUPS

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The expansion of extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* (EC) isolates has significantly been driven by the wide dissemination of particular clones in different geographic areas. Although a high occurrence of ESBL-producing EC isolates has been reported in Portuguese clinical settings, data regarding population structure are scarce. We aimed to characterize recent ESBL-types and EC clones circulating in Portuguese hospitals.

One hundred and seventy-four ESBL-producing EC isolates recovered from a general hospital in the North (A, n=120 from 2006–7 and 2010) and 2 local hospitals from the Centre (B and C, n=54 from 2006–7) were analysed. They were mainly recovered from urine samples (85%). Bacterial identification and antibiotic susceptibility testing were performed by standard methods. Production of ESBLs was investigated by DDST, PCR (*bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>) and sequencing. Clonal characterization included PCR for identification of O25b-ST131 clone and *E. coli* phylogroups, *Xba*I-PFGE and MLST.

CTX-M-15 was the most prevalent ESBL (n=118, 68%) detected in all hospitals and time periods, although other CTX-M-types (CTX-M-1, -2, -9, -14, -32, -79) were also identified (24%; mainly in 2010). SHV-12 and TEM-52 were less frequent (6% and 2%, respectively). ESBL-producing EC isolates belonged to phylogenetic groups B2 (n=117, 67%; *bla*<sub>CTX-M-1,-2,-14,-15,-32</sub>; *bla*<sub>SHV-12</sub>), A (n=20, 12%; *bla*<sub>CTX-M-1,-2,-14,-15,-32</sub>; *bla*<sub>SHV-12</sub>; *bla*<sub>TEM-52</sub>), B1 (n=21, 12%; *bla*<sub>CTX-M-1,-9,-14</sub>; *bla*<sub>SHV-12</sub>; *bla*<sub>TEM-52</sub>) or D (n=16, 9%; *bla*<sub>CTX-M-1,-14,-15,-79</sub>; *bla*<sub>SHV-12</sub>; *bla*<sub>TEM-52</sub>). Most of B2 isolates (n=115, 66%, 7 PFGE-types) were identified as B2-ST131EC, detected since 2006 in different hospitals harbouring *bla*<sub>CTX-M-15</sub> (97%), *bla*<sub>CTX-M-1</sub> (1%), *bla*<sub>CTX-M-32</sub> (1%), *bla*<sub>CTX-M-14</sub> (1%) or *bla*<sub>SHV-12</sub> (1%). A high clonal diversity was observed among other phylogroups, although a few worldwide spread clones or clonal complexes such as D-ST117, D-ST648, A-CC10, A-CC23 and B1-CC155 were detected carrying diverse *bla*<sub>ESBL</sub> genes (*bla*<sub>CTX-M-1,-9,-14,-15,-32,-79</sub>; *bla*<sub>SHV-12</sub>; *bla*<sub>TEM-52</sub>).

We describe the current spread of a high diversity of ESBLs (mostly CTX-M-types) among EC clinical isolates in Portugal, associated with particular widespread clones from different phylogroups, including the pandemic B2-ST131 clone. The genetic diversity (ESBL-types and PFGE-patterns) observed for ST131 and other EC clones suggests intracolon evolution by both genomic and plasmid diversification.

## Epidemiology

## P207

DISCRIMINATION OF CLINICALLY RELEVANT *KLEBSIELLA PNEUMONIAE* CLONES BY FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

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Multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) have been pivotal for the population structure characterization of multidrug resistant *Klebsiella pneumoniae* isolates and the recognition of particular high-risk clonal groups (e. g. CG15, CG258). We aimed to explore the potential of Fourier Transform Infrared Spectroscopy in attenuated total reflectance (FTIR-ATR) to quickly and at low cost discriminate diverse *K. pneumoniae* clones. They include forty-nine previously characterized *K. pneumoniae* isolates, most of them producing diverse ESBLs (n=42; CTX-M-15, SHV-2/-12/-28/-55/-106, TEM-24), AmpCs (n=3; DHA-1) or carbapenemases (n=3; VIM-1/-34) from different Portuguese hospitals (n=4) between 2003 and 2012. They belonged to ST15 (n=24, 5 PFGE-types), ST147 (n=9, 1 PFGE-type), ST336 (n=7, 1 PFGE-type), ST14 (n=3, 3 PFGE-types), ST11 (n=3, 2 PFGE-types), ST916-like (n=2, 1 PFGE-type) and ST459 (n=1, 1 PFGE-type). Colonies grown on Mueller-Hinton agar (37°C, 18h) were transferred from the agar plates to the ATR crystal and air-dried. Spectra were acquired from 4000–600 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> and 32 scan co-additions and each isolate was tested in triplicate in three independent subcultures. Spectra were processed and analysed with Partial Least Square Discriminant Analysis (PLSDA) considering the phospholipids/DNA/RNA and polysaccharide regions (1500–900 cm<sup>-1</sup>).

Isolates were grouped by PLSDA in six distinct clusters corresponding to: i) CG15, which includes heterogeneous ST15 and ST14 (a single locus variant of ST15) isolates producing a wide diversity of  $\beta$ -lactamases (CTX-M-15, SHV-2, -12, -28, -55, -106, TEM-24, VIM-34); ii) ST147 (SHV-12), iii) ST336 (CTX-M-15), iv) ST11 (DHA-1), v) ST916-like (DHA-1) and vi) ST459 (VIM-1). The analysis of spectra corresponding to ST15 isolates evidenced three distinct clusters which overall matched with those assigned by comparison of PFGE-patterns. In one of the clusters we observed three subgroups corresponding to the different PFGE-types.

FTIR demonstrated a reliable and reproducible discrimination of *K. pneumoniae* clones, including some associated with a high genomic diversity by genotypic methods. Further studies with a larger sample are needed in order to validate this approach as a useful epidemiological typing tool with application at a large-scale basis.

## Epidemiology

P208

### FIRST DESCRIPTION OF *ACINETOBACTER PITTII* CLINICAL ISOLATES HARBORING BLAOXA-58 AND BLAOXA-23 IN A PORTUGUESE HOSPITAL

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*Acinetobacter* species have emerged as significant nosocomial pathogens. *A. baumannii* (Ab), in particular, has been associated with multi and pandrug resistant phenotypes and a significant attributable mortality. Although other related species such as *A. pittii* (Ap) have also been implicated in nosocomial infections and associated with the carriage of carbapenem hydrolyzing class D  $\beta$ -lactamases (CHDL) genes, there are no data on the clinical importance of this species in our country.

We aimed to understand the relevance of Ap species in a Portuguese clinical setting by characterizing antimicrobial susceptibility, presence of CHDL genes and clonality.

All Ap isolates (n=5) collected from different wards of a Northern Portuguese Hospital (2010–2012) were included. Identification was performed by Maldi-Tof and *rpoB* gene sequencing. Antimicrobial susceptibility was performed by disc diffusion and E-test methods and carbapenemase production was evaluated by a phenotypic assay. CHDLs genes and plasmid replicases were searched by PCR and confirmed by sequencing. Clonality was studied by Apal-PFGE. Genetic location for CHDL and replicases genes was determined by I-Ceul-hybridizations.

Three Ap (Ap1–Ap3) were susceptible to all antibiotics tested: imipenem (IMP=0.19–0.25mg/L), meropenem (MEM=0.25–0.38mg/L) cefotaxime (CTX), ceftriaxone (CRO), cefepime (FEP), ceftazidime, amikacin, gentamicin, tobramycin, ciprofloxacin and tetracyclin. One (Ap4) was susceptible to most of the antibiotics tested including carbapenems (MEM=0.75mg/L, IPM=1.5mg/L) and intermediate to CTX and CRO. Carbapenem and FEP resistance was only observed in Ap5 isolate (IMP>32mg/L, MEM >32mg/L), which was intermediate to CTX and CRO and susceptible to the remaining antibiotics. Only Ap5 was positive for carbapenemase production. CHDL genes observed were *bla*<sub>OXA-23</sub> (Ap5) and *bla*<sub>OXA-58</sub> (Ap4). Ap4 and Ap5 belonged to the same PFGE clone and differed from the other 3 isolates. *bla*<sub>OXA-23</sub> was chromosomally located and *bla*<sub>OXA-58</sub> was present in a ~90 kb plasmid carrying a new Rep3-superfamily replicase (73,14% homology with rep p3ABSDF002, NC\_010398).

To our knowledge, this is the first report of CHDL genes-carrying Ap in Portuguese Hospitals. Although considered less virulent and more susceptible, this study highlights the potential of Ap, or, probably, Ap particular clones, to acquire and act as a reservoir of CHDL resistance genes, underscoring the need for a tight surveillance of non-Ab species.

## Epidemiology

## P209

FIRST DESCRIPTION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN CAPE VERDE ISLANDS

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of infections worldwide. In a previous surveillance study in Cape Verde undertaken in 1997 we reported a 41% nasal carriage rate of *S. aureus* among patients and health care workers (HCW) in the two largest hospitals in the archipelago. Although no MRSA isolates were detected in 1997, an unusually high prevalence (35%) of Panton-Valentine leukocidine (PVL) was found among methicillin susceptible *S. aureus* (MSSA). Here we describe the results of a new surveillance study performed in 2013.

In February 2013, 312 individuals (181 inpatients and 131 HCW) from Hospital Agostinho Neto (HAN) located in Praia, and Hospital Baptista de Sousa (HBS) located in Mindelo, were screened for *S. aureus* nasal carriage. The presence of *mecA* and 11 virulence determinants including PVL was tested in all isolates by PCR. Moreover, isolates were characterized by pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing (MLST) and *SCCmec* typing (for MRSA).

A total of 62 individuals were *S. aureus* nasal carriers (19.9%), out of which two patients (3.2%) from HAN were colonized with MRSA. These two MRSA isolates belonged to PFGE L, *spa* t186/t12827, ST88-IVa. Almost half of the MSSA isolates (n=31, 49.2%) were clustered into three major clonal lineages: PFGE A, *spa* t355/t774, ST152 (n=11); PFGE B, *spa* t589/t861/t1510/t2771/t12826, ST508 (n=11); and PFGE C, *spa* t084, ST15 (n=9). The remaining isolates were distributed into seven minor groups (n=27), and five singletons. Ten of the MSSA clonal lineages, including PFGE B and C were previously detected in 1997. Although *S. aureus* isolates presented a high variability of virulence factors, PVL prevalence decreased from 35% in 1997 to 16.9% of the MSSA isolates in 2013.

MRSA nasal carriage in hospitals in Cape Verde seems to remain low. However, MRSA clonal lineage ST88-IVa, prevalent in other countries in Africa, has entered the archipelago. Additional infection control measures are warranted to avoid a massive MRSA spread within the country.



## Epidemiology

### P210

#### INSIGHT INTO THE MOLECULAR EPIDEMIOLOGY OF MULTIRESTANT NOSOCOMIAL *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM*

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The clinical relevance of *Enterococcus faecalis* and *Enterococcus faecium* relies in their capacity to acquire resistance to several antimicrobials, and to the presence of virulence determinants. While *E. faecalis* is the main cause of nosocomial infections, resistance to vancomycin is more prevalent among *E. faecium*. This inconsistency between nosocomial dissemination and glycopeptide resistance is not well understood and seems to be related to the bacterial genetic background.

The aims of this study were to establish and compare the prevalence and clonal structure of high-level gentamicin-HLGR and glycopeptide-GR resistant enterococcal isolates recovered from clinical samples during 2007 and 2008 from two different hospitals of Lisbon (Portugal) and to address the hypothesis that resistance to high concentrations of aminoglycosides may be considered as background for the subsequent acquisition of vancomycin resistance. A total of 806 *E. faecalis* and *E. faecium* isolated from infection products were studied. Antimicrobial susceptibility testing to ampicillin-Amp, ciprofloxacin-Cip, erythromycin-E, gentamicin- CN-120 microg, linezolid-LZD, quinupristin/dalfopristin-QD, tetracycline-Te, teicoplanin-Tec and vancomycin-Va was performed for all the isolates. Clonal relatedness was established by Pulsed Filed Gel Electrophoresis (PFGE) and by virulence determinants profiling among all HLGR and/or GR isolates and a subset of 10% *E. faecalis* and 30% *E. faecium* that were susceptible both to high level concentrations of gentamicin and to glycopeptides (HLGS/GS). Resistant isolates were screened for the presence of aminoglycoside and/or glycopeptide resistance genes. All glycopeptide resistant isolates were tested by Multilocus Sequence Type (MLST) and the plasmid localization of the *vanA* gene was performed by S1-digestion of genomic DNA followed by PFGE separation.

Among *E. faecalis* the HLGR prevalence was 40% and GR was 2%. Among *E. faecium*, HLGR prevalence was 35% and GR was 13%.

Among *E. faecalis*, resistance to vancomycin seem to be linked to a particular clone (PFGE AO) – ST6 belonging to the clonal complex CC2, which is recognized as a genetic lineage well-adapted to the nosocomial setting, and related with the presence of multiple virulence determinants.

*E. faecalis* isolates non-resistant to vancomycin and aminoglycosides showed a polyclonal structure and unrelated with the one of the resistant isolates. In contrast, related clonal types were found among *E. faecium* resistant and susceptible to vancomycin and aminoglycosides.

Our results seems to indicate that resistance to vancomycin among *E. faecium* isolates does not seem to be linked to a particular lineage and is not necessarily associated with resistance to aminoglycosides. Among this species, frequent replacement of sporadic clones was observed suggesting that each hospital has its own population. The presence of a polyclonal population is also evidenced by the variety of STs found among these isolates: ST17, ST18, ST125 and ST280 and variability in the number and size of the plasmid carried by these isolates (1 plasmid – 97kb size among *E. faecalis* isolates and 1 to 6 plasmids with a size between 48,5 kb – 291 kb ) among *E. faecium* isolates

Our results seem to suggest that the clonal spread and aminoglycoside resistance pool contribute to the dissemination of vancomycin resistance among *E. faecalis* and not among *E. faecium*.

## Epidemiology

### P211

#### SEROPREVALENCE OF HUMAN LEPTOSPIROSIS IN LUBANGO (ANGOLA)

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Leptospirosis is an infectious disease caused by pathogenic bacteria of genus *Leptospira*, occurs mainly in developing countries, and in tropical and subtropical regions. Lately, it has become an emerging problem for public health, affecting a wide range of mammals, including humans. There have been an increased number of cases with changes in the known epidemiological pattern and also a resurgence of epidemic outbreaks due to climate change. The nonspecific clinical presentation of the infection, can lead to a wrong diagnosis, where similar febrile diseases are common and confused with other disorders such as malaria. In Angola (Lubango), laboratory evaluation and diagnosis is not practiced due to lack of availability and implementation of specific tests, thus its prevalence is unknown.

This work aims to determine leptospirosis seroprevalence in patients/users assisted at the Central Hospital of Lubango, between September to December 2012, and to contribute for an inclusion of this pathology at differential diagnosis of patients with indeterminate febrile syndrome, contributing to disease prevention and control.

A total of 300 blood samples was collected from patients whose principal signs and symptoms were fever. A clinical/epidemiological questionnaire was applied, according to the ethics committee Angolan Health.

Samples were tested at Leptospirosis Reference Lab in Lisbon/Portugal (IHMT/UNL), by serological and molecular approaches: MACROLepto (screening test), and MAT (reference test), both for detection of antibodies anti-*Leptospira* spp, and through a PCR protocol targeting the *hap1* gene in order to detect *Leptospira* DNA, respectively.

According to questionnaire responses, the principal risk source for leptospirosis was the contact with rodents, observed in 91% of patients, and the main clinical manifestations were headache (84%) and myalgia (59%). From the serological results, 23% of the samples were positive and 37% doubtful by MACROLepto test, 40% of the samples were confirmed by MAT. Leptospiral DNA was detected in 30/193 (16%) of the samples. Six samples were positive for the three tests.

The results show leptospire presence in Lubango, and the existence of human contact with these bacteria. Thus, is important to develop the control and prevention measures against the disease, but also to perform more epidemiological studies to better understand the transmission patterns between humans and animals, and the range of hosts/reservoirs involved.

## Epidemiology

### P212

#### SPREAD OF CTX-M-15 AMONG DIVERSE B2-ST131 AND OTHER A, B1 AND D *ESCHERICHIA COLI* CLONES RESPONSIBLE FOR COMMUNITY-ONSET INFECTIONS IN PORTUGAL

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The worldwide spread of extended-spectrum beta-lactamases (ESBLs) has been fuelled by a few extraintestinal pathogenic *E. coli* (ExPEC) clonal variants, particularly disseminated in the clinical setting. We aimed to investigate the diversity of ESBL-producing *E. coli* (ESBL-EC) responsible for community-onset infections in order to identify potential extra-hospital reservoirs and elucidate transmission pathways. Thirty six extended-spectrum cephalosporins resistant *E. coli* from urinary tract infections (UTIs) identified in a community laboratory from the North of Portugal (Aug-Sep 2012) were characterized. Bacterial identification, antimicrobial susceptibility testing and ESBL production were assessed by standard methods. *E. coli* phylogenetic groups (PhG) were determined by a multiplex PCR and clonal relatedness by PFGE and InfoQuest. ExPEC virulence factors (38 VFs) and ST131, ST69 and ST95 clones were screened by PCR. Most isolates belonged to PhG B2 (47%), but also A (25%), D (22%) or B1 (6%). Isolates produced CTX-M-15 (47%), CTX-M-1 and CTX-M-14 (22% each) or SHV-type (8%). B2 isolates were grouped in two clusters (I and II, 75.4% and 68.2% homology, respectively) presenting variable ESBLs, AbR and virulence profiles. Cluster I grouped highly related ST131 isolates (n=15), sharing a common virulence (*usp-PAI-fimH-fyuA-iutA-traT-ompT-kpsMTII-K5-sat*) and AbR profile (quinolones, sulfonamides, tetracycline, trimethoprim and streptomycin). Three variants were identified: i) n=8 (1 PFGE-type) CTX-M-15 producers, additionally resistant to other aminoglycosides and variable presence of *iha*; ii) n=6 (3 PFGE-types, 77.7% homology) isolates producing CTX-M-15 (n=4) or -14 (n=2) carrying frequently *afa/draBC*, *pap* alleles and/or *hlyA*; and iii) n=1 CTX-M-14 producer carrying *afa/draBC*, *pap* alleles and *hlyA*. Cluster II B2-isolates were diverse (n=2, CTX-M-14 or SHV-12, variable VFs profile) and were only streptomycin resistant. PhG A, D and B1 isolates (n=19, 19 PFGE-types) produced mostly CTX-M-1 (n=7), CTX-M-15 (n=6), CTX-M-14 (n=4) or SHV-like (n=2). The low diversity of ESBLs (mostly CTX-M-15) identified currently in the community setting seems to have been driven by the amplification of EC clonal variants widespread in Portuguese hospitals. Moreover, the identification of diverse ESBL-EC clones with variable virulence potential highlights the risk for further dissemination and/or persistence.

## Epidemiology

### P213

#### UPDATE ON THE EPIDEMIOLOGY OF *STREPTOCOCCUS PNEUMONIAE* SEROTYPE 6C CARRIED BY YOUNG CHILDREN IN OEIRAS (2009–2012)

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*Streptococcus pneumoniae* (pneumococcus) lives asymptotically in the human nasopharynx. However, it is responsible for high rates of morbidity and mortality worldwide. The major virulence factor of pneumococcus is the polysaccharide capsule. Over 90 capsular types (serotypes) have been described to date. Serotype 6C, in particular, was first described in 2007 [1]. Soon after the discovery of this serotype, we described its epidemiology in carriage, from 1996–2007, in Oeiras, Portugal [2]. Here, we aimed to update that study to monitor how serotype 6C is evolving in the population. Between 2009 and 2012, 1,824 nasopharyngeal swabs were obtained from children participating in pneumococcal carriage cross-sectional studies. Pneumococci were isolated by routine procedures and serotyped by PCR and/or by the Quellung reaction. Antibiotic susceptibility was tested by disc diffusion or Etest. Genotyping was performed by multilocus sequence typing (MLST). Pneumococcal carriage remained stable during the study period (range 60.2%–63.8%). The prevalence of serotype 6C in 2009–2012 was 8.2%, 17.5%, 7.4% and 8.6% in consecutive years being higher than reported before (between 1996–2007 ranged between 0.2%–5.8%). The most common resistance pattern was non-susceptibility to penicillin, and resistance to macrolides, lincosamides streptogramins and tetracycline, which was found in 3.3% (2009), 17.0% (2010), 28.6% (2011) and 0% (2012) of the isolates. MLST of 42 selected representative isolates of pneumococci expressing serotype 6C identified three main clonal complexes (CC) – CC156, CC315 and CC395 – all described in the previous study. Multiresistance was associated to CC315, which was only detected in a single isolate in 2012. We conclude that between 2009–2012, serotype 6C has remained in circulation among young carriers due to the maintenance of lineages previously described. We highlight the importance of studying the epidemiology of this serotype since it is thought that the new 13-valent pneumococcal conjugate vaccine may confer cross-protection to this serotype.

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## Health Biotechnology

## P214

ANTIOXIDANT PROPERTIES OF *AMANITA PONDEROSA* CULTURES AND ANTIPROLIFERATIVE EFFECT AGAINST MDA-MB-231 HUMAN BREAST CANCER CELLSCátia Salvador<sup>1</sup>; M.R. Martins<sup>2</sup>; M.F. Duarte<sup>3</sup>; A.T. Caldeira<sup>4</sup><sup>1</sup>Chemistry Department, Évora Chemistry Centre (CQE), Évora University, Évora, Portugal;<sup>2</sup>Chemistry Department, Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM), HERCULES Laboratory, Évora University, Évora, Portugal;<sup>3</sup>Centre of Agricultural and Agri-Food Biotechnology of the Baixo Alentejo e Litoral (CEBAL), Beja Polytechnic Institute, Beja, Portugal; Centre for Research in Ceramics & Composite Materials (CICECO), Aveiro University, Aveiro, Portugal;<sup>4</sup>Chemistry Department, Évora Chemistry Centre (CQE), HERCULES Laboratory, Évora University, Évora, Portugal

Edible mushrooms are much appreciated, due to their texture and flavour as well for their chemical composition and nutritional properties. Some mushrooms have been reported as functional foods as a source of bioactive compounds with antioxidant properties, such as phenolic compounds, carotenoids and polysaccharides, important to prevent some diseases, such as hypertension, hypercholesterolemia and cancer [1, 2]. The medicinal properties of several species of edible mushrooms have been extensively investigated and reported their antitumoral activity.

*Amanita ponderosa* are wild edible mushrooms, which grow in Mediterranean forests in mountained areas with holm oaks and cork trees, namely in Alentejo region (Southern Portugal) and Andalusia (Southern Spain). There are few studies about biological properties of this species, however with this work it was possible to obtain *A. ponderosa* pure cultures from strains collected from different areas of Alentejo [3].

The aim of this study was to evaluate the antioxidant properties of *A. ponderosa* cultures and characterize their anti-tumoral activity of cultures against human breast cancer cell (MDA-MB231) *in vitro* model.

The antioxidant properties were screening by the evaluation of DPPH radical scavenging activity and lipidic peroxidation by the  $\alpha$ -carotene linoleate system. Cultures and mushrooms showed antioxidant activities as well capacity to mimetize catalase and superoxide dismutase enzymatic activities. Cultures (mycelia and supernatants) present a strong anti-proliferative ability, decreasing the percentage of viability of MDA-MB-231 human breast cancer cells.

Further studies are required to investigate pharmacological and toxicological properties of bioactive compounds produced by *A. ponderosa* cultures, with further medicinal potential and functional foods.

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## Health Biotechnology

P215

### DISPOSABLE IMMUNOSENSOR FOR DIAGNOSIS OF HUMAN CYTOMEGALOVIRUS CONGENITAL INFECTION

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Human Cytomegalovirus (HCMV) is a herpes virus that establishes a lifelong latent infection that, in the most of the immunocompetent individuals does not represent a serious problem. However, in immunosuppressed individuals, as transplanted ones, individuals infected by HIV or with an immature immune system (as fetuses and newborns) the infection can be severe or even fatal. For the diagnosis of HCMV, there are several methods, but these are expensive, need skilled operators or/and require long time to perform, so a method to detect HCMV that could potentially be done as a sensitive, cheap and rapid test will be most welcome.

In the acute infection by HCMV, specific antibodies are generated against to a large number of structural and non-structural proteins. Nevertheless glycoprotein B (gB) is the dominant antigen existing in the capsule of HCMV and approximately 100% of infected individuals with HCMV develop antibodies against this protein. Thus, gB can be viewed as a promising component to help the development of new tests. In this work, an immunosensor for HCMV diagnosis, by gB detection, is developed. This device combines the excellent stability of nanogold labels with the convenient stripping analysis of silver nanoparticles (Ag NPs) and disposable electrode. The developed electrochemical sandwich type immunosensor allows the ultrasensitive gB detection by the catalytic deposition of AgNPs on the surface of gold nanoparticles (AuNPs) attached to captured antibodies.

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## Health Biotechnology

### P216

#### GENOMIC INSTABILITY ASSOCIATED TO FE-S CLUSTERS BIOGENESIS IMPAIRMENT IN *SACCHAROMYCES CEREVISIAE* CELLS

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Iron-sulfur clusters (ISCs), biogenesis of which occurs in mitochondria, play an important role in essential cell functions such as respiration, ribosome biogenesis, regulation of gene expression and DNA-RNA metabolism. Mutations in the mitochondrial genome and instability of the nuclear genome cause multiple human diseases. Recent studies have connected defective ISC biogenesis, transport and/or cytosolic ISC protein assembly to nuclear genomic instability. Based on these works, our objective is to gain further insight in the relation between both processes by studying *S. cerevisiae grx5* mutant cells (lacking mitochondrial glutaredoxin 5) as a model of ISC impairment. This mutant displays increased genetic instability as indicated by elevated spontaneous mutation frequency, higher recombination rate, hypersensitivity to DNA damaging agents (UV, hydroxyurea and MMS), and significant increase of Rad52-associated foci formation, all of which indicate the presence of non-repaired DNA lesions. Sensitivity studies against DNA damaging agents have been performed in DNA repair mutants simultaneously lacking Grx5, in order to determine possible impairment of diverse DNA repair pathways due to defective ISC biogenesis machinery.

## Health Biotechnology

P217

### IN VITRO CONTROLLED DRUG RELEASE FROM CONTACT LENS MATERIALS UNDER PHYSIOLOGICAL OCULAR TEAR FLOW

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Nowadays, over 90% of currently administered ocular drugs are administered topically through eye drops [1], but the residence time of the drug in the eye is short, and only 1% to 7% of the administered drug is absorbed by the eye, leading to a final poor drug bioavailability and in some instances to side effects [2].

In the last few years, efforts have been made to develop more efficient drug delivery systems. Therapeutic soft contact lenses have demonstrated to be an ideal platform for the controlled delivery of numerous drugs as well as comfort molecules [3].

Typically, drug release experiments are conducted at infinite sink conditions, but, under normal physiological conditions, the human eye has a reduced tear volume and a tear turnover rate that varies between 1 and 4  $\mu\text{L}/\text{min}$ . Thus, infinite sink conditions are not the most appropriate to study the drug release kinetics on the eye.

The present work involves the development of a novel microfluidic device to simulate the physiological conditions (temperature, tear volume and flow rate) and to test contact lens materials as controlled drug release vehicles. The microfluidic device was produced from Poly(methyl methacrylate) (PMMA) and engineered to possess an inner chamber with a volume of 45  $\mu\text{L}$ . Isotonic saline solution was allowed to flow directly over the therapeutic contact lens with a volumetric flow rate of approximately 3  $\mu\text{L}/\text{min}$ , similar to that of the human eye. Two types of hydrogels were prepared as contact lens materials: a poly-hydroxyethylmethacrylate (pHEMA) based hydrogel and a silicone hydrogel. Both hydrogels were loaded with an antibiotic (levofloxacin) and an antiseptic (chlorhexidine) by soaking in the drug solutions. Results demonstrated extended drug release for 2–3 days, while the experiments performed in infinite sink conditions, showed a release time of 10 hours. This demonstrates that the hydrodynamic conditions significantly affect the drug release kinetics of therapeutic contact lenses and that extrapolation to *in vivo* behavior should be done with care.

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## Health Biotechnology

P218

### LOW COST LAB-ON-PAPER PLATFORMS FOR GLUCOSE TESTING AND MONITORING

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There is a strong interest in the use of biopolymers in the electronic and biomedical industries, mainly towards low-cost applications. The possibility of developing entirely new kinds of products based on cellulose is of current interest, in order to enhance and to add new functionalities to conventional paper-based products. We present our preliminary results towards the development of paper-based microfluidic devices for diagnostics, using glucose testing as a model [1]. This proof-of-concept platform will ultimately allow the measurement of glucose levels in human blood and other biological fluids. The devices are based on the definition of microchannels and reaction zones on hydrophilic paper via the patterning of walls of hydrophobic polymers using an eco-friendly wax-printing technology. Colorimetric glucose quantification was achieved through enzymatic reactions performed within specific reaction zones of the paper-based device. Briefly, the action of glucose oxidase decomposes glucose into hydrogen peroxide that is then utilized by a second enzyme, peroxidase. Peroxidase is responsible for the oxidation of colorimetric indicators generating a visible colour change. Different indicators were added to distinct reaction zones on paper matrices (4-aminoantipyrine + 3,5-dichloro-2-hydroxy-benzenesulfonic acid, potassium iodide and a mixture of both). The coloration achieved increased with growing glucose concentration and was highly homogeneous, covering all the surface of the paper reaction zones in a 3D sensor format. These devices showed a major advantage when compared to the 2D lateral flow glucose sensors, where some carryover of the coloured products usually occur. We can print a calibration colour gradient scale directly in the paper device in order to compare with test results, allowing to infer an approximate concentration of glucose in the sample. The results obtained with the proof-of-concept 3D glucose sensors are promising towards the future development of simple and cost-effective paper-based diagnostic devices.

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## Health Biotechnology

P219

### MOLECULAR RECOGNITION OF DNA HYBRIDS ON PAPER VIA THE ANCHORING OF ANTIBODIES WITH A FUSION OF ZZ-DOMAIN WITH CARBOHYDRATE-BINDING MODULES

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Microfluidic paper-based analytical devices fabricated by wax-printing are suitable platforms for the development of simple and low-cost molecular diagnostic assays, especially in resource-limited settings, because paper is a ubiquitous material that is inexpensive, biodegradable and compatible with biological and chemical assays [1, 2]. Paper devices can be modified for biological assays by adding appropriate reagents to the test areas. For this purpose, the use of affinity immobilization strategies can be a good solution for bioactive paper fabrication [2].

This study explores a methodology developed to capture labeled-DNA strands and hybrids on paper via the anchoring of antibodies with a fusion protein that combines a carbohydrate binding module from *Clostridium thermocellum* CipA (CBM3a), with high affinity to cellulose, and the ZZ fragment of the staphylococcal protein A, which recognizes IgG antibodies via their Fc portion [3, 4]. Immobilized antibodies via CBM-ZZ were able to capture labeled-DNA strands and hybridized DNA using a fluorescein-labeled-DNA as probe.

The ability of an antibody specific to biotin to discriminate complementary from non-complementary biotin-labeled targets was demonstrated in both spot and wax-printed microchannel assays. Hybridization was detected by fluorescence emission of the DNA probe. The efficiency of the capture of labeled-DNA by antibodies immobilized on paper via CBM-ZZ construct was significantly higher when compared with a physical adsorption method where antibodies were simply spotted on paper without the intermediation of other molecules.

This strategy provides an effective means of immobilization of antibodies on paper chips for labeled-DNA detection.

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## Health Biotechnology

### P220

#### POTENTIAL OF BIOETHANOL AND COFFEE PRODUCTION RESIDUES TO REDUCE ACRYLAMIDE IN BISCUITS

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The technology of coffee production is associated with a release of valuable residues containing favourable fatty acids. Similarly, fermentation and distillation steps in bioethanol processing produce by-products with high content of protein and fibre. These residues can enhance nutritional values of cereal based products by a replacement of margarine or flour in their formulation. On the other hand, acrylamide as an undesirable potentially carcinogenic compound is formed in cereal products during baking from amino acid asparagine and reducing sugars. In this study, the impact of both of these residues on acrylamide content in biscuits was investigated. The supplementation of margarine by coffee oil up to 4 g.kg<sup>-1</sup> of dough resulted in 12 % reduction of acrylamide. The addition of by-products of bioethanol production in amount of 10 g.kg<sup>-1</sup> of flour resulted in 24 % decrease of acrylamide.

However, the addition of residues is limited by their influence on organoleptic properties of final products. A sensory evaluation of enriched biscuits revealed that presented replacement of margarine by coffee oil up to 2 % (w/w) and 1 % (w/w) addition of by-product from bioethanol production were acceptable.

This approach seems to be a promising way how to improve both dietary properties and safety of cereal based products. It could be recommended also for an innovation and production of cereal based products with a potential health benefit.

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## Health Biotechnology

P221/F11

### BIOACTIVE COMPOUNDS FROM MARINE MICROALGAE — HEALTH APPLICATIONS

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Some microorganisms, namely marine microalgae and cyanobacteria, are very rich in bioactive compounds that, due to their benefits, can find applications in human health (Raposo et al., 2013). As a matter of fact, some of these microalgae produce high amounts of polyunsaturated fatty acids (PUFA), some of them being essential for humans. Some PUFA have hypolipidaemic properties, some others are even very important in the development and functioning of the nervous system and facilitate normal growth.

Another important group of compounds synthesized by marine microalgae are sterols, and phytosterols have already been approved by the Bureau of Nutritional Sciences–Health Canada (2010) because of their hypocholesterolaemic properties. Phytosterols have also some benefits on heart and coronary diseases.

Pigments, such as phycocyanin, astaxanthin and  $\beta$ -carotene, produced by *Arthrospira platensis*, *Haematococcus* and *Dunaliella salina*, respectively, have also proved to be effective as antioxidants, protecting against oxidative stress, and as therapeutic agents against atherosclerosis, coronary heart and degenerative diseases.

Some marine unicellular algae are also very rich in protein, the aminoacid profile being similar to that of soya, as it happens with *Arthrospira*. In addition, it was already demonstrated that plant protein, such as that from soybean, can have hypocholesterolaemic and hypoglyceridaemic properties.

In addition, there are many other biochemical compounds produced by marine microalgae that have also great potential in health applications, as therapeutics or nutraceuticals.

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## Drug Resistance Targets and Biotechnology

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### ASSESSMENT OF THE POTENTIAL OF DIETARY ISOTHIOCYANATES ON QUORUM SENSING INHIBITION

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The increased resistance of bacteria to conventional antibiotics led to a high interest in the development of new antimicrobial products. In most of the cases, the limited efficacy of antibiotics in treatment of infections is related to the presence of biofilms [1]. Consequently, new measures are required for the development of new antimicrobials that act on novel bacterial target of microorganisms that grow in biofilms. Quorum sensing (QS), a form of cell-to-cell communication in bacteria, is an important regulatory mechanism in biofilm formation and differentiation. The interference with QS can affect the biofilm development and make the bacteria more susceptible to antimicrobials [2]. In this context it is recognized that some dietary phytochemicals such as isothiocyanates (ITCs) have health benefits including antimicrobial activity against clinical important microorganisms [3]. The purpose of the present study was to evaluate the potential of three ITCs (allylisothiocyanate–AITC), benzylisothiocyanate–BITC and 2-phenylethylisothiocyanate–PEITC), at several concentrations on QS inhibition (QSI). A disc diffusion assay based on the pigment inhibition of *Chromobacterium violaceum* CV12472 was performed. In addition was extracted and quantified the amount of violacein produced after exposition to phytochemicals. The interference with bacterial QS is indicated by the lack of pigment production of *C. violaceum*. AITC, BITC and PEITC demonstrated capacity for QSI in addition to antimicrobial activity. AITC caused QSI halos of 5, 14 and 45 mm. For BITC and PEITC was found halos of QSI ranging from 5 to 13 mm and 5 to 35 mm, respectively. The percentage of violacein inhibition by ITCs was more than 80%. The results of this study emphasize the potential of ITCs as emergent molecules for biofilm control thought QSI.

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## Drug Resistance Targets and Biotechnology

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### CAFFEINE EXTRACTION FROM GUARANÁ SEEDS WITH AQUEOUS IONIC LIQUID SOLUTIONS

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Common products used in agriculture for pest control present serious drawbacks to human health, namely a high toxicity and volatility. Caffeine, a well-known alkaloid, presents antibacterial and antifungal properties. Therefore, caffeine could be used as a potential and more environmentally safe natural pesticide for the control of pest on food crops. Classically, the best attempts to extract caffeine from biomass have resulted in low efficiency and in a large consumption of hazardous organic solvents and/or energy requests. Thus, the extraction of caffeine from natural plants using benign and more economic processes is of great interest. In this work, we report an enhanced extraction of caffeine from guaraná (*Paullinia cupana*, Sapindaceae) seeds using aqueous solutions of ionic liquids. Guaraná is a climbing plant particularly common in Brazil which possesses around twice the caffeine typically found in coffee beans. Several operational parameters (ionic liquid structural features and concentration, contact time of extraction, solid-liquid ratio, particle size, and temperature of extraction) were investigated with the goal of achieving an improved process. To this end, a surface response methodology was employed. The data obtained reveal a high potential of ionic-liquid-water systems for the extraction of caffeine from guaraná seeds providing extraction yields up to 9 wt %. Moreover, moderate temperatures and short extraction times were employed. Further, the recyclability and reusability of the ionic liquids were successfully demonstrated supporting the economic and environmental viability of the process. In summary, it was shown that aqueous solutions of ionic liquids are in fact a good alternative for the solid-liquid extraction of caffeine from biomass samples.<sup>1</sup>

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### CONTRIBUTION OF EFFLUX SYSTEMS TO CLARITHROMYCIN RESISTANCE IN *MYCOBACTERIUM AVIUM* COMPLEX

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Emergence of resistance to macrolides in *Mycobacterium avium* complex (MAC) is problematic. Clarithromycin (CLA) is crucial for MAC therapy; but resistance emerges promptly. Previously, we have demonstrated that efflux pumps (EP) play a significant role in MAC resistance to macrolides. In this work, we aimed to i) evaluate the ability of efflux inhibitors (EIs) to reduce CLA resistance in MAC strains, ii) assess efflux activity by real-time fluorometry, and iii) evaluate the expression level of genes coding for EPs. For that, we used two reference strains, *M. avium* 104 and *M. intracellulare* ATCC, and one clinical strain, *M. intracellulare* 253/07 with a mutation in the 23S rRNA (CLA'). Strains were characterized by CLA susceptibility testing (in presence/absence of EIs) and minimum inhibitory concentration determination by broth microdilution. The capacity of the EIs verapamil, thioridazine and chlorpromazine to inhibit efflux was assayed by a semi-automated method that detects the accumulation and extrusion of ethidium bromide. MAC strains were exposed to subinhibitory concentrations of CLA and the expression level of 8 EP genes assessed by RT-qPCR. The results obtained shown that i) the resistance to CLA was significantly reduced in the presence of the EIs thioridazine, chlorpromazine and verapamil; (ii) increased efflux activity and overexpression of all EPs genes was observed in *M. avium* 104 and *M. intracellulare* 253/07 but not in *M. intracellulare* ATCC; and iii) the efflux activity was inhibited in the presence of the same EIs. In spite of the presence of a mutation in *M. intracellulare* 253/07, EP overexpression and significant efflux activity was detected, demonstrating that high-level resistance to clarithromycin it is a balance between active efflux and the genetic resistance. Overexpression of all EP genes indicates that induction of efflux pumps can be a general stress response and mutations mediate the resistance only up to a certain level, above which, resistance is efflux-driven. In conclusion, we demonstrate that emergence of resistance to CLA in MAC it is a balance between the mutation and efflux (in *M. avium* an early stress response; in *M. intracellulare* a late response when exposed to CLA) and this increased efflux/resistance can be reduced by EIs as adjuvants of the conventional therapeutic regimen that will reduce the probability of acquired resistance.

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### CORRELATION BETWEEN PHENOTYPIC AND GENETIC RESISTANCE TO FIRST AND SECOND LINE ANTITUBERCULOSIS DRUGS IN A HIGH TUBERCULOSIS ENDEMIC AREA

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The spread of multi and extensively drug resistant *Mycobacterium tuberculosis* (MTB) poses a serious problem to the control of tuberculosis (TB). Accurate drug susceptibility testing (DST) is essential to reduce the dissemination of the disease. The purpose of this study was to determine the resistance patterns of MTB strains circulating in Lisbon and its correlation with mutations associated with resistance. 20 multidrug resistant strains were characterized by 1st and 2nd line DST using the MGIT960/TB eXIST system. Genotypic characterization focused on the analysis of the genes involved in drug resistance with the aid of the line-probe assays Genotype MTBDR<sub>plus</sub> and MTBDR<sub>sl</sub> or DNA sequencing. The results show 100% agreement between the presence of a target gene mutation and the resistance phenotype: mutations in *rpoB* were associated with high level resistance to rifampicin and rifabutin; mutations in *inhA* promoter and ORF were associated with high level resistance to isoniazid and ethionamide (ETH); mutations in *ethA* were found in strains without mutations in *inhA* and associated with ETH high level resistance; mutations in *katG* were associated with INH high level resistance. Low level resistance to ethambutol was associated with the mutation *embB306*. Resistance to pyrazinamide was correlated with mutations in *pncA* gene; high level resistance to streptomycin was associated with mutations in *rpsL* and low level of resistance associated with mutations in *gidB*. For amikacin (AMK), mutation in *rrs* was associated with high level resistance. Mutations in *tlyA* correlate with capreomycin (CAP) low level resistance. Simultaneous resistance to AMK (high level) and CAP (low level) was associated with mutations in *rrs*. Resistance to ofloxacin and moxifloxacin was correlated with mutations in *gyrA* gene and associated with low level resistance. Resistance to para-amino-salicylic acid was associated with mutations in *thyA* and concerning linezolid, no resistant strains were detected. In conclusion, the description of the resistance profiles of drug resistant MTB strains circulating adds new knowledge for the design of more effective tools for the diagnosis of TB. Together, DST and the detection of mutations associated with resistance will allow the early administration of appropriate therapy since strains with low level resistance may still respond to the treatment despite the presence of the mutation.

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### EXPOSURE OF *C. KRUSEI* TO VORICONAZOLE: CHARACTERIZATION OF INDUCED RESISTANCE

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We assessed the voriconazole (VOR) resistance mechanisms acquired *in vivo* and *in vitro* by clinical *C. krusei* strains. Five *C. krusei* successive isolates were recovered from the urine of a kidney transplant patient under VOR treatment: isolate 628 L (before therapy), isolates 633L and 640L (at 9<sup>th</sup> and 16<sup>th</sup> day of VOR therapy), 657L and 671L (at 10<sup>th</sup> and 20<sup>th</sup> days after VOR discontinuation). We genotyped the isolates using both microsatellite analysis and intergenic repeat-PCR (CKRS-1). In addition, four independent *C. krusei* clinical isolates, VOR-susceptible, were grown in the presence of VOR 1 µg/l, for 30 days, in order to obtain VOR-resistant derivatives. VOR MICs were assessed in the absence/presence of 100 mg/l of the efflux pump inhibitor FK506, according to CLSI M27-A3 protocol. The expression of *ABC1* and *ABC2* genes (coding for ATP dependent efflux pumps) and *ERG11* gene (coding for lanosterol-14 $\alpha$ -demethylase target enzyme) was assessed by RT-qPCR using *ACT1* gene (coding for actin) as the reference. VOR-resistant isolates with an expression of the target genes higher than 2-times that of its susceptible parent were considered as an overexpressing isolate. All the strains isolated from the kidney transplant patient were clonal. VORMIC increased during therapy from 0.25 (isolate 628L) to 4.0 mg/l (isolates 633L and 640L) and decreased after VOR discontinuation (isolates 657L and 671L, VOR MICs 0.25 mg/l). *In vitro* exposure of VOR-free *C. krusei* clinical strains to low VOR concentrations generated VOR-resistant derivatives with MICs ranging from 4 to 8 mg/l. In both groups of strains the efflux inhibitor FK506 restored the susceptibility of all the VOR-resistant isolates (from 8 to 0.06 mg/l). The expression of *ABC1* and *ERG11* successively increased in *in vivo* VOR-resistant isolates (633L, 640L) and returned back to basal level in post-therapy isolates (657L, 671L). Surprisingly, the four *in vitro* induced VOR-resistant isolates showed different gene expression profiles: P21 and P34 overexpressed exclusively *ABC1* and *ERG11*, respectively, while P1 and P24 did not overexpress any of the tested resistance genes. Our results highlight the diversity of resistance mechanisms to azole in *C. krusei*. Interestingly, two VOR-resistant isolates did not overexpress *ABC1*, *ABC2* or *ERG11* suggesting yet unknown resistance mechanisms.

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### INVOLVING OF SGS 1 IN RESPONSE TO RAPAMYCIN TREATMENT IN YEAST *SACCHAROMYCES CEREVISIAE*

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In *Saccharomyces cerevisiae*, rapamycin exposure inhibits the TOR signaling pathway causing a profound alteration in the transcription pattern of many genes including those involved in ribosome biogenesis and nutritional changes. Deletion of the *RRD1* gene encoding a peptidyl prolyl isomerase resulted in mutants that are resistant to rapamycin.

The spot test analysis revealed that deletion of the *SGS1* gene in the *rrd1Δ* mutant partially suppresses the rapamycin resistant phenotype of the single *rrd1Δ* mutant. The *SGS1* gene encodes a helicase that functions in many biological processes including a role in recombinational DNA repair, transcription, and stabilizing the replication fork including transcriptional regulation. We check if the requirement of *Sgs1* in promoting resistance to *rrd1* mutant is dependent upon the helicase function of *Sgs1*, which is important for transcription. Introduction of a single copy plasmid carrying the native *SGS1* gene restored the rapamycin resistance to the *sgs1 rrd1* double mutant. In contrast, introduction of the plasmid *sgs1-hd* carrying a point mutation that inactivates the helicase function of *Sgs1* did not restore rapamycin resistance to the double mutant. This finding strongly suggests that the helicase function of *Sgs1* is required to promote rapamycin resistance in the absence of *Rrd1*.

A fluorescence-activated cell sorting analysis of synchronized cultures with  $\alpha$ -factor revealed that deletion of the *SGS1* gene in the *rrd1D* mutant resulted in double mutants that were unable to rapidly progress to the G2 phase as the single *rrd1D* mutant in the presence of rapamycin. Since *Sgs1* has a role in maintaining gene expression, we interpret these data to suggest that in the absence of *Rrd1*, *Sgs1* might be required to maintain the expression of genes needed for these cells to enter the G2 phase.

Finally, RT-PCR showed that genes such as *HSP42* and *RSP2* known to be respectively upregulated and downregulated in the parent in response to rapamycin were not induced or inhibited in the *rrd1Δ* mutant, but only if *SGS1* was deleted. Since *Sgs1* is involved in transcriptional regulation, we propose that it acts as a repressor or an inducer of a subset of genes and that its *Rrd1*-dependent degradation promotes induction of these genes in response to rapamycin.

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### IONIC-LIQUID-BASED AQUEOUS BIPHASIC SYSTEMS FOR THE IMPROVED DETECTION OF ETHINYL ESTRADIOL IN SEWAGE STREAMS

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Natural and synthetic steroid hormones have been recognized as some of the most prominent endocrine disruptor compounds found in aqueous streams. Ethinyl estradiol (EE2) is a synthetic steroid hormone widely used in oral contraceptives and hormone replacement therapies. It is excreted by humans and animals and it is released into the environment through the discharge of domestic sewage effluents as well as by the disposal of animal wastes. Nowadays, there is a reasonable number of techniques for the degradation and/or removal of these hormones in sewage treatment plants. However, none of these procedures is able to completely remove EE2 from the aqueous effluents and their presence, even at low concentrations (ng/L), represents serious risks for the aquatic population. Therefore, the monitoring of the EE2 content is crucial. However, the very low concentrations of EE2 in wastewater make its identification and quantification unviable due to the relatively “high” detection limits of conventional analytical equipment. Thus, it is necessary to develop specific methods to concentrate EE2 from aqueous media further allowing its proper identification and quantification.

Aqueous biphasic systems (ABS) are currently recognized as efficient strategies for concentrating metabolites from complex matrices and aqueous phases. In this context, novel ABS composed of hydrophilic ionic liquids combined with  $\text{KNaC}_4\text{H}_4\text{O}_6$  were tested for extracting and concentrating EE2 from aqueous samples. Extraction efficiencies of EE2 between 92 and 100 % were attained in all the investigated systems. Moreover, by a proper manipulation of the phase forming components and their concentration it was possible to increase up to 200 times the solubility of EE2 in the ionic-liquid-rich phase. The gathered data show the remarkable ability of ionic-liquid-based ABS to extract and concentrate EE2 from water samples in a single-step procedure, with the further possibility of reusing the ionic liquid and salt.

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### IONIC-LIQUID-BASED AQUEOUS TWO-PHASE SYSTEMS FOR THE EXTRACTION OF DYES

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Dyes are used in different industries for coloring purposes. The textile industry is one of the main industries which discharges a heavy load of chemicals during the dyeing process. As a result, the release of large contents of dyes for the aqueous effluents leads to both environmental and economic concerns. The extensive use of dyes also guides to further problems in human health and in the ecosystem. Most of the dyes are carcinogenic, mutagenic, allergenic and toxic. Therefore, new environmental laws are being implemented, and the removal of textile dyes from wastewater streams has been a subject of great interest in the past few years.

As a novel approach to remove dyes from aqueous effluents, in this work, ionic-liquid-based aqueous two-phase systems (ATPS) were investigated. The studied ATPS are composed of several ionic liquids (ILs) and distinct inorganic salts to evaluate the influence of the ionic liquid structural features, the nature and salting-out ability of the salt employed, and the pH of the aqueous medium. The extraction efficiencies of several dyes, namely sudan III, indigo blue and chloranilic acid using different ATPS were experimentally determined. The results obtained reveal that a proper selection of the ionic liquid and of the inorganic salt can lead to the complete extraction and concentration of the several dyes in a single-step procedure.

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#### NOVEL MUTATIONS IN *PNCA*, ASSOCIATED WITH CATALYTIC CENTRE, MIGHT BE RELATED WITH PZA HIGH RESISTANCE PROFILE

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Tuberculosis (TB) remains a serious health problem worldwide, and the greatest threat to TB control is the emergence of multidrug (MDR) *Mycobacterium tuberculosis* (Mtb) strains. Pyrazinamide (PZA) is an important first-line anti-TB drug. The emergence of isolates resistant to PZA, associated with MDR resistance, difficult TB treatment options. In order to evaluate mutations that might be responsible for PZA resistance, 30 clinical isolates collected in Lisbon Health Region, were screened for mutations in *pncA*, pyrazinamidase (PZase) coding gene, which convert the pro-drug PZA into its active form. We found 12 mutations in 23 resistant isolates. Among these mutations, six occurred in residues from catalytic centre or play an important role in PZase-PZA binding. We highlighted mutations that have never been described before: 3bp deletion (Del) at position 412, and 13 base pair (bp) deletion between residues Ala146 and Gly150. The Del 412 3bp observed corresponds to entire deletion of Cys138 residue. Cys138 is a conserved residue in the active site, responsible for maintaining a functional conformation and is directly involved in drug donor binding. We also found a 13bp deletion between residues Ala146 and Gly150, resulting in a frameshift mutation plus four residues absence (Val147, Arg148, Asn149 and Gly150). There are no references for mutations in these residues, however it has been reported that residue Ala146 interacts with Cys138 to establish a functional folding. Evidences from mutational directed studies showed that amino acid substitution in catalytic centre residues produced some degree of conformational changes, but substitutions in Cys138 was associated with large folding alterations. Altogether, the results let us to hypothesize that the novel mutations presented might confer significant differences in protein folding. The loss of four amino acid residues plus frameshift near Ala146 residue could alter stability of PZase, and interfere with Cys138 normal interaction, resulting in a diminished PZase activity. Moreover, absence of the entire Cys138 residue would drastically alter the catalytic centre conformation, resulting not only in a different folding and reduction of protein stability but could also depleted PZase activity, and culminate with mycobacteria survival in the presence of PZA. Nevertheless, more studies are needed to evaluate the influence of such mutations in resistance to PZA.

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### PROTEOMIC ANALYSIS OF *AEROMONAS SALMONICIDA* CECT894<sup>T</sup> WITH EXPERIMENTALLY INDUCED RESISTANCE TO AMPICILLIN

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The genus *Aeromonas* includes species that are common in drinking waters and in food, particularly seafood. Several species are recognized as etiological agents of a wide spectrum of diseases in humans and animals. The members of this genus often exhibit resistance to  $\beta$ -lactam antibiotics (penicillins, cephalosporins and carbapenems), mainly due to the coordinated expression of inducible chromosomal  $\beta$ -lactamases. Recent works indicate that the antibiotic resistance does not depend only on expression a  $\beta$ -lactamases, but is the result of the interplay of proteins from several metabolic networks. Often, the expression of these molecules occurs in response to the stress imposed by the antibiotic (adaptive response).

In the present study we were able to identify changes in the intra and extracellular proteome of a laboratory-induced ampicillin-resistant strain derived from the type strain *Aeromonas salmonicida* CECT894<sup>T</sup> (minimal inhibitory concentration [MIC] 128  $\mu\text{g.mL}^{-1}$ ) when compared with the original strain *Aeromonas salmonicida* CECT894<sup>T</sup> ([MIC] 0.5  $\mu\text{g.mL}^{-1}$ ). We used 2D gel electrophoresis, followed by MALDI-TOF/TOF for protein identification. Both strains were characterised in terms of biofilm formation, cytotoxicity, activity of hemolysin, proteases and  $\beta$ -lactamases, in the presence and absence of ampicillin.

Both strains, when exposed to antibiotic, displayed improved ability to form biofilms, to cause cytotoxic effects and displayed enhanced activity of hemolysins, proteases and  $\beta$ -lactamases. For the resistant strain, proteins associated to virulence, antibiotic resistance and DNA protection were overexpressed while lower expression levels were found for proteins involved in glycolysis, protein biosynthesis, amino acid transport and cell division. Our data puts in evidence the metabolic changes occurring when this species is challenged by a  $\beta$ -lactam: the response involves reduction of metabolic activity, synthesis of virulence factors as well as activation of mechanisms for protection of genetic integrity and for defense against the antibiotic.

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### THE ROLE OF TYPE-II NADH-MENAQUINONE OXIDOREDUCTASE (NDH-2) ON *MYCOBACTERIUM TUBERCULOSIS* RESISTANCE TO DRUGS

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Tuberculosis (TB) remains a global health problem, claiming millions of human lives every year. The emergence of drug resistant TB, particularly the one caused by multi- and extensively drug-resistant *Mycobacterium tuberculosis*, and the lack of therapeutic options, demand for new therapeutic strategies and targets. The objective of this work was to study the type-II NADH-menaquinone oxidoreductase (NDH-2) of *M. tuberculosis*, a key enzyme in the respiratory chain of this bacterium, which has been suggested as target for the design of new anti-TB drugs. We are particularly interested in understanding the functional correlation between the NDH-2 activity and the energy demand of efflux systems, which have been recently recognized as key elements in the early stages of development of the antimicrobial resistance.

The *ndh-2* gene, coding for *M. tuberculosis* NDH-2 was cloned into *M. smegmatis* strain mc<sup>2</sup> 155 and the transformed strain used to overexpress the protein. Both wild-type and transformed strains were characterized by determination of minimum inhibitory concentration (MIC) of antibiotics (isoniazid, rifampicin, ethambutol, clarithromycin, erythromycin, ofloxacin, kanamycin, amikacin, gentamicin, capreomycin and tetracycline) in the presence of compounds known as efflux inhibitors (EIs), namely thioridazine, chlorpromazine, verapamil (VP), flupentixol (FPX), haloperidol (HAL), orthovanadate, arylpiperazine and carbonyl cyanide m-chlorophenyl hydrazone. The efflux inhibitor HAL, followed by FPX and VP, promoted the highest reductions in the MICs of the antibiotics, particularly clarithromycin, erythromycin and rifampicin. The effect of EIs on the enzymatic activity of NDH-2 was also evaluated by measurement of the NADH-menaquinone oxidoreductase activity on membrane suspensions obtained from cells overexpressing NDH-2. The results obtained showed that the enzymatic activity of NDH-2 is also more affected in the presence of efflux inhibitors HAL, FPX and VP. Overall, the results indicate that NDH-2 is a promising target for the development of new anti-tuberculosis compounds.

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### EFFLUX INHIBITORS AGAINST DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS*: ANTIMICROBIAL AGENTS AND ENHANCERS OF MACROPHAGE KILLING ACTIVITY

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The main mechanisms associated with drug resistance in *M. tuberculosis* (MTB) involve mutations in target genes and increased efflux. Here, we study the antimycobacterial activity of efflux inhibitors (EIs) against drug resistant MTB, *in vitro*, and assess their effect on the enhancement of the killing activity of human monocyte-derived macrophages. The EIs verapamil, flupenthixol, haloperidol, chlorpromazine and thioridazine were tested against eight MTB strains: H37Rv, H37RvΔ*katG*, four multidrug resistant, and two isoniazid mono-resistant. The strains were characterized by antibiotic susceptibility testing, with and without EIs, and analysis of drug resistance associated genes plus genes coding efflux pumps (EPs). The ability of EIs to inhibit efflux was assayed by real-time fluorometry. The bactericidal activity of the compounds was investigated through time-kill studies. The expression level of genes coding EPs was determined by qRT-PCR. The antimycobacterial activity of the compounds against intracellular MTB and their potential to induce phagolysosome acidification were evaluated. The results showed that the resistance levels for isoniazid and rifampicin can be reduced from high to low level in presence of EIs. The inhibition of efflux by EIs leads to intracellular accumulation of antibiotics and increased susceptibility despite the presence of a mutation conferring resistance. Time-kill studies demonstrated that these compounds are bactericidal and potentiate antibiotic activity. The majority of EP genes were upregulated in response to antibiotic exposure indicating that antibiotics act like inducers that stimulate a general stress response. This supports the notion that the different resistance levels are a balance between the induction of several EPs and due to the mutation. Albeit EPs showed substrate promiscuity, our results point out to the existence of a verapamil sensitive EP associated with rifampicin and isoniazid transport. Moreover, these compounds enhance the killing activity of the macrophage against intracellular MTB and induce phagolysosome acidification thereby contributing to the elimination of the internalized mycobacteria. In conclusion, due to their dual role as antimicrobial agents and enhancers of macrophage killing activity, this work highlights the value of EIs as adjuvants of drug resistant tuberculosis therapeutics.

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### A NEW APPROACH FOR THE EXTRACTION OF PHARMACEUTICALLY ACTIVE COMPOUNDS FROM AQUEOUS STREAMS USING IONIC LIQUIDS

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In the modern era of human life, there is an increased worldwide consumption of active pharmaceutical ingredient (API), such as ciprofloxacin, naproxen and ibuprofen. Nevertheless, API are not completely metabolized by humans and are inherently excreted into domestically effluents. Numerous studies have been showing that a wide variety of pharmaceuticals are present in wastewater effluents and are a matter of great concern for the public health [1]. Albeit wastewater treatment plants (WWTPs) use advanced technologies for pollutants/contaminants removal, none of these processes was specifically designed for API [2]. In this context, this work addresses the use of liquid-liquid extraction, employing a novel class of compounds – ionic liquids (ILs), for the removal of API from aqueous streams. In particular, aqueous biphasic systems (ABS) composed of ILs and aluminium-based salts were tested in the extraction of ciprofloxacin, naproxen and ibuprofen from aqueous media. Depending on the API under study the complete extraction was achieved either by employing imidazolium- or phosphonium-based ILs. Due to the outstanding results, the implementation of these new alternatives in WWTPs can be foreseen.

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ANTAGONISTIC ACTIVITIES OF KEFIRGEL AND KERFIRGEL PRODUCTS AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATED FROM DIABETIC FOOT ULCERSSoraia Zenão<sup>1</sup>; Alfredo Aires<sup>2</sup>; Carla Dias<sup>3</sup>; Maria José Saavedra<sup>3</sup>; Conceição Fernandes<sup>1</sup>

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Diabetic foot ulcers are often complicated by infection and among pathogens the Gram-positive *Staphylococcus aureus* is the most common isolated. Also concomitantly, the high prevalence of methicillin-resistant *S. aureus* (MRSA) was significant impact on successful treatment of infected foot ulcers. In this context, the purpose of the present study was to evaluate the antibacterial properties of Kefigel®, a natural product composed by nettle (*Urtica dioica* L), lavender (*Lavandula angustifolia* Mill) and kefir grains, which have been reported as to having antibacterial activity against several diseases. Here, antibacterial effect of Kefigel® and its components were investigated against 20 *S. aureus* isolates (10 MSSA and 10 MRSA) collected from several diabetic foot ulcers. To assess antibacterial activity, the disk diffusion assay method, minimum inhibitory concentration (MIC), minimal bacterial activity and effects on specific growth rate, were applied. Results showed that by diffusion method, only etanolic extracts of nettle and lavender showed antibacterial activity and their effects were mainly bacteriostatic. Compared to the antibiotic gentamicin, the nettle and lavender extracts showed an efficacy between 50 and 100% relative to the antibiotic. Generally, the MRSA isolates showed higher inhibition halos comparing MSSA isolates. In turns, Kefigel® (40mg.mL<sup>-1</sup>) affects the specific growth rate of *S. aureus* isolates, since after incubation for 9h almost MSSA isolates growth were inhibited. A bactericide effect was observed only against one isolates MRSA. These findings indicate a potential use of Kefigel® as a natural product having an effective effect against *Staphylococcus aureus*. The topical use of this product for prevention of diabetic foot ulcers infections can be useful, however further investigation will be made, namely tests with high Kefigel® concentrations and synergetic effects with antibiotics.

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## ANTIBACTERIAL SCREENING OF ACTIVE ETHANOLIC FRACTIONS OF URTICA DIOICA L AGAINST PATHOGENIC BACTERIA FROM GASTROINTESTINAL AND RESPIRATORY TRACT

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Medicinal plants are largely explored source of drug repository. Although a huge number of antibiotics are available for treatment of microbial infections, development of resistance mechanism against antibiotics is nowadays a major health concern. Therefore the needs of discover new antimicrobial compounds with high safety index is always recurrent and medicinal plants have great potential for providing novel drug leads with novel mechanism of action. Historically, this plants have always been a source of inspiration for new drug compounds. In this context we evaluate the antimicrobial potential of various extracts (produced by a partial purification in a silica gel column) of *Urtica dioica* L (stinging nettle) (Urticaceae) collected from Vila Real region (Portugal), against 10 different bacteria isolates collected from clinical patients (gastrointestinal segments and respiratory tract), namely 4 Gram positive [2 *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (MSSA), *Staphylococcus saprophyticus*, and *Enterococcus faecalis*] and 5 Gram negative [*Salmonella thyphi*, *Echerichia coli*, *Klebsiela pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis*]. The current study represents the investigation of antimicrobial activity of *U. dioica* from four fractions (Hexane, Ethyl–acetate, ethanol and water) that were prepared using sequential fractionating method by column chromatography. The antibacterial activity was assessed by disk diffusion and minimum inhibitory concentration methods. The phytochemical composition of *U. dioica* fractions were assessed by HPLC–UV–DAD. Our results showed that only ethanolic fractions had antibacterial activity but only in *S. aureus* (MRSA and MSSA), *S. saprophyticus* and *E. faecalis* isolates. Thus, only the Gram positives were affected by *U. dioica* extracts. The inhibition zone diameter halos ranges from 0 to 23 mm and the minimum inhibitory concentration (MIC) were 6.25 mg.mL<sup>-1</sup> for MRSA isolates and 0.78 mg.mL<sup>-1</sup> for *S. saprophyticus* and *E. faecalis*. Our results show a direct association between the antibacterial activities and high content of phytochemicals detected in the ethanolic fractions particularly with phenolic acids (chlorogenic and ferulic acids) and flavonols (rutin, isoquercetin and quercetin isomers). Our observations suggest the importance of ethnomedicinal use of *U. dioica*, which could be used by the pharmaceutical industry as source of a natural antimicrobial agents and antioxidant compounds.

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### ANTIOXIDANT AND ANTIFUNGAL ACTIVITIES OF HALOGENATED ARYLAMIDE-ACETALS: POTENTIAL DRUGS FOR NEURODEGENERATIVE DISEASES

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Antioxidants behave as major defence agents against reactive oxygen species (ROS) which are prominent in many serious illnesses, including neurodegenerative diseases such as: Alzheimer's and Parkinsons diseases [1]. Oxidative stress has been implicated in the pathophysiology of many neurodegenerative diseases, and also fungal infections. Antioxidant defense mechanisms are responsible for the removal of O<sub>2</sub>, scavenging of reactive oxygen species or inhibition of reactive oxygen species formation [2]. Therefore the discovery of small molecules with antioxidant properties is important in the fight against neurodegenerative diseases and fungal infections [2].

In this project a small library of small halogenated arylamide-acetal compounds was screened for antioxidant capacity and antifungal activity: N-(2,2-Dimethoxyethyl)-2-iodobenzamide, N-(2,2-Dimethoxyethyl)-2-bromo-5-methoxybenzamide, N-(2,2-Dimethoxyethyl)-2-bromo-5-methyl-benzamide and N-(2,2-Dimethoxyethyl)-2-bromo-4-methylbenzamide.

The four compounds showed a considerably antioxidant capacity with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene systems, values between 2,5 ± 0,7% to 10 ± 0,8%. The highest antioxidant activity was obtained with N-(2,2-Dimethoxyethyl)-2-bromo-5-methoxybenzamide with both methods.

The potential antifungal activity was determined using an agar disc diffusion method against three different filamentous fungi stains, *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium* sp. and a yeast strain, *Candida albicans*. The results allow to verify that the compounds have in fact inhibition capacity for the fungi tested, confirmed by the formation of an inhibition halo around the discs and a visible inhibition of sporulation.

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RATIONAL DESIGN AND FUNCTIONAL CHARACTERIZATION OF A BIOACTIVE ALANINE-RICH PEPTIDE ANALOGUE FROM *PLEURONECTES AMERICANUS*

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In last decades, several peptides have been observed in the host defense against pathogenic microorganism, which are able to act in different targets. This ability allows the development of novels strategies for rational design of unusual bioactive compounds. The present work focus on structural and functional evaluation of the palindrome analogue peptide, named *Pa*-MAP2, designed with base in the peptide *Pa*-MAP previously synthesized and characterized as a multifunctional peptide from *Pleuronectes americanus*. After chemical synthesis, *Pa*-MAP2 showed an improvement activity against *Escherichia coli* ATCC 8739 with a MIC value of 3.2  $\mu$ M. Moreover was any deleterious effects were observed against *Staphylococcus aureus* ATCC 25923. Furthermore, the peptide did not showed cytotoxicity against erythrocytes and RAW 264.7. *Pa*-MAP2 at standard concentrations of 1 mg.kg<sup>-1</sup> was also evaluated *in vivo* through intraperitoneally *E. coli* infected mice with a sub-lethal concentration. *Pa*-MAP2 was able to prevent *E. coli* infection and further improve mice survival with total weight gain of 2.5%. In contrast mice treated with ampicillin concentration 2 mg.kg<sup>-1</sup> lost 5.6% of their weight. For a better understanding of functional-structure relations analysis molecular modeling was realized. Secondary structure model of *Pa*-MAP2 was constructed using Modeller v9.12 and evaluated with Procheck program. Ramachandran plot demonstrated that 100% of amino acid residues are in favorable regions. In addition, *Pa*-MAP2 showed a structure pH dependent in the range between pH 3.0 to 7.0 the structure observed was random with a negative band in 198 nm and from pH 8.5 to 11.0 the secondary structure presented clearly  $\alpha$ -helice conformation with two negative bands in 208 and 222 nm. *In silico* docking studies demonstrated six amino acid residues positive charged in the positions Lys<sup>2</sup>, Lys<sup>9</sup>, Lys<sup>13</sup>, Lys<sup>16</sup>, Lys<sup>20</sup> and Lys<sup>27</sup> that probably permit an electrostatic attraction with the negatively charged membrane constructed by using CHARMM-GUI server in a proportion of 9:1 (DPPE: DPPG) based in Gram-negative bacterial membrane. The structural conformations observed *in vitro* and *in silico* for *Pa*-MAP2 were complementary and similar for others antimicrobial peptides in hydrophilic and hydrophobic environment. Data here reported indicated that *Pa*-MAP2 could be a potent candidate for *E. coli* bacterial control.

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### **SALVIA OFFICINALIS FROM SPAIN: AROMATIC PROFILE BY ENANTIOSELECTIVE GAS CHROMATOGRAPHY–MASS SPECTROMETRY**

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**Objectives.** The identification and determination of biochemicals of the essential oil of *Salvia officinalis*, grown from organic farming in Murcia (Spain). The oil has been eco-extracted, by steam distillation with a portable still, next to cultivation, and with a boiler feed with biomass, from plants previously distilled.

**Methodology** ([www.um.es/genz/e00605/serv03.htm](http://www.um.es/genz/e00605/serv03.htm)). Fast Gas Chromatography on a non-polar fast column (SLB-5ms) 15m x 0.1mm x 0.1µm, and Enantioselective Gas Chromatography on a chiral column Chiraldex B-DM 30m x 0.25mm x 0.12µm, were carried out on a GC-chromatograph (Agilent 7890), with hydrogen as gas carrier (PDH). The Mass Spectrometry Detector used an electronic impact ionizer (70 eV), and a single quadrupole analyzer (Agilent 5975 MSD). Sandwich split/splitless injections, with hexadecane as inner standard, and calibration straight with standards (Gerstel MPS-2XT autosampler).

**Results.** The chromatographic results showed that the *S. officinalis* oil from Murcia is especially rich in some biomolecules like (mM concentration, % enantiomers): Camphor (2413, +90, -10), a-Thujone (2383, +4, -96), Z-Ocimene (642), Camphene (531, +42, -58), b-Thujone (433), Eucalyptol (425), a-Pinene (281, +41, -59), Borneol (275, +67, -33), Linalool (247, +61, -39), Limonene (151, +52, -48), Bornyl acetate (148, +3, -97), a-Humulene (131), b-Caryophyllene (124, +4, -96), b-Pinene (120, +41, -59), Myrcene (92), a-Terpineol (45, +3, -97), g-Terpinene (33), Terpinen-4-ol (25, +50, -50), Linalyl acetate (21), and other minor components.

**Conclusions.** *Salvia officinalis* oil has a very high concentration of Camphor, a-Thujone, Z-Ocimene, Camphene, b-Thujone and Eucalyptol, about 10-20 times higher than that of many other biomolecules. Near pure (-)-enantiomers are present for a-Thujone, Bornyl acetate, b-Caryophyllene and a-Terpineol. There are mainly (+)-Camphor, (+)-Borneol and (+)-Linalool, whereas there are mainly (-)-Camphene, (-)-a-Pinene and (-)-b-Pinene. Furthermore, Limonene and Terpinen-4-ol have similar proportions of both enantiomers. This biochemotype is markedly different than that of *Salvia officinalis* oil, with similar cultivation and extraction procedures in Castilla-La Mancha (Spain). The essential oil from Murcia has higher concentrations than that stated in the corresponding ISO standard for Eucalyptol, Camphene, Limonene and Linalool. This oil is a good source of biomolecules for pharmaceuticals, cosmetics, fragrances and food industries.

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## P240

TOXICOLOGICAL AND PHARMACOLOGICAL EVALUATION OF THE ESSENTIAL OIL AND AQUEOUS EXTRACT OF *LAVANDULA STOECHAS* L. SUBSP. *LUISIERI*S.A. Arantes<sup>1</sup>; M. F. Candeias<sup>1</sup>; M. Pereira<sup>2</sup>; O. Lopes<sup>3</sup>; M. Lima<sup>4</sup>; M. R. Martins<sup>1</sup>

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Lavenders belong to the family *Labiatae* and represent some of the most popular medicinal plants extensively used in aromatherapy [1]. In this study we selected *Lavandula stoechas* L. subsp. *luisieri* (Rozeira) Rozeira, commonly known as rosemary, endemic of Portugal and collected in Évora (Alentejo).

The aim of this study was to evaluate the acute toxicity and the analgesic and anti-inflammatory activities of the essential oil and aqueous extract from leaves of *L. luisieri*. The essential oil was obtained by hydrodistillation in *Clevenger* type apparatus and the resulting hydrolate was lyophilized. Chemical composition of essential oil was evaluated by GC–FID and extract was characterized by phytochemical tests and total phenolic and flavonoid quantification. Brine shrimp *in vitro* lethality was evaluated (LC<sub>50</sub>) [2]. Pharmacological screening and acute toxicity (LD<sub>50</sub>) were determined in *Swiss* mice, according to Up–and–Down OECD Procedure [3]. This approach was complemented with liver and kidney histopathological studies. Pharmacological properties, namely, analgesic and anti–inflammatory activities were evaluated in *Wistar* rats [4].

The essential oil showed toxicity to *A. saline* (LC<sub>50</sub> = 100 mg/mL), while hydrolate don't present toxicity (LC<sub>50</sub> >> 3500 mg/mL). The essential oil and hydrolate of *L. luisieri* showed very low toxicity in *Swiss* mice (LD<sub>50</sub> values >> 2000 mg/kg), with no clinical symptoms, however, a passive behavior was registered for the animals administered with 2000 mg/kg of essential oil. Histopathological studies showed some changes in the structure of hepatocytes and in the epithelial cells of renal tubules only for the highest doses of essential oil. The essential oil (100 mg/kg) and hydrolate (500 mg/kg) showed analgesic and anti–inflammatory activities. In previous studies performed in our laboratory, essential oil presented also important antioxidant and antimicrobial properties. Studies will continue with the toxicological evaluation of hepatic and renal function, by quantification of serum enzymes and metabolites and by the assessment of mechanisms involved in anti–inflammatory activities, in order to clarify the possible application of essential oil and aqueous extract as nutraceutical and/or phytotherapeutic agent.

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### TRAFFICKING STUDIES OF PLASMID DNA THROUGH FÖRSTER RESONANCE ENERGY TRANSFER

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The low efficiency of gene expression has hampered clinical application of plasmids in DNA vaccination. Plasmid trafficking from extracellular space up to nucleus is prevented by several barriers which include degradation of the plasmid DNA by extracellular or cytosolic nucleases, low efficiency of plasmid uptake, degradation in the endosome or inability to escape from it as well as low effectiveness in the transcription/translation process. One possible solution can be the use of liposomes that could protect the DNA from degradation and optimize the delivery route and/or method, leading to enhanced pDNA immunogenicity.

The aim of this work is to study the intracellular dissociation of liposomes-pDNA complexes (lipoplexes) in living cells by using Förster Resonance Energy Transfer (FRET). This methodology has the advantage of providing resolution to close interactions, and is expected to be able to detect the onset of dissociation of DNA from liposomes. This dissociation is of paramount importance since it allows ensuring that once the plasmid is complexated with the adjuvant it will enter the cell by endocytosis being able to escape efficiently from the endosomes. In addition, the complex must be dismantled in order for the plasmid to become transcriptionally active.

The results have shown that the dissociation of DNA from liposomes occurs immediately after liposome internalization and increases over the time. By 24 hours post-transfection, the FRET signal is residual, nuclear plasmid uptake is visible and protein expression is observed.

*In-house* liposomes seems to be efficient carriers for pDNA transfection, protecting efficiently the DNA from the nucleases attack, helping in the cell uptake and escape from the endosome, and optimizing the delivery route/method. The lipoplex was correctly dismantled, with DNA dissociating from the liposome shortly after entering the cell, which allows for it to enter the nucleus and then express GFP protein. These results suggest that the *in-house* procedure to produce liposomes is effective for DNA delivery.



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## VIOLURIC ACID-LACCASE MEDIATOR SYSTEM: KINETIC OPTIMIZATION OF THE ENZYMATIC SYNTHESIS OF CINNABARINIC ACID

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**Objectives.** Cinnabarinic acid is a fungal antioxidant with potential bioactivities as topoisomerase inhibitor, and as modulator of immune response in autoimmune disorders. The aim of this work is the study of the enzymatic synthesis of cinnabarinic acid, using as biocatalyst a laccase mediator system with violuric acid.

**Methodology** ([www.um.es/genz/e00605/serv03.htm](http://www.um.es/genz/e00605/serv03.htm)). *Trametes villosa* laccase (TvLac) was provided by Novozymes, 3-hydroxyanthranilic (3HAA), cinnabarinic (CA) and violuric (VLA) acids were purchased from Sigma, other reagents were from Merck. An Agilent 1200 Rapid Resolution UHPLC-DAD chromatograph, was used for determination of products, with a 100 mm x 4.6 mm x 2.6 mm Phenomenex Kinetex Core-Shell C-18 reverse phase column. The synthetic assays were carried out in 50 mM pH 5.0 sodium acetate buffer, with continuous stirring and an open system to oxygen from air.

**Results.** The chemical synthesis of 1 mM CA from 3HAA with p-benzoquinone requires 5 hours, with several side products and low yield. The enzymatic synthesis with TvLac ends in 3 hours without side products. The enzymatic synthesis with TvLac and VLA generates a mediator radical (VLAR), which originates de conversion of 3HAA, in 1 hour, without side products and with full yield in CA production. The concentration of all species have been determined using UHPLC-DAD, with commercial reagents as external standards, from aliquots at several times during each assay. The process follows a uniexponential kinetics. The amplitude of uniexponential does not vary with the enzyme neither mediator concentration, whereas it increases in a linear way with the 3HAA concentration. The argument of uniexponential rises linearly with the enzyme concentration, does not change with the mediator concentration, and decreases with the 3HAA concentration. These experimental dependences agree with the kinetic analysis of the reaction mechanism proposed, for the enzymatic synthesis.

**Conclusions.** VLA and TvLac are an efficient laccase mediator system for enzymatic synthesis of 3HAA in CA. The assay conditions have been optimized for quick and full yield of the conversion, in an eco-friendly bioprocess. This procedure can be useful for enhance the healthy properties of the antioxidant CA, with increasing interest for treatment of multiple sclerosis, rheumatoid arthritis, myasthenia gravis and other autoimmune disorders.

**Acknowledgements.** This work has been partially supported by grants from several Spanish organizations. Projects BIO2009-12956 (MINECO, Madrid) and 08856/PI/08 (Fundación Seneca, CARM, Murcia).

## Session 4

# Molecular Microbiology and Microbial Physiology

# Plenary Lecture

## YEAST DEALING WITH STRESS IN FERMENTATIVE ENVIRONMENTS: REVISITING PHYSIOLOGICAL MODELS IN LIGHT OF MOLECULAR BIOLOGY

Cecília Leão

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During must fermentation, yeast cells deal with numerous environmental stresses such as progressive nutrient depletion, temperature variation and high alcohol concentration. These stress factors could have adverse effects on cell growth, cell viability and fermentative activity, leading to the so called stuck and sluggish fermentations that are still major problems for the wine and bioethanol industries. Thus, one of the main challenges is to understand, at biological and molecular level, how the temperature, the nutrients and the fermentation end-products, particularly ethanol, can affect the performance of the fermenting microorganism.

This lecture will be focused on alcoholic fermentation carried out by *Saccharomyces cerevisiae* addressing the triangulation “nutrient consumption, growth/fermentation, cell death/aging”. The Professor Nicolau Van Uden’s physiological models on yeast performance in fermentative environments will be revisited, highlighting their relevance on the study of the identification of the molecular players involved in the response of the yeast to fermentative environments. The last part of this presentation will explore those physiological and molecular integrative models on the peculiar behavior of the wine spoilage yeast *Zygosaccharomyces bailii*.

# Keynote Lecture

## KL04

### THE YEAST CHRONOLOGICAL AGING MODEL: PROTEOTOXIC STRESS AND AGE-RELATED DYSFUNCTIONS

Paula Ludovico

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Yeast provides a simple and powerful model for cellular aging research. A combination of genetics, cell biology, and biochemistry has unraveled the complexities of numerous cellular processes conserved in evolution and governing, at least to some extent, the rate of aging. Several common denominators of aging found in different models, as yeast, include genomic instability, loss of proteostasis, deregulated nutrient sensing and mitochondrial dysfunction. One of the major challenges is now to understand their interconnection and relative contributions to age phenotypes. Yeast chronological aging model has been used to get new insights on these questions and results will be presented regarding the emergent phenomenon of hormesis promoted by hydrogen peroxide. We have shown that elevated levels of the reactive oxygen species (ROS), hydrogen peroxide, are generated during caloric restriction, which extends life span and retards age-related phenotypes in a variety of species, reducing the accumulation of superoxide anions. These findings established a role for hormesis effects of hydrogen peroxide in promoting longevity that has now been observed in several model systems (1,2). The beneficial role of ROS in lifespan extension challenges the free radical theory but is consistent with the essential role of these molecules in cell signaling.

The study of another aging hallmark, loss of proteostasis, was performed in yeast aged cells expressing the Parkinson's disease related protein, alpha-synuclein. Alpha-synuclein is a naturally prone to misfold protein in high concentrations impacting on cell clearance routes and protein quality control systems such as the ubiquitin-proteasome system and autophagy. The results to be presented will show that the increased autophagy and mitophagy observed in yeast cells expressing alpha-synuclein accelerates chronological aging (3). The regulation of autophagy and its crosstalk with genomic instability will be discussed. Overall, the data to be presented gives new insights on cellular mechanisms of aging. "All the models are wrong but some are useful" and this is the case of the yeast chronological aging model.

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# Oral Presentations

## OP14

### ROS-MEDIATED LINKS BETWEEN PRIMARY AND SECONDARY METABOLISM IN STREPTOMYCES: THE EXAMPLE OF PIMARICIN PRODUCTION IN STREPTOMYCES NATALENSIS ATCC 27448

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*Streptomyces* are Gram-positive soil-dwelling filamentous bacteria well known for their ability to produce numerous secondary metabolites that display a wide range of chemical diversity and biological activities. The biosynthesis of these compounds occurs in a growth-phase dependent manner and is controlled by environmental and physiological factors. Being an aerobic process, streptomycete secondary metabolism is strongly affected by oxygen availability. However, high levels of oxygen can enhance the formation of reactive oxygen species (ROS) that are potentially hazardous to the cell.

Pimaricin is a 26-membered polyene antibiotic produced by *Streptomyces natalensis* ATCC 27448. It is widely used in the food industry to prevent mould contamination of non-sterile foods, and in human antifungal therapy. Despite the general interest on polyenes as a major class of antifungal agents, very little is known about the factors that modulate their biosynthesis.

We have shown the existence of a crosstalk between intracellular ROS homeostasis and secondary metabolite production in *S. natalensis*. An effective imbalance of the intracellular ROS homeostasis resulted in a modulation of pimaricin production: the strains defective for the H<sub>2</sub>O<sub>2</sub> detoxifying proteins KatA1 and AhpCD revealed a pimaricin overproducing phenotype, while the strain defective for the superoxide dismutase protein, SodF, was a pimaricin underproducer.

In order to unveil, at the molecular level, the mechanisms that lie behind this crosstalk we compared the transcriptomes of the mutant strains CAM.02 ( $\Delta$ sodF) and CAM.04 ( $\Delta$ ahpCD) with the wild-type by interspecies microarrays. Using a genome-wide approach we have identified the metabolic pathways that are in the interface between intracellular ROS homeostasis, primary and secondary metabolism.

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## OP31

FUNCTIONING, REGULATION AND GENETICS OF AN ESAT-6-LIKE SECRETION SYSTEM IN *BACILLUS SUBTILIS*Catarina Baptista<sup>1</sup>; Hugo Condessa Arreto<sup>1</sup>; Carlos São-José<sup>1</sup><sup>1</sup>Faculdade de Farmácia da Universidade de Lisboa, DMI, CPM-URIA

The recently discovered Type VII/Esat-6 secretion systems seem to be widespread among bacteria of the phyla *Actinobacteria* and *Firmicutes*. In some species they play an important role in pathogenic interactions with eukaryotic hosts (1). Several studies have predicted that the locus *yukEDCByueBC* of the Gram-positive model bacterium *Bacillus subtilis* would encode an Esat-6-like secretion system (Ess). The *B. subtilis* Ess (BsEss) could constitute an attractive model to study molecular details of this secretion pathway. We have recently reported the first evidences for the functioning of BsEss in an undomesticated *B. subtilis* strain (2). We showed that YukE, a small protein with the typical features of the secretion substrates from the WXG100 superfamily is actively secreted to culture media. YukE secretion depends on intact *yukDCByueBC* genes, whose products share sequence or structural homology with known components of the *Staphylococcus aureus* Ess. Biochemical characterization of YukE indicates that it exists as a dimer both *in vitro* and *in vivo*. The *B. subtilis* Ess essentially operates in late stationary growth phase in absolute dependence of phosphorylated DegU, the response regulator of the two-component system DegS-DegU. Interestingly, the system is poorly functional in the domesticated and classic lab strain *B. subtilis* 168, although it carries an intact *BsEss* locus. We found that mutations already reported for strain 168, such as those affecting *sfp* and the promoter sequence of gene *degQ* contribute to the defective operation of the Ess. Further genetic analysis disclosed the involvement of other genes such as *swrB* and *yeeF* in BsEss operation. These studies aim to decipher the genetic makeup of the *B. subtilis* Ess and thus to contribute to a better understanding of its functioning and regulation.

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## OP32

### PHOSPHOPROTEOME ANALYSIS TO UNRAVEL THE ROLE OF THE CERAMIDE-ACTIVATED PROTEIN PHOSPHATASE SIT4P IN MITOCHONDRIAL FUNCTION AND LIFESPAN IN YEAST

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The *Saccharomyces cerevisiae* Sit4p is the catalytic subunit of a ceramide-activated serine-threonine protein phosphatase, with multiple cellular roles as regulation of cell cycle progression, mitochondrial function, stress resistance and life span [1–3]. Sit4p is activated in cells lacking Isc1p, a protein with a role in sphingolipid metabolism. Moreover, *SIT4* deletion abolishes the premature ageing and oxidative stress sensitivity of *isc1D* mutants by suppressing mitochondrial dysfunction [3]. As such, the identification and characterization of Sit4p downstream targets will extend our knowledge on Sit4p function and provide insights into the mechanism linking sphingolipid metabolism and modulation of mitochondrial function.

With this objective, *S. cerevisiae* BY4741 (wild type) and *sit4D* cells were used to search for changes in expression of proteins and in phosphorylation levels that may allow to identify targets of Sit4p-mediated dephosphorylation. Proteins from whole cells or a mitochondrial fraction were analyzed by two dimensional gel electrophoresis. Phosphoproteins were then detected by Western blotting and identified by mass spectrometry.

The analysis of the phosphoproteome in whole cell extracts revealed 6 proteins differentially phosphorylated in *sit4Δ* cells. Notably, hexokinase 2 (Hxk2p) is hyperphosphorylated in this mutant and the nonphosphorylatable Hxk2p-S15A mutation suppresses phenotypes associated with *SIT4* deletion, suggesting that Hxk2p functions downstream of Sit4p in the control of cell cycle, mitochondria function, oxidative stress resistance and chronological lifespan. A phosphoproteomic analysis of isolated mitochondria is still undergoing to identify potential Sit4p targets and evaluate their impact, particularly in the modulation of mitochondria function.

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## OP42

TOXICITY OF SUGAR PHOSPHATES IN A *BACILLUS SUBTILIS* STRAIN DEFECTIVE IN *ARA*RLia Marques Godinho<sup>1</sup>; Isabel de Sá-Nogueira<sup>1</sup>

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*Bacillus subtilis* ability to metabolize L-arabinose is dependent on three intracellular enzymes encoded by the *araA*, *araB* and *araD* genes that convert L-arabinose to D-xylulose 5-phosphate, which enters the pentose phosphate pathway. The transcription of these genes is induced by arabinose and negatively controlled by AraR, a repressor allosterically regulated by arabinose.

The expression of the arabinose catabolizing enzymes in an *araR*-null mutant is constitutive and higher than that observed in the wild-type strain, and addition of arabinose to an exponentially growing culture of the mutant strain results in immediate cessation of growth[1]. We believe that this phenotype is caused by the accumulation of phosphorylated sugar intermediates of arabinose metabolism, however the mechanisms that underlie such toxicity are yet to be fully understood. Uptake and metabolism of carbohydrates are usually tightly controlled and a sudden increase in carbohydrate uptake causes an imbalance between flux through the upper branch of glycolysis and the capacity of the lower branch of glycolysis[2]. To bypass this increased uptake of carbohydrate, synthesis of methylglyoxal from dihydroxyacetone phosphate is thought to function as an overflow mechanism, preventing accumulation of phosphorylated intermediates. Methylglyoxal is highly cytotoxic and its presence in the medium is known to inhibit growth in *B. subtilis*[2].

In an *araR*-null mutant, addition of either arabinose or ribitol to an exponentially growing culture results in immediate cessation of growth. The toxic effect of arabinose is suppressed in a strain bearing a large deletion of all genes downstream *araD* in the metabolic operon *araABDLMNPQ-abfA*. However, toxicity is not suppressed in the presence of ribitol, a non-catabolized pentose alcohol. In this work we show that in the latter mutant the suppression of the toxicity is caused by a decrease in the mRNA level of the arabinose catabolizing genes and most probably a reduction of intracellular levels of phosphorylated sugar intermediates. Furthermore, we determined the accumulation of intracellular methylglyoxal in the different strains in the presence of arabinose or ribitol. The results hint at distinct mechanisms underlying toxicity and will be discussed.

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This work was partially supported by PEst-OE/BIA/UI0457/2011 from FCT and grant SFRH/BD/73109/2010 from FCT to LMG

# Poster Presentations

## Epigenomics

P243

### EPIGENETIC MODULATION OF PLANT CELL CULTURES FOR THE PRODUCTION OF BIOPHARMACEUTICALS

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Molecular Farming – the use of plant based systems for production of recombinant proteins – is an emerging field with a high potential impact in the pharmaceutical sector. Plant production platforms offer significant advantages when compared to traditional expression systems, namely in terms of safety and cost. However, a major challenge remains so that plants become truly competitive, which is to improve the low yields of product accumulation that are generally obtained.

The aim of this work is to increase the final yield of recombinant proteins produced in plant cell cultures by adding small molecule enhancers, mainly histone deacetylase (HDAC) inhibitors to the culture medium. We expect these compounds will promote histone hyperacetylation and alter the epigenetic status of the cells, by opening the chromatin conformation. This in turn will promote a higher transcription level of the transgene that encodes the protein of interest leading to a higher yield of the recombinant product.

We used tobacco BY2 cell cultures producing a human recombinant protein, Prostaglandin D Synthase (PGDS), to which we added sodium butyrate – a compound that inhibits HDAC activity. Preliminary results show an increase in both cell growth and PGDS yield. Other compounds were synthesized and tested. These compounds have different functional groups, such as hydroxamic acid, carboxylic acid and substituted aromatic amides, known to be important for the inhibition of HDAC, for which results will be discussed.

This work will be most relevant for the establishment of plant cell cultures as viable platforms for the cost-effective production of economically important recombinant proteins.

## Microbial Physiology

P244

## AMMONIUM TOXICITY IN AGING YEAST CELLS IS INFLUENCED BY AMINO ACID DEPRIVATION, THROUGH DIFFERENT SIGNALING PATHWAYS

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When studying the impact of nutrient–signaling pathways in aging of yeast, by culturing *Saccharomyces cerevisiae*, the composition of culture media has proven to be an extrinsic factor affecting the chronological life span (CLS). In this context, we aimed to study ammonium ( $\text{NH}_4^+$ ) as a nutrient capable of regulating CLS of *S. cerevisiae* and uncover the signaling pathways involved. We have previously shown that cells starved for auxotrophic–complementing amino acids and aged in water are particularly sensitive to ammonium–induced cell death, this process being mediated through the regulation of the evolutionary conserved pathways PKA, TOR and SCH9 and accompanied by an initial apoptotic cell death followed by a fast secondary necrosis. Here, we report that the effect of  $\text{NH}_4^+$  on aging yeast depends on the specific auxotrophic–amino acid they are deprived of. Compared with no amino acid starvation, starvation for leucine alone or in combination with histidine resulted in the most pronounced  $\text{NH}_4^+$ –induced CLS shortening, whereas starvation for lysine, alone or in combination with histidine, resulted in the least sensitivity to  $\text{NH}_4^+$ . We also show that  $\text{NH}_4^+$ –induced CLS shortening is mainly mediated by Tor1p in cells starved for leucine or histidine, but by Ras2p in cells starved for lysine and in non–starved cells. Sch9p, contrary to Tor1p and Ras2p mediates cell survival in response to  $\text{NH}_4^+$  in all starvation conditions, through the phosphorylation of Hog1p. We further report that the CLS of prototrophic strains is also decreased by  $\text{NH}_4^+$ , in comparison to other nitrogen sources. In summary, herewith we show that the toxic effects of  $\text{NH}_4^+$  on CLS shortening are regulated by a starvation–dependent and a starvation–independent component, being mediated essentially by Tor1p and by Ras2p, respectively. In amino acid starved cells the presence of leucine can ameliorate  $\text{NH}_4^+$  effects while lysine has the opposite effect. The use of  $\text{NH}_4^+$  as a nitrogen source decreased the CLS of prototrophic strains, showing that its CLS–shortening activity is not limited to auxotrophic strains with amino acid biosynthesis defects. Our data indicate that the modulation of nitrogen sources supplied to cells can drastically modulate CLS. Since  $\text{NH}_4^+$ –induced cell death is involved in different human disorders that are accompanied by hyperammonemia, our results may also afford new insights into the understanding of the cell molecular bases triggering cell death in such pathologies.

## Microbial Physiology

### P245

#### BIOFILM FORMATION BY *CAMPYLOBACTER* SPP. IN ABIOTIC SURFACES: ELUCIDATION OF ENVIRONMENTAL DETERMINANTS ASSOCIATED TO PERSISTENCE IN THE FOOD CHAIN

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Campylobacteriosis is the most notified zoonosis in developed countries, *Campylobacter jejuni* being the most frequent etiological agent and poultry meat consumption representing an important risk factor in the transmission of pathogenic strains to humans. Despite the efforts to reduce the number of human cases, the incidence rates remain high and standard biosecurity measures remain ineffective in *Campylobacter* control. Therefore, clarification of the physiology and of the survival mechanisms of microaerophilic *Campylobacter* spp. during processing and storage of meat products is essential. In this context, we sought to determine the conditions that promote *in vitro* biofilm formation by *C. jejuni* and *C. coli*. Based on *flaA*-RFLP pattern and on erythromycin and ciprofloxacin susceptibility phenotypes, determined previously for 179 *Campylobacter* isolates obtained from broilers at slaughter in a nation-wide prevalence study (Carreira et al., 2012), we selected nine strains to characterize their attached growth to different hydrophilic and hydrophobic abiotic surfaces: stainless steel, glass and polystyrene. Several environmental variables that may play a key role in the transition from planktonic to biofilm growth, such as temperature, incubation time, atmosphere (aerobiosis/microaerobiosis) and substrate, were considered. Selected isolates varied in their abilities to attach and grow onto the different surfaces, which was apparently independent of resistance phenotype or *flaA* genotype. Biofilm formation occurred essentially at the air-liquid interface and was favored by longer incubation periods under aerobiosis and higher temperatures. Preliminary results suggest that the switch from planktonic to biofilm growth might be an adaptive response of *Campylobacter* bacteria to environmental stress, contributing to the spread and persistence of these microorganisms along the food chain. Moreover, we identified two strains forming exceptionally high amounts of biofilm on steel and glass slides, which will be useful in the future to study the molecular determinants involved in this process.

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## Microbial Physiology

P246

### CHARACTERIZATION OF BACTERIAL POPULATIONS UNDER SHEAR STRESS

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Bacteria suffer constant exposure to external mechanical forces such as the continuous shear stress generated by flowing blood in the host or by flowing liquids in industrial settings. Furthermore, while in natural settings bacteria tend to organize in complex biofilms or aggregates, in the laboratory studies are usually performed using planktonic cultures and the formation of tri-dimensional structures goes unnoticed.

To characterize the dynamics and mechanical properties of planktonic populations, we monitored by rheology, the growth of *Staphylococcus aureus* strain COL. The viscosity of the culture was measured as a function of time along the growth curve. Unexpectedly, we observed extensive changes in viscosity, allowing to establish an analogy with the three phases of the growth curve, measured by optical density: during the lag phase, the viscosity increased scarcely but during the exponential phase, this parameter steeply augmented over tenfold, presenting consistent drops and immediate full recoveries, strongly suggesting transient structural alterations in the bacterial population. The viscosity profile was consistent with a percolated structure, probably formed due to transitory aggregation effects between adjacent cells, resulting from incomplete separation of daughter cells upon division and/or from secondary adhesion events between mature cells. The development of such a percolated structure is consistent with exponential growth since cell division is at its maximum rate and adhesion factors are over-expressed in this stage.

While entering stationary phase, the viscosity of the culture reached its maximum and then suffered a definitive drop, a behavior with no counterpart in the optical density curve. This observation indicated that the tri-dimensional percolated structure could no longer be formed after this stage, although no decrease was observed in the cell viability.

Moreover, distinct rheological patterns were obtained for other bacterial species, suggesting that this approach may allow to differentiate at the species level or even different strains.

We propose that a tri-dimensional picture of a bacterial population may be drawn using rheology, uncovering physiological information on the status of the population which went undetected until now.

\* These authors contributed equally.



## Microbial Physiology

### P247

#### FUNCTIONALITY OF FOREIGN COMPATIBLE SOLUTES SYNTHESIZING GENES IN A *THERMUS THERMOPHILUS* MUTANT DEFICIENT IN THE SYNTHESIS OF MANNOSYLGLYCERATE

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Among *Thermus thermophilus* isolates, strain HB27 is the only trehalose-negative naturally occurring strain. It is unable to grow in media containing more than 2% NaCl and in these growth conditions it accumulates mannosylglycerate (MG). MG, in this thermophilic organism, is synthesized by a two-step pathway that converts GDP-mannose and 3-phosphoglycerate into mannosyl-3-phosphoglycerate (MPG) by MPG synthase which is then hydrolyzed to MG by MPG phosphatase. Additionally, there is a single-step pathway for the synthesis of MG, where GDP-mannose is condensed with D-glycerate into MG in a reaction catalyzed by mannosylglycerate synthase (MGS). Thus far, *Rhodothermus marinus* is the sole organism possessing the two pathways for synthesis of MG where the correspondent genes and respective enzymes have been characterized, however the existence of a salt-dependent control mechanism of gene expression is not established.

To achieve the ideal candidate for expression of genes encoding compatible solutes synthesis, a HB27 *mpgs*-null mutant was constructed by insertion-deletion directed mutagenesis. Experimental results demonstrate that this HB27 $\Delta$ *mpgs* mutant was unable to grow in media containing more than 1% NaCl and did not accumulate any compatible solutes, being a suitable thermophilic host to express genes with thermophilic origin.

Preliminary results using the mutant transformed with a shuttle-vector for constitutive expression of *mgs* gene from *R. marinus* showed the rescue of salt-sensitive phenotype of the 127 $\Delta$ *mpgs* mutant by the ability to grow in concentrations of salt up to 2% with the accumulation of MG but only when D-glycerate was externally provided revealing the unavailability of this MG precursor in the HB27 strain. The successful complementation of the mutation in 127 $\Delta$ *mpgs* by the expression of the *mgs* gene revealed its function as osmoprotectant for *T. thermophilus* HB27 and supports the use of *T. thermophilus* strains as host systems to access the functionality of foreign genes encoding compatible solutes synthesis.

## Microbial Physiology

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MANNOSYLGLYCERATE AND DI-MYO-INOSITOL-PHOSPHATE HAVE INTERCHANGEABLE ROLES DURING STRESS ADAPTATION OF *PYROCOCCUS FURIOSUS*Ana M. Esteves<sup>1</sup>, Sanjeev K. Chandrayan<sup>2</sup>; Patrick M. McTernan<sup>2</sup>; Michael W. W. Adams<sup>2</sup>; Nuno Borges<sup>1</sup>; Helena Santos<sup>1</sup><sup>1</sup>*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal;*<sup>2</sup>*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA*

Marine hyperthermophiles accumulate small organic compounds, known as compatible solutes, in response to supra-optimal temperature or salinity. *Pyrococcus furiosus* is a marine hyperthermophilic archaeon that grows optimally at temperatures near 100°C. This organism accumulates mannosylglycerate (MG) and di-*myo*-inositol phosphate (DIP) in response to osmotic and heat stress, respectively [1]. The ability of these ionic organic solutes to stabilize proteins *in vitro* has been extensively demonstrated[2]. Therefore, it was assumed that MG and DIP could protect cellular components against heat damage *in vivo*. However, a definite proof of the role of these solutes in stress adaptation of hyperthermophiles is still missing, largely due to the lack of genetic tools to produce suitable mutant strains. Recently, tools for the genetic manipulation of *P. furiosus* became available [3], making this organism a promising target for our purpose. In this work, genes pfc\_02085 and pfc\_04525 coding for key enzymes in the synthesis of MG and DIP, respectively, were deleted by double-crossover homologous recombination. As expected, the resulting mutant strains did not accumulate MG or DIP, respectively. The growth profile and solute pattern of the mutant strains were investigated under optimal and stressful growth conditions. DIP is a perfect replacement for MG in heat stress adaptation, but MG confers slightly better performance under osmotic stress. The results show the functional versatility of MG and DIP; on the other hand, the cascade of events leading to MG synthesis is tuned specifically for osmotic stress, while the induction of DIP synthesis has greater flexibility.

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## Microbial Physiology

P249

ROS-BASED REGULATION OF TACROLIMUS BIOSYNTHESIS: THE OXYR-AHPCD SYSTEM IN *S. TSUKUBAENSIS*.Sílvia D.S. Pires<sup>1</sup>, Rute Oliveira<sup>2</sup>; Pedro Moradas-Ferreira<sup>1</sup>; Marta V. Mendes<sup>2</sup><sup>1</sup>IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150 Porto;

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Tacrolimus (FK-506) is a macrolide widely used to prevent graft rejection in organ transplanted patients. The biosynthesis of tacrolimus in *Streptomyces* submerged cultures is controlled by networks regulated by different factors such as dissolved oxygen (DO). Exposure of microorganisms to high levels of DO increases the formation of reactive oxygen species (ROS) that are harmful to cell components. To counteract these effects, microorganisms have response mechanisms at different levels, from modulation of gene expression to changes in enzymatic and non-enzymatic activities in order to sense, detoxify and repair the damage caused by ROS.

The characterization of *S. tsukubaensis* NRRL 18488, the producer of tacrolimus, showed high levels of total catalase specific activity in submerged cultures throughout the growth curve. Moreover, a temporal overlap is observed between the growth-phase dependent increase in catalase activity, the decrease in H<sub>2</sub>O<sub>2</sub> intracellular levels and the onset of tacrolimus production.

We have identified the genes coding for the main anti-oxidant defence in *S. tsukubaensis* namely the alkyl hydroperoxide system (AhpCD), the primary defence against endogenously generated H<sub>2</sub>O<sub>2</sub>, and OxyR, a LysR redox-sensitive transcriptional regulator, that has been described in many bacteria as the positive transcriptional regulator of the AhpC encoding gene. In order to address the role of intracellular ROS homeostasis in tacrolimus biosynthesis, knock-out mutants defective on the OxyR and AhpC proteins were generated and fully characterized regarding tacrolimus production, intracellular ROS levels, anti-oxidant enzymatic activities and gene transcription.

This study revealed a novel regulation of the OxyR-AhpCD system in streptomycetes and helped us to establish a link between the oxidative stress response and tacrolimus biosynthesis in *S. tsukubaensis*.

## Acknowledgements.

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## Microbial Physiology

### P250/F33

#### INTERACTIONS BETWEEN *SACCHAROMYCES CEREVISIAE* AND *HANSENIASPORA GUILLIERMONDII*: CELL-CELL CONTACT MECHANISM

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Several studies have shown that the early death of non-*Saccharomyces* during wine fermentations are due to yeast-yeast interactions induced by *Saccharomyces cerevisiae* (Sc) through different mechanisms such as growth arrest mediated by a cell-cell contact mechanism (Nissen et al. 2003) and death by killer-like toxins (Pérez-Nevado et al 2006; Albergaria et al. 2010). Besides, previous work also showed that death of non-*Saccharomyces* in co-cultivation with Sc is always triggered at the end of exponential growth phase (Pérez-Nevado et al 2006). In order to investigate the role of cell-cell contact in the early death of non-*Saccharomyces*, we performed assays in which Sc cells pre-grown at enological growth conditions for 12 and 48 h, respectively, were in direct contact with *Hanseniaspora guilliermondii* (Hg) cells at high cellular density ( $10^7$ - $10^8$  cells/ml) in a carbon-free medium. As a negative control we performed similar assays in which Sc and Hg cells were separated by a dialysis tube (pore cut-off of 1000 kDa) and as a positive control a single Hg culture. Results showed that Hg cell density decreased by 2 orders of magnitude (i.e. from  $10^8$ - $10^6$  cells/ml) in contact with 48 h-grown Sc cells, while its viability remained unchanged ( $10^8$ cfu/ml) in the presence of 12 h-grown Sc cells. Moreover, Hg viability was not affected both in the dialysis tube experiments and single culture, which confirmed the death-induced cell-cell contact phenomenon.

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## Molecular Microbiology

## P251

ACTIVATION OF THE PROTEIN PHOSPHATASE SIT4P IMPAIRS MACROAUTOPHAGY, MITOPHAGY AND MITOCHONDRIAL DYNAMICS IN *ISC1Δ* CELLSVitor Teixeira<sup>1</sup>, Tânia C. Medeiros<sup>1</sup>; Rita Vilaça<sup>1</sup>; Pedro Moradas-Ferreira<sup>1</sup>; Vitor Costa<sup>1</sup>

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Sphingolipids are important signalling mediators by regulating important biological processes, such as cell growth and apoptosis. Sphingolipid metabolism in *S. cerevisiae* is similar to that of its mammalian counterpart, but yeast use ceramide to synthesize other class of complex sphingolipids, namely inositolphosphosphingolipids (1). In turn, these complex sphingolipids are hydrolyzed by Isc1p, an inositol-phosphosphingolipidphospholipase C, encoded by the *ISC1* gene to produce ceramide. Recent studies have implicated Isc1p in the regulation of oxidative stress, mitochondrial function and chronological lifespan (CLS). The mutant strain displays shortened CLS, increased hydrogen peroxide sensitivity and mitochondrial dysfunctions (1,2). Importantly, the disruption of the *SIT4* gene, which encodes for the catalytic subunit of type 2A ceramide-activated protein phosphatases (PP2A), suppresses these phenotypes (2).

Recent findings have provided new understanding of how autophagy, mitochondria and redox signalling are interconnected and the impact of autophagic dysfunction on oxidative stress in *S. cerevisiae*. Notably, the regulation of dynamic mitochondrial processes such as fusion, fission, and mitophagy has also been shown to be an important mechanism controlling cellular bioenergetics and fate (3). On this basis, this work intends to evaluate Sit4p-driven alterations in these quality control mechanisms impacting on mitochondrial defects of *isc1Δ* cells. The results demonstrated that Isc1p-deficient cells presented impaired autophagic induction and autophagic flux and defects on the selective cargo-delivering cytoplasm-to-vacuole targeting (*Cvt*) pathway, implicating macroautophagic dysfunction in the etiology of mitochondrial defects of the mutant strain. In parallel, we also demonstrate that mitophagy was impaired in *isc1Δ* cells and this was closely related with mitochondrial fragmentation and shortened CLS. The deletion of *SIT4* abolished macroautophagic and mitophagic defects and mitochondrial fragmentation of *isc1Δ* cells, therefore contributing to reestablish proper mitochondrial function in the mutant strain.

Taking together, this study demonstrates that ceramide signaling through the Sit4p protein phosphatase is also involved in the regulatory circuit integrating macroautophagy, mitophagy and mitochondrial dynamics inputs. We also provide significant evidence of a common crosstalk of sphingolipid dynamics, redox signalling and cell homeostatic processes.

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## Molecular Microbiology

P252

### ANALYSIS OF GENE EXPRESSION VARIABILITY IN STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

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*Staphylococcus epidermidis*, a normal inhabitant of a human skin and mucosa, has emerged as one of the principle bacterial agents involved in nosocomial infections, particularly, in patients with indwelling medical devices. It's pathogenesis is related with the ability to adhere and form biofilms on the surface of those medical devices and is also associated with patients' immune system that can be compromised. This pathologic condition leads to a high morbidity and, uncommonly, mortality.

In the last decade, the quantification of gene expression has been one of the major areas of research in progress. The use of molecular biology techniques, such as quantitative (q) PCR, allowed the study of the process of biofilm formation, which is very complex and involves elaborated genetic regulation. When determining the quantification of genetic expression, there are two critical experimental steps that can impact the outcome of the experiment: RNA extraction, traditionally considered the crucial step of the whole experience, and reverse transcriptase reaction. We have previously shown that in *S. epidermidis* biofilms, RNA extraction procedure has a strong influence on the outcome of gene expression quantification. Here we evaluated the individual contributions of all experimental steps in the outcome of reliable gene expression determinations. To achieve that, we determined the expression of *aap*, *fmtC* and *lrgB* genes using the type strain RP62A, by performing technical duplicates of each experimental step, and evaluating the coefficient of variability. Interestingly, our results showed that the bulk of the variability of the gene expression quantification derived from the biological replicates, and not from any of the experimental steps. Furthermore, variability from RNA extraction was not significantly different from the variability obtained from reverse transcriptase or qPCR experiments. This study further confirms that biofilms are difficult biological samples with enhanced difficulties in gene expression determinations.

## Molecular Microbiology

## P253

## CHARACTERIZATION OF A MULTIRESTANCE PLASMID FROM A METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS CLINICAL STRAIN

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*Staphylococcus aureus* is a major pathogen, responsible for infections in the hospital and in the community. Development of resistance to antimicrobials is of paramount importance in *S. aureus*, in particular, acquisition of exogenous resistance genes mediated by mobile genetic elements, such as plasmids. In a previous work, screening for biocide resistance determinants (*qacA/B*) in a collection of 53 *S. aureus* clinical strains isolated at a Lisbon hospital, identified *qacA* in a plasmid harbored by a methicillin-resistant *S. aureus* (MRSA) strain, SM39. We characterized this plasmid, pSM39, aiming to understand its contribution to SM39 antimicrobial resistance phenotype.

The MRSA strain SM39 was characterized by MLST typing. Plasmid pSM39 was isolated and fully sequenced by next-generation sequencing. After assembly, pSM39 nucleotide sequence was analyzed using the freeware programs BLAST and ORF Finder, available at National Center for Biotechnology Information (NCBI).

MLST typing revealed that strain SM39 belonged to ST88, a clonal lineage generally associated with community-onset infections, with a sporadic occurrence in Europe. pSM39 is a 26 kb plasmid harboring 30 putative ORFs, encoding replication and maintenance functions or resistance to antimicrobial agents. No genes for mobilization or conjugation were identified. pSM39 was found to be linked to resistance to  $\beta$ -lactams (*blaZ* in a full Tn552 copy), to biocides (*qacA* and *qacR* genes) and to the heavy-metals cadmium (*cadA* and *cadC*) and mercury (*mer* operon flanked by two copies of IS257). Comparative analysis revealed that ORF8 had no homology with known *S. aureus* proteins, yet it was identical to a hypothetical protein from the *Staphylococcus epidermidis* plasmid pSK105. The surrounding regions, including the recombinase gene *sin* also showed higher homology with pSK105, suggesting that pSM39 resulted from a rearrangement between a *S. aureus* plasmid and a pSK105-like plasmid.

This study reports the characterization of pSM39, a large non-conjugative multiresistance plasmid present in a clinical MRSA strain that belongs to a clonal lineage usually associated with community acquired infections. This plasmid conveys resistance to  $\beta$ -lactams, biocides and heavy-metals, highlighting the important role of antimicrobials co-selection on the survival of such strains in different noxious environments. It also emphasizes the role of other staphylococci as reservoirs for antimicrobial resistance genes.

## Molecular Microbiology

P254

### CLONING AND EXPRESSION OF CLOSTRIDIUM THERMOCELLUM CBM3 IN PICHIA PASTORIS

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Cellulose-binding modules (CBMs) have been used to improve the surface/interface properties of cellulose fibers. Glycosylation in fungal CBMs has been suggested essential for the modification of paper pulps properties. In a previous work, the CBM3 from the bacterium *Clostridium thermocellum* scaffolding protein (CipA) was expressed in *Escherichia coli* and conjugated with polyethylene glycol (PEG) to mimetize glycosylation [1]. Recombinant CBM3-PEG conjugate improved the drainability of *Eucalyptus globulus* and *Pinus sylvestris* pulps, but not recombinant CBM3 alone (i.e. non-conjugated CBM3), without affecting the physical properties of the papersheets [1]. Nevertheless, the role of glycosylation on CBMs activity still needs to be elucidated.

The aim of this work is to produce glycosylated CBM3 in the methylotrophic *Pichia pastoris* KM71H yeast strain and to study the effect of the glycans of recombinant CBM3 on the surface properties of cellulose fibers. Two versions of the *cbm3* gene, both containing codons in preference in *P. pastoris*, were cloned into the pPICZ $\alpha$ A plasmid: one with three native potential N-glycosylation sites (N12; N68; N124), and the other with no potential N-glycosylation sites (amino-acid substitutions: N12Q; N68Q; N124Q), to serve as control [2]. pPICZ $\alpha$ A is an integrative vector that contains the strong methanol inducible *AOX1* promoter and the *Saccharomyces*  $\alpha$ -factor preprosequence to direct the recombinant protein into the secretory pathway. The integration of the CBM3 coding sequences in the yeast genome was carried out by electroporation and confirmed by colony PCR. Multi-copy recombinants were selected in YPD plates containing 1000  $\mu$ g/ml Zeocin. Induction was conducted in shake-flasks at 30 °C and 200 rpm, using buffered minimal medium supplemented daily with 0.5% (v/v) methanol, during 3 days. The expression of the recombinant proteins in the culture supernatants was analyzed by SDS-PAGE. The non-glycosylated version of recombinant CBM3 presented a band with its calculated molecular weight (18 kDa), while the putative glycosylated version presented a band with higher molecular weight. N-Glycosylation was confirmed by the reduction of this molecular weight to 18 kDa after digestion with Endoglycosidase H. Both recombinant proteins showed high affinity for cellulose in Avicel-binding assays. The characterization and application of the glycosylated recombinant CBM3 for the modification of pulp and paper properties are now being conducted.

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## Molecular Microbiology

P255

## CONTRIBUTION OF MS6 GP1 TO MYCOBACTERIUM SMEGMATIS LYSIS

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Mycobacteriophage Ms6 is a temperate double-stranded DNA (dsDNA) phage that infects the non-pathogenic *Mycobacterium smegmatis*. Similarly to what happens with all other dsDNA phages studied so far, Ms6 must compromise host cell integrity in order to release its progeny at the end of the lytic cycle. Ms6 lytic operon is organized into five genes. In addition to the endolysin (*lysA*) and holin-like genes (*gp4* and *gp5*), two accessory lysis genes are found, *gp1* and *gp3* (*lysB*), which reflects a novel mechanism of phage mediated lysis. *lysB* encodes an enzyme with lipolytic activity whereas *gp1* encodes a chaperone-like protein. Gp1 interacts with LysA and enables its access to the peptidoglycan layer in a holin-independent manner. However, some aspects concerning Gp1 role in the lytic process are not completely clear. In this work we present data obtained using two different recombinant mycobacteriophages constructed by the recently developed Bacteriophage Recombineering of Electroporated DNA (BRED) technology. Infection of *M. smegmatis* with a *gp1* defective Ms6 shows decreased plate efficiency as well as lower burst-size when compared to the wild-type phage. Cell fractionation assays upon infection of *M. smegmatis* with Ms6 carrying *gp1* and *lysA* fused to tag sequences reveals that Gp1 is present in every cell compartment, while LysA seems to be restricted to the cell wall. Furthermore, Gp1 expression precedes LysA production along the infectious cycle. Overall, these results show that Gp1 has an important role in the whole process, being required for an efficient lysis of *M. smegmatis*. In addition, Gp1 ubiquitous distribution is in agreement with its chaperone features and with the fact that it assists LysA translocation across the cytoplasmic membrane. The study of bacteriophages opens new perspectives regarding the treatment of bacterial infections and, in this case, it may also contribute to the understanding of the secretion pathways used by mycobacteria.

## Molecular Microbiology

P256/F15

### DIRECTED EVOLUTION OF THE LANTIBIOTIC LICHENICIDIN

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Lichenicidin is a lantibiotic naturally produced by *Bacillus licheniformis* strains. It was the first lantibiotic to be produced totally *in vivo* in *Escherichia coli*, using a fosmid containing the entire *lic* gene cluster. Since it is a two-peptide lantibiotic, lichenicidin activity against clinically relevant bacteria (e.g. methicillin-resistant *Staphylococcus aureus*) is due to the synergistic action of 2 structurally different peptides (Bli $\alpha$  and Bli $\beta$ ). Lantibiotics are ribosomally synthesized, which makes them more amenable to bioengineering approaches. We used a directed evolution strategy to construct random mutagenesis libraries for Bli $\alpha$  and Bli $\beta$  structural genes. To achieve this, the *licA1* and *licA2* genes were amplified independently with GeneMorph II Random Mutagenesis Kit (Agilent). Each amplification product was cloned into the pUC19a vector and transformed in *E. coli* BLic5 $\Delta$ A1A2 strain (containing all *lic* biosynthetic genes but the structural genes). The antagonistic activity of about 3000 and 2200 clones of the Bli $\alpha$  and Bli $\beta$  libraries, respectively, was evaluated against *Micrococcus luteus*, using agar medium supplemented with the complementary peptide (either Bli $\alpha$  or Bli $\beta$ ). The results showed that approximately 50% of the clones obtained on both libraries exhibited reduced or null bioactivity. Among these, 100 clones from each library were selected for sequencing. Results showed that most of the mutations occurred in amino acids involved in the formation of lanthionine and methyllanthionine thioether rings, which are essential for the structure and bioactivity of these compounds. MS-analysis will help to understand if these peptides are not produced or if they are inactive. Increased activity was detected for 90 (Bli $\alpha$ ) and 73 (Bli $\beta$ ) mutants. Presently, these clones are under study, for confirmation of such phenotype. Herein a method to generate and screen a library of lantibiotic mutants using the Gram-negative host *E. coli* was developed and will be used to acquire new insights regarding the structure-function relationship activity of lantibiotics.

## Molecular Microbiology

### P257

#### EFFLUX AS A FIRST-LINE RESPONSE TO FLUOROQUINOLONES AND OTHER ANTIMICROBIALS IN *STAPHYLOCOCCUS AUREUS*

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*Staphylococcus aureus* is an important human pathogen, for which efflux-mediated resistance, in particular to fluoroquinolones (FQ), has been neglected in comparison with target-based mutation. The aim of this work was to study the balance between efflux and target mutation during the development of resistance to FQs and other antimicrobials in *S. aureus*.

Two FQ-resistant *S. aureus* clinical strains, carrying the same set of mutations but differing in efflux capacity, and the susceptible strain ATCC25923 were subjected to a 20-day exposure to constant concentrations, sub-inhibitory or inhibitory, of the efflux inducers, CIP, ethidium bromide (EtBr) and cetrimide. The cell responses were monitored throughout the exposure processes by determination of minimum inhibitory concentrations of FQs, biocides and dyes in the absence/presence of efflux inhibitors, assessment of EtBr efflux activity, expression analysis of the main multidrug efflux pump (EP) genes and screening of mutations associated with FQ resistance.

The three strains developed a multidrug resistance (MDR) phenotype, with increased resistance to FQs, biocides and dyes, in the course of all exposure processes, independently of the inducer or its concentration. Emergence of the MDR phenotypes was correlated with an increase of efflux capacity of the strains. Gene expression assays disclosed a temporal pattern for expression of EP genes; an early-response with high expression levels of several EP genes followed by a late-response, with overexpression of specific genes (*mepA* and *nor* genes). The overall cell response varied according to the strains original efflux activity, being more pronounced for the strains with an initial basal efflux activity. Increased resistance to FQs in the two CIP-resistant strains was due solely to increased efflux, as no additional target-based mutations were acquired. For ATCC25923, exposure to ciprofloxacin resulted in a first efflux-mediated response, followed by the occurrence of one mutation in the *grlA* target gene that lead to phenotypic resistance to fluoroquinolones.

This study provides evidence that efflux mediates a first-line response to antimicrobials capable of conferring a MDR phenotype. Moreover, it reveals that resistance to FQs occurs in a two-step process, a first response with augmented efflux followed by the occurrence of target-based mutations that provide the cell with a stable resistance phenotype.

## Molecular Microbiology

### P258

#### EMERGENCE OF CARBAPENEM RESISTANCE AMONG GRAM-NEGATIVE BACTERIA WITHIN CENTRO HOSPITALAR DO BAIXO VOUGA, AVEIRO

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Nowadays, bacterial infections are a threat especially due to emergence of multidrug resistant (MDR) bacteria. So, the use of last resort antibiotics, mainly carbapenems, to treat these bacterial infections is increasing. The use and misuse of these compounds in the hospital environment, led to the development of carbapenem-resistant (CR) bacteria, which can also be the origin of nosocomial infections. We investigated the prevalence of CR bacteria isolated in the Centro Hospitalar do Baixo Vouga, Aveiro. Bacteria were isolated from biological samples from inpatients and from hospital surfaces. Clonal relationship was evaluated by rep-PCR and analysed with Gel Compar II 5.0 program (Applied Maths, Kortrijk, Belgium). Identification and antibiotic susceptibilities were determined using the automatic VITEK2 AES system (BioMérieux, Marcy L'Étoile, France) and confirmed by diffusion disk according EUCAST. Molecular characterization of CR mechanisms were performed by PCR. Mutations in outer membrane proteins were detected by qPCR.

CR bacteria were isolated from: i) biological samples (*C. freundii* (2), *K. pneumoniae* (7) and *P. aeruginosa* (40)); ii) inanimate surfaces (*K. pneumoniae* (1), *K. oxytoca* (1) and *P. aeruginosa* (3)). Resistance to carbapenems was confirmed by E-test. KPC-3 carbapenemases were detected in two *K. pneumoniae* isolated from urine and two *K. pneumoniae* collected from bed rails and bedside table. Mutations in *ompK* genes were also studied, and amino acid substitutions were identified: *ompK35* differ in Val345Ser; *ompK36* differ in Leu8Ser; and addition of one nucleotide in *ompK36* originated a new amino acid (Asp137). Metallo-beta-lactamases were detected in sixteen *P. aeruginosa* from urine and in three *P. aeruginosa* collected from bed rails. In some of these isolates, a mutation in the *oprD* gene was detected with one amino acid substitution (Thr103Ser). Carbapenemases were not found in *C. freundii*. Nonetheless mutations in the OM proteins were detected: *ompA* (Thr59Ala) and *ompC* (Val24Phe). *ompF* is absent in these isolates.

Since bacteria have the ability to survive in hospital surfaces and can therefore be implicated in nosocomial infections, improvement of surfaces sanitation is important to control their dissemination to avoid serious hospital outbreaks. Emergence of CR bacteria is a serious problem since it decreases the antibiotic options, which can ultimately be restricted to colistin.

## Molecular Microbiology

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### EXPRESSION AND ANALYSIS OF ENDO-1,3- $\beta$ -GLUCANASE GENE FROM THE PLANT PATHOGEN PHYTOPHTHORA CINNAMOMI

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*Phytophthora cinnamomi* is considered one of the most widespread and destructive plant pathogen of the planet. It presents a wide geographical and host range distribution. Cause huge economic damage to many cultures worldwide. All this facts contribute to their relevance as model organisms for research.

In oomycetes, germination, sporulation, cell growth and pathogenesis processes have been associated to the structure of the cell wall. Despite its apparent rigidity to the cell wall is a very dynamic structure, suffering several modifications in the composition and structure throughout the organism life cycle.

1.3- $\beta$ -glucan is the polysaccharide responsible of the shape and rigidity of the cell wall of oomycetes. In multiple processes such us morphogenesis and pathogenesis the hydrolysis of the 1.3- $\beta$ -glucans are mediated by *endo-1,3- $\beta$ -glucanases*.

This fact suggests that those enzymes could play a key role in the above mentioned processes. The identification, study and analysis of the expression of genes involved in the biological development of *Phytophthora cinnamomi* and their interaction with hosts is a fundamental step for understanding all kind of events.

The purpose of this study was to isolate and clone the gene *endo-1,3- $\beta$ -glucanase* of *Phytophthora cinnamomi* in two expression vectors, *E. coli* and *Pichia pastoris* respectively. These two different expression systems can be used to obtain pure enzyme in large quantities. The gene, clone in the appropriated vectors, was used for the transformation of *Escherichia coli* and *Pichia pastoris* and the protein expression we analyzed at different times of incubation.

The results obtained revealed a successful expression of the gene in both vectores and in both organisms *Escherichia coli* and *Pichia pastoris*.

The gene expression was studied during growth in different carbon sources and was also performed a time course of endo-1,3- $\beta$ -D-glucosidase production. In vivo infection of *Castanea sativa* revealed changes in expression, with a times increase and decrease, suggesting that a compensatory mechanism must occur in plant defense.

## Molecular Microbiology

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### FUNCTIONAL ANALYSIS OF MATING IN THE FUNGAL PATHOGEN *PARACOCIDIoidES* SPP.

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The thermodimorphic fungus *Paracoccidioides* is the etiological agent of paracoccidioidomycosis (PCM), a mycosis prevalent in Latin America. The fungus undergoes a morphological transition from non-pathogenic mycelium at environmental temperatures to a pathogenic yeast form at the mammalian host temperature. Sexual reproduction in *Paracoccidioides* has not been observed, yet isolates encode homologues of all mating-related genes from other Ascomycetes, mating signaling pathway components and *MAT1-1/MAT1-2* locus. This supports the occurrence of sexual reproduction in the *Paracoccidioides* genus. We present data showing low basal expression levels of the *a*- and  $\alpha$ -pheromone receptor genes (*PREA* and *PREB*), the  $\alpha$ -pheromone gene (*PB $\alpha$* ) and the *MAT* genes, in both forms of *Paracoccidioides*. Addition of synthetic *PB $\alpha$*  in *P. brasiliensis* *MAT1-2* strains failed to elicit transcriptional activation of mating-related genes, suggesting that the strains tested are insensitive to *PB $\alpha$* . Functionality of *P. brasiliensis* mating genes was further assessed by their heterologous expression in the corresponding *Saccharomyces cerevisiae* null mutants. We show that *S. cerevisiae* strains expressing *PREB* respond to *PB $\alpha$*  isolated from *Paracoccidioides* culture supernatants and its synthetic form, by shmoo formation, growth and cell cycle arrests. While the complementary *a*-pheromone could not be identified in *Paracoccidioides* genomes, we demonstrate likewise that *a*-pheromone-specific extracts from *Paracoccidioides* culture supernatants elicit shmoo formation and growth arrest in *S. cerevisiae* strains expressing *PREA*. This indicates that *Paracoccidioides* species secrete active  $\alpha$ - and *a*-pheromones into the culture medium that are capable to activate their cognate receptor. Overall, our results provide new insights into the existence of a functional mating signaling system in *Paracoccidioides*, by demonstrating signal activation by the mating pheromones and receptors system in a yeast model. Additional experiments are in progress to address the functionality of other mating signaling components. We are also constructing *Paracoccidioides* strains overexpressing *MAT1-2* or *NSD* (positive regulator of sexual reproduction), and gene-silenced strains for *GPRD* (negative regulator of sexual reproduction) to clarify their effect on mating-gene expression levels and pheromone sensitivity. Finally, we are identifying the environmental conditions that stimulate sexual reproduction in *Paracoccidioides*.

## Molecular Microbiology

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HETEROLOGOUS EXPRESSION OF LICHENICIDIN: IMPROVEMENT OF THE BIOSYNTHESIS LEVELS IN *E. COLI*Joana Barbosa<sup>1</sup>, Tânia Caetano<sup>1</sup>; R. D. Suessmuth<sup>2</sup>; Sónia Mendo<sup>1</sup><sup>1</sup>Department of Biology and CESAM, University of Aveiro, Portugal;<sup>2</sup>Department of Chemistry, Technical University of Berlin, Germany

The dissemination of drug-resistant pathogenic bacterial strains has increased the need for new antibiotics with specifically targeted activities. Lichenicidin is a two-peptide lantibiotic (Bli $\alpha$  and Bli $\beta$ ), naturally produced by *Bacillus licheniformis* I89, with activity against MRSA, *Enterococcus faecium*, *Haemophilus influenza* and *Listeria monocytogenes*, which are clinically relevant strains. Lichenicidin was the first lantibiotic to be totally produced *in vivo* using a Gram-negative heterologous expression system (*Escherichia coli*). This allowed the application of novel bioengineering approaches to lantibiotics, namely the incorporation *in vivo* of nonproteinogenic amino acids in Bli $\alpha$  and Bli $\beta$ . That was achieved by using a fosmid as vector, where all the lichenicidin biosynthetic genes are placed under the control of *Bacillus licheniformis* regulatory determinants (promoters, RBS, etc.). Despite the success of this approach, it is believed that the production levels of lichenicidin can be improved. In this context, it is our aim to select for the best system to achieve such a feat in *E. coli*. In a first approach, the essential biosynthetic genes of Bli $\alpha$  and Bli $\beta$  were cloned separately and under the control of *E. coli* transcription and translation determinants. Also, two different plasmids were used: pET24a (low copy) and pUC19a (high copy). All the constructs were performed in *E. coli* DH5 $\alpha$  cells and then transformed in BL21(DE3)Gold. Using this approach, the production of active Bli $\alpha$  and Bli $\beta$ , in separate, was achieved only with the low copy number plasmid. The exact production levels will be determined by HR-ESI-MS/MS and compared with those of the fosmid-expression system. This will allow the selection a better system for Bli $\alpha$  and Bli $\beta$  production in *E. coli*. Moreover, other *E. coli* strains (Origami 2(DE3) and BL21(DE3) Star) were transformed with the constructed plasmids and the production levels of lichenicidin will also be evaluated as above.

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### IDENTIFICATION OF THE GENOMIC PACKAGING SEQUENCE OF CHIKUNGUNYA VIRUS

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Chikungunya virus (CHIKV) is a 12 kb positive-strand RNA virus of the *Togaviridae* family transmitted to humans by *Aedes* mosquitoes. It causes an acute infection in humans characterized by severe prolonged arthralgia. Indigenous to tropical Africa for several decades, CHIKV has been spreading throughout Africa and Asia, causing massive outbreaks of increasing severity, and reached some parts of Southern Europe [1]. In spite of the resurgence of interest in CHIKV, many aspects of its replicative-cycle remain to elucidate, *inter alia* the mechanism involved in packaging of the viral genome and nucleocapsid assembly. It is known that a signal sequence on the alphavirus genomic RNA allows its selective packaging in the host cell cytoplasm crowded with other RNAs. Unlike most of the alphavirus, whose packaging sequence is found in the nsP1 coding region, in CHIKV its location was assigned to the nsP2 gene [2].

The main purpose of this work was the identification of the CHIKV packaging sequence (PS) by analysis of RNA sequences present in virus-like particles (VLPs) and nucleocapsids (NCs) produced in cells transfected with expression vectors harboring putative PS.

Eight fragments of the CHIKV nsP2 gene, amplified by RT-PCR from genomic RNA, were cloned downstream of the green fluorescent protein (EGFP) gene in the expression vector pIC113. Recombinant plasmids and expression vectors for CHIKV structural polyprotein and capsid-poli(His) protein were cotransfected into HEK293T cells to produce VLPs and NCs, respectively. The analysis of the packaged mRNAs was performed by reverse transcription and real-time PCR targeting the EGFP transcripts.

In this study we identified a CHIKV 350 bp sequence (nt 2501-2855 in the CHIKV S27 strain) that is preferentially up-taken into extracellular VLPs. The EGFP transcripts encapsidated were successfully translated upon cell inoculation with the VLP preparation. The selective packaging of this sequence was further confirmed in pull-down assays specific for capsid-poli(His) protein-RNA complexes in the cytoplasm of cotransfected cells. The CHIKV PS identified includes five stem-loop structures characterized by a GUG motif in the base of the apical loop. The role of secondary structures and conserved GUG motifs present in this sequence deserve further analysis for a better understanding of the CHIKV assembly process.

[1] Schwartz O and ML Albert. *Nat Rev Microbiol* (2010) **38**:7388-7399

[2] Kim DY *et al.* *J Virol* (2011) **85**:8022-8036



## Molecular Microbiology

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INSIGHTS INTO THE LICHENICIDIN SELF-PROTECTION MECHANISM USING *E. COLI* AS MODEL ORGANISMTania Caetano<sup>1</sup>, Roderich D. Suessmuth<sup>2</sup>; Sónia Mendo<sup>1</sup><sup>1</sup>Departament of Biology & CESAM, University of Aveiro; <sup>2</sup>Department of Chemistry, Technical University of Berlin

Lantibiotics are lanthipeptides with antibacterial activity. Thus, their producers possess self-protection mechanisms that help them to cope with the produced compounds. Lichenicidin is a lantibiotic produced by *B. licheniformis* strains, which is active against several Gram-positive bacteria. Lichenicidin biosynthetic cluster possesses 5 genes putatively related with its self-immunity system. These genes were previously annotated as *licFGEHI* and their predicted protein sequences were analysed in this study. Based on the presence of conserved motifs and transmembrane domains, they were reclassified to *licF<sub>1</sub>GEF<sub>2</sub>I*, where i) *licF<sub>1</sub>* and *licF<sub>2</sub>* encode 2 ATP-binding domains of an ABC transporter, ii) *licG* and *licE* encode 2 membrane proteins, that should constitute the transmembrane domains of an active ABC transporter and ii) *licI* encodes a protein of unknown function. Generally, lantibiotics are not effective against Gram-negative strains and lichenicidin is not an exception. However, it was previously shown that *E. coli* *lptD4213* is sensible to lichenicidin. Thus, this strain was herein used as model organism to understand the involvement of *licF<sub>1</sub>EF<sub>2</sub>I* genes in the self-protection mechanism of lichenicidin. Firstly, *licF<sub>1</sub>GEF<sub>2</sub>I* genes were cloned in pUC19a plasmid and transformed into *lptD4213* strain. The evaluation of lichenicidin extracts activity against the obtained strain showed that *licF<sub>1</sub>GEF<sub>2</sub>I* genes conferred full immunity to *E. coli* *lptD4213*. Using the same approach, the impact of some of these genes on self-protection was assessed constructing plasmids containing the following arrays *licF<sub>1</sub>GEF<sub>2</sub>*, *licF<sub>1</sub>GE*, *licGEF<sub>2</sub>I* and *licI*. After well-diffusion agar testing, the results showed that *licI* alone does not contribute to lichenicidin resistance. The absence of *licF<sub>2</sub>* resulted in a more sensitive strain than with the absence of *licF<sub>1</sub>*. This suggests that the assembly of an active ABC-transporter is more dependent of LicF<sub>2</sub> than of LicF<sub>1</sub>. However, ABC-transporters involved in lantibiotic immunity possess two homodimeric ATP-binding domains (LanF) for two heterodimeric transmembrane domains (LanGE). Thus, the presence of two different *lanF* genes among the lichenicidin immunity determinants is unique. Moreover, our results suggest that, albeit at different extent, both *licF* genes contribute to lichenicidin protection. Therefore, this mechanism seems to be different from those already characterized and it will be focus of further investigation.

## Molecular Microbiology

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### MOLECULAR CHARACTERIZATION OF THE TRANSCRIPTIONAL REGULATOR CHR<sub>B</sub> FROM *OCHROBACTRUM TRITICI* 5BVL1

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*Ochrobactrum tritici* 5bvl1 is able to resist to high concentrations of chromate through the expression of an inducible chromate-resistant determinant, found in a mobile element (Tn*OtChr*), which carries the genes, *chrB*, *chrA*, *chrC* and *chrF*. This work shows the characterization of the *chr* operon regulation, present in Tn*OtChr*, which is controlled by a transcriptional regulator, ChrB.

Fusions of *chr* promoter, or *chr* promoter and *chrB* gene, upstream of a *gfp* reporter gene, identified the most probable promoter sequence within the *tnpR-chrB* intergenic region. This region contains an AT-rich imperfect inverted repeat sequence. The results of the in vitro DNA-binding assays with purified ChrB (His- or no-tagged) showed that the protein binds directly to the *chr* promoter region. In order to identify the ChrB functional domain for sensing chromate stress and for DNA-binding, site-directed mutagenesis of ChrB was performed. Among several single amino acid mutants, three mutants (R180; R187 and H229) prevented chromate induction without any modification to the protein's stability. Two ChrB mutants (R18 and R23) were constitutively active, regardless of chromate stress conditions, indicating that the residues most probably belong to the protein-DNA binding site.

As such, the ChrB was classified as a transcriptional regulator that recognizes a specific DNA sequence, regulating the expression of a chromate resistance determinant.

## Molecular Microbiology

### P265

#### MOLECULAR EPIDEMIOLOGY AND RISK FACTORS BY EXTENDED-SPECTRUM $\beta$ -LACTAMASES PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* IN URINARY TRACT INFEC

Maria José Espinar<sup>1</sup>, Isabel M. Miranda<sup>1</sup>; Sofia Costa-de-Oliveira<sup>1</sup>; Nuno Gomes<sup>1</sup>; Acácio G. Rodrigues<sup>1</sup>; Cidália Pina-Vaz<sup>1</sup>

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Infections caused by ESBL-producing bacteria are often associated with increased mobility, mortality and healthcare associated costs. Reports in Europe have been increasing and rather than just being related to nosocomial infections, these infections are more often community-acquired. Acquired resistance to beta-lactams is mainly mediated by extended-spectrum beta-lactamases (ESBLs) that confer bacterial multiresistance to all beta-lactams except carbapenems and cephamycins. The aim of this study was to characterize the extended-spectrum  $\beta$ -lactamases (ESBLs) *Escherichia coli* and *Klebsiella pneumoniae* isolated from urinary infection (UTI) in the population of kidney transplant patients (KTPs) of Hospital São João, Porto (Portugal) using a molecular approach and perform a risk factors analysis. Retrospective case-control study was designed in order to compare 92 patients with UTI, 50% of reported cases were caused by ESBL producing bacteria and the other half involved patients with UTI originated by non ESBL-producing bacteria. Variables like: previous urinary infections, creatinin and ureia serum determination, nitrites, immunosuppression regimen, prophylaxis, prior antibiotic exposure within previous two months, susceptibility profiles and comorbidity were studied. From the 49 patients with UTI by ESBL producing bacteria, eighty one strains were isolated from urine, two isolates from each patient, separated in time, were genotyped by random amplified polymorphic DNA (RAPD). Samples were screening for the presence of ESBLs-encoding genes, namely CTXbla, TEMbla, SHVbla using a multiplex PCR. Diabetes Mellitus, prophylaxis and previous use antibiotic revealed to be independent risk factors of infection by ESBL positive. High resistance to gentamicine, ciprofloxacin and trimetopim-sulfamethoxazol was found. Interestingly, resistance profile could be associated with several types of genes co-existing in the same strain. *E. coli* showed predominance in CTXbla (25%) TEMbla/CTXbla (37.5%) while *K. pneumoniae* showed TEMbla (35.3%) followed by CTX/TEM/SHVbla (29.4%). Genotyping of strains isolated from a single patient shown that 62.5% of UTIs were recurrent, while in 37.5% of the cases were caused by different types of bacteria, which strongly indicates that recurrent urinary infections. UTI by ESBL producing bacteria is a serious problem in KTPs and patient treatment must be chosen based on an analysis of their specific resistance pattern.

## Molecular Microbiology

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### NATURAL MUTATIONS LEADING TO DISRUPTION OF THE BAC PROTEIN PRODUCTION IN *STREPTOCOCCUS AGALACTIAE*

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*Streptococcus agalactiae* (the Group B *Streptococcus*, GBS) is able to colonize numerous tissues employing different mechanisms of gene regulation, particularly via two-component regulatory systems (TCS). These systems sense the environmental stimuli and regulate expression of the genes including virulence genes. The *bac* gene transcription encoding for Bac antigen, an important virulence factor, has recently been shown to be under the control of an two-component system Bgr (*bac* gene regulatory system), comprising a DNA-binding response regulator (Sak189 – BgrR) and a sensor histidine kinase (Sak188 – BgrS) [1]. A mutant strain in which this TCS genes were inactivated showed decreased *bac* gene transcription / Bac antigen expression and a moderate increase in virulence in a mouse intraperitoneal infection model. Bac antigen expression is likely to be closely coordinated during infection and further studies are needed to elucidate this process.

In this work a total of 140 GBS strains isolated from human in China, Russia, Sweden and Portugal, of serotypes Ia (33 strains), Ib (32 strains), II (37 strains), III (15 strains), V (21 strains) and NT (2 strains) and associated with diverse clinical origins were screened by PCR, sequencing analysis of PCR products, SDS-PAGE and western-blotting for detection of both *bgrR* and *bgrS* genes and for selection and further studies of *bac* gene positive strains lacking the Bac protein. Transformation of the strains by recombinant plasmids derived from the vector pAT29 was performed using Gene Pulser Xcell (Bio-Rad Laboratories, USA).

Among 53 out of 140 strains of serotypes Ib (n=27; 50.9%), II (21; 39.6%), Ia (n=3; 5.7%) and NT (n=2; 3.8%) detected as *bac* positive, five did not produce the Bac protein. Four of these natural strains were collected in Portugal (3 invasive strains and 1 colonization strain) and in China (1 colonization strain). Disruption of the Bac protein production in these strains was associated with: i) integration of the mobile element *IS<sub>Sa4</sub>* upstream the *bac* gene; ii) point mutation in the *bac* gene resulting a stop codon TGA and a truncated form of the Bac protein; iii) synonymous point mutations in *bgrS* gene; iv) point mutations causing single aminoacid substitutions in *bgrR* gene: Arg560Lys and Arg755Gln; v) single nucleotide deletion in the *bgrR* gene resulting in a frameshift and changing of aminoacid sequence of the BgrR protein.

Transformation assays with a recombinant plasmid pBgrRS(P), comprising a full-size genes under the control of the natural promoter and constructed based on the vector pAT29 resulted in the restoration of protein expression. These data confirm the pivotal role of two-component system BgrRS in the activation of *bac* gene expression.

[1] Rozhdestvenskaya AS, Totolian AA, Dmitriev AV (2010) Inactivation of DNA-Binding Response Regulator Sak189 Abrogates beta-Antigen Expression and Affects Virulence of *Streptococcus agalactiae*. PLoS ONE 5(4): e10212. doi:10.1371/journal.pone.0010212

## Molecular Microbiology

P267

### SCREENING FOR PKS AND NRPS GENES AND PREDICTION OF BIOACTIVE COMPOUNDS FROM MARINE CYANOBACTERIAL ISOLATES

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Cyanobacteria are important primary producers and many are N<sub>2</sub>-fixers, playing a key role in the marine environment. They are a prolific source of bioactive compounds and some of these products are toxic to a wide array of organisms, including animals and humans. However, cyanobacteria can also produce secondary metabolites with promising therapeutic applications such as anticancer, antibiotic and anti-inflammatory activities. In a previous work, several cyanobacteria strains were isolated from intertidal zones on the Portuguese coast and characterized using a polyphasic approach [1]. In this study, a preliminary screening indicated that these cyanobacteria did not have the genes encoding proteins involved in the production of conventional cyanotoxins. However, earlier it was shown that extracts of *Synechocystis* and *Synechococcus* were toxic to invertebrates, with crude extracts causing stronger effects than partially purified ones [2]. To further evaluate the potential of our isolates to produce bioactive compounds, a PCR screening for the presence of genes encoding non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS), targeting the adenylation (A) and ketosynthase (KS) domains respectively, was performed. DNA fragments were obtained for more than 80% of the strains tested, and the results revealed that PKS are more ubiquitous than NRPS genes. The sequences obtained were used to an *in silico* prediction of the compounds that can be produced by these strains. To understand if the genes were transcribed under routine laboratory conditions, RT-PCR analyses were performed for selected strains. Metabolomic studies were initiated.

[1] Brito et al. (2012). *Syst. Appl. Microbiol.* 35: 110–119.

[2] Martins et al. (2007). *Toxicon.* 50: 791–799.

## Molecular Microbiology

### P268

#### SEQUENCE ANALYSIS AND EXPRESSION OF GENES INVOLVED CYTOSKELETON AND INFECTION MECHANISMS IN THE PLANT PATHOGEN *PHYTOPHTHORA CINNAMOMI*

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Oomycetes from the genus *Phytophthora* are plant pathogens that are devastating for agriculture and natural ecosystems. The biggest productivity and yield break occurs due to the ink disease; caused by *Phytophthora cinnamomi*, which is one of the most widely distributed *Phytophthora* species, with nearly 1000 host species. The knowledge about the molecular mechanisms responsible for its pathogenicity is an important tool in order to fight diseases associated with this pathogen.

Complete open reading frames (ORFs) of *act1*, *act2* and *tub1*, genes that participate in cytoskeleton formation in *P. cinnamomi*, were achieved by high-efficiency thermal asymmetric interlaced (HE-TAIL) polymerase chain reaction (PCR). *act1* gene comprises a 1128 bp ORF, encoding a deduced protein of 375 amino acids (aa) and 41,972 kDa. *act2* ORF comprises 1083 bp and encodes a deduced protein of 360 aa and 40,237 kDa. *tub1* has a total length of 2263 bp and encodes a 453 aa protein with a molecular weight of 49.911 kDa.

With this technique we also identified and characterized some proteins involved in mechanisms of infection by *Phytophthora cinnamomi*: endo-1,3-beta-glucanase (complete cds), exo-glucanase (partial cds); glucanase inhibitor protein (GIP) (complete cds); necrosis-inducing Phytophthora protein 1 (NPP1) (complete cds), transglutaminase. The genes of *P. cinnamomi* revealed significant homologies with correspondent genes of species of the genus *Phytophthora*. This genes isolated from *P. cinnamomi*, were successfully expressed in *E. coli* and *P. pastoris*.

The expression of these genes was monitored during growth in different carbon sources (glucose, cellulose and sawdust) by RT-qPCR and its level of expression was evaluated at five time points. The highest expression of genes occurred in sawdust at 8 hours of induction. In vivo infection of *Castanea sativa* revealed an increase of their expression from 12 to 24 hours. After 36 hours their expression decreased suggesting that a compensatory mechanism may occur in the host plant.

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## Molecular Microbiology

P269/F18

### THE IMPACT OF LYSB IN MYCOBACTERIA PHAGE-MEDIATED LYSIS

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Nowadays bacterial resistance to antibiotics is a disturbing reality and casts a dark veil over the future combat to infectious diseases. It is urgent to find and develop new molecules that can be used as alternative therapeutics. In this sense, bacteriophages have been explored due to their ability to destroy essential structures of bacteria.

We have been studying the lysis properties of mycobacteriophage Ms6, a phage that infects *Mycobacterium smegmatis*, a non-pathogenic strain used as a model to study mycobacteria, which includes *M. tuberculosis*, the causal agent of tuberculosis, one of the leader causes of death by a single pathogen. Ms6 encodes a lytic enzyme, named LysB, capable of hydrolyzing important lipid reach components of the mycobacterial cell envelope. In this work we show that using a new method, named Bacteriophage Recombineering of Electroporated DNA (BRED) we efficiently constructed an Ms6 mutant deleted in gene *lysB*. We show that LysB has an important role on host lysis. One-step growth curve assays revealed that lysis is less efficient in absence of LysB, resulting in a delay of the timing of lysis, as a consequence of a deficient release of the phage particles which are trapped in cell debris.

Understanding the bacteriophage-host interactions at the molecular level, will contribute to the discovery of new antibacterial therapeutic targets and for the design of phage-based products with medical and biotechnological applications.

## Molecular Microbiology

### P270

## THE IMPORTANCE OF MITOCHONDRIAL FUNCTION IN THE ROLE OF THE YEAST CATHEPSIN D IN ACETIC ACID-INDUCED APOPTOSIS

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Cathepsin D (CatD) is a lysosomal protease that, in response to an apoptotic stimulus can be released into the cytosol and play a prominent role in the mitochondrial apoptotic cascade (1–2). We previously showed that, like its mammalian CatD ortholog, the yeast vacuolar protease Pep4p translocates to the cytosol during acetic acid-induced apoptosis (3). We also showed Pep4p was required for efficient mitochondrial degradation and for increased cell survival (3), which depends on its catalytic activity (4). Nevertheless, its precise role in the mitochondria-dependent death pathway is still elusive. We therefore sought to elucidate the importance of mitochondrial proteins/function in the protective role of Pep4p in acetic acid-induced apoptosis. We showed that this phenotype depends on the presence of the yeast mitochondrial AAC proteins, the yeast adenine nucleotide translocator, but is independent of the voltage-dependent channel Por1p (4). We now show that it also depends on an active mitochondrial function, as deletion of *PEP4* in a W303 rho<sup>0</sup> background actually resulted in increased resistance to acetic acid-induced cell death, in contrast with the rho<sup>+</sup> strain. Consistently, deletion of *PEP4* in the BY4741 strain background, which has lower mitochondrial mass than the W303 strain (5), also resulted in increased cell survival in the presence of acetic acid. In contrast, like in W303 *pep4Δ* cells, BY4741 *pep4Δ* displayed a delay in mitochondrial degradation in response to acetic acid. Our results thus show that the pro-survival role of Pep4p in acetic acid-induced cell death is dependent on mitochondria function, whereas its role in mitochondrial degradation likely is not. This study shed more light on the role of yeast CatD in mitochondrial degradation, its dependence on mitochondrial proteins/events and may contribute to an enhanced understanding of the role of CatD in mammalian apoptosis.

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## Molecular Microbiology

P271

## THE ROLE OF PLASMID DETERMINANTS IN THE RESISTANCE OF STAPHYLOCOCCUS AUREUS TO ANTIMICROBIAL AGENTS

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The impact of *Staphylococcus aureus* infections and the increasing number of strains resistant to multiple antimicrobial compounds, has kept the interest on this microorganism over the years. Plasmids play an important role in the *S. aureus* ability to manage with constant environmental challenges, as one of the main ways of gene transmission, including resistance and virulence determinants.

The aim of this study was to determine the importance of the plasmids carried by clinical *S. aureus* strains to their susceptibility profile towards a range of antimicrobial compounds.

The study collection comprised 52 *S. aureus* clinical isolates from a hospital laboratory in Lisbon, 50 of which were resistant to methicillin (MRSA). The antimicrobial susceptibility profile to an assortment of antibiotics and biocides, including heavy metals, was evaluated by the Kirby-Bauer method and determination of minimum inhibitory concentrations. Plasmids were extracted by a modified alkaline lysis procedure and assigned to a restriction profile, according to their restriction pattern with enzymes *AccI* and *EcoRI*. Three strains (SM1, SM11 and SM26), carrying plasmids with representative restriction profiles, were selected for plasmid curing. Comparison of the resistance profiles for both original and cured strains allowed a correlation of the different plasmids with resistance profiles, which was then analyzed by screening specific resistance genes by PCR.

Plasmids were identified in 43 out of the 52 (82.7%) isolates, of which 25 carried a single plasmid and 18 carried two or more plasmids. The plasmids identified were assigned into eleven restriction profiles (P1 to P11). The curing of plasmids pSM1 (P1 – large plasmid), pSM11 (P2 – large plasmid) and pSM26 (P10 – small plasmid) followed by PCR screening allowed the correlation of plasmid pSM1 to resistance to  $\beta$ -lactams (carriage of the *blaZ* gene), plasmid pSM26 to inducible resistance to macrolides/lincosamides/streptogramins (*ermC* gene) and plasmids pSM1 and pSM11 to decreased susceptibility to cadmium (*cadD* and *cadA*, respectively).

This study demonstrates the impact of plasmid determinants to the resistance profile to antimicrobial compounds of clinical *S. aureus* strains. The finding of a large percentage of strains harboring resistance plasmids highlights their essential role in the survival of this major pathogen in hostile environments and spreading of antimicrobial resistance.

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## Molecular Microbiology

P272

### TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF YEAST LACTATE AND ACETATE TRANSPORTERS BY A RNA HELICASES

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*DHH1* plays a fundamental role in mRNA decay and transcription and controls the turn over of this nucleic acid. Mutants deleted for *DHH1*, showed a deficient mRNA decay, longer half-live times of several mRNAs and accumulated capped deadenylated transcripts, indicating that Dhh1 is crucial for an efficient decapping, after deadenylation, interacting with the decapping machinery and increasing its efficacy.

Previous experiments revealed that the gene *DHH1* complemented the phenotype of a *Saccharomyces cerevisiae* mutant (named Ace8), affected in the expression of the genes coding for monocarboxylic-acids transporters, *JEN1* and *ADY2* (Paiva et al., 1999). In wild type cells, *JEN1* expression is undetectable in the presence of glucose or formic and propionic acids, and induced in the presence of lactate. In glucose, *JEN1* has been shown to be subjected to transcriptional catabolic repression.

Northern blot analysis showed that the deletion of Dhh1 led to the accumulation of *JEN1* mRNA when formic or propionic acids were used as sole carbon and energy sources. Dhh1 is known to interact with the decapping activator Dcp1 and with the deadenylase complex. This led to the hypothesis that *JEN1* expression is post-transcriptionally regulated by Dhh1 in presence of formic acid. Analyses of *JEN1* mRNAs decay in the wild type and in the mutant strain confirmed this hypothesis. Interestingly, we found that the *JEN1* mRNAs accumulating in the *dhh1* mutant in presence of formic acid were not translated into a functional Jen1 protein. In these conditions, no measurable activity for the acetate and lactate carrier was found either in transport assays or at expression of Jen1::GFP fusion. These results revealed the complexity of the expression regulation of *JEN1* in *S. cerevisiae* and evidenced the importance of *DHH1* in this process. More generally, microarray analyses of *dhh1* mutants indicated that Dhh1 plays a large role in metabolic adaptation and suggested that carbon source changes trigger a complex balance between transcriptional and post-transcriptional effects.

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## Molecular Microbiology

P273

### TRANSCRIPTIONAL REGULATION OF AUTOPHAGY DURING YEAST CHRONOLOGICAL AGING BY NUTRIENT-SENSING PLAYERS

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Aging is a complex and multifactorial process driven by genetic, environmental and stochastic factors that lead to the time-dependent physiological decline of biological systems. Given the complexity of the aging process, the research into the molecular determinants of aging has progressed rapidly mainly due to studies in simple model organisms as *Saccharomyces cerevisiae*. Several common denominators of aging found in different models, as yeast, include loss of proteostasis and deregulated nutrient sensing. Consistent with the relevance of deregulated nutrient sensing as a longevity modulator, caloric restriction (CR) is the only non-genetic intervention that delays the onset of aging in all model organisms. By negatively regulating the nutrient signaling pathways, CR also modulates autophagy. However, the mediators of autophagy regulation promoted by CR are still debated. Our data demonstrated that during yeast chronological life span, cells submitted to proteotoxic stress experienced a premature aging precipitated by exacerbated autophagy. The increased autophagy was found to be mediated by the metabolic sensor sirtuin 2 (Sir2) through the transcriptional regulation of the ATG8 gene (1). In contrast, yeast cells under CR are able to deal with the imposed proteotoxic stress displaying an increased longevity that is associated with the homeostatic regulation of autophagy. Our findings highlighted that Sir2 impacts on yeast chronological aging through the transcriptional regulation of autophagy. Additionally, we also unraveled that the longevity promoting effects of CR are partially dependent on the maintenance of autophagy at homeostatic levels. Together, the data suggests that autophagy is an important determinant of longevity but it has to be maintained at physiological levels to exert the desired anti-aging effects.

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## Molecular Microbiology

P274

UNDERSTANDING THE MOLECULAR DETERMINANTS OF UNISEXUAL BEHAVIOR IN THE YEAST *PHAFFIA RHODOZYMA*Márcia David-Palma<sup>1</sup>, Marco A. Coelho<sup>1</sup>; Diego Libkind<sup>2</sup>; José Paulo Sampaio<sup>1</sup>; Paula Gonçalves<sup>1</sup>

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*Phaffia rhodozyma* (teleomorph *Xanthophyllomyces dendrorhous*) is basidiomycete yeast with biotechnological relevance due to its ability to produce the carotenoid astaxanthin, which is broadly used in food and in the pharmaceutical industry. In most basidiomycete yeasts the sexual cycle is initiated by the mating of two compatible strains of distinct mating types (heterothallism) followed by the formation of a dikaryotic mycelium, but in the case of *Phaffia* no such compatibility system appears to be necessary and no mycelium is formed. *Phaffia*'s sexual cycle is characterized by the conjugation of mother cell and bud (homothallism or unisexual behavior), followed by the formation of a slender aerial basidium in which meiosis supposedly occurs and from which apical basidiospores arise. In heterothallic basidiomycetes two classes of genes are known to determine sexual identity: pheromone/receptor (*P/R*) genes and homeodomain (*HD*) transcription factor genes. The *P/R* locus encodes lipopeptide pheromones and pheromone receptors that mediate cell-cell recognition leading to cell fusion, while the *HD* locus encodes homeodomain transcription factors that control the progression through the sexual cycle. In homothallic basidiomycetes the presence/absence and function of these genes has not hitherto been fully characterized at the molecular level. Using a draft genome sequence from the *Phaffia* strain CBS 7918<sup>T</sup>, we identified putative mating type genes, representing both *MAT* loci. Two putative pheromone and pheromone receptor genes were identified, as well as a pair of divergently transcribed homeodomain genes. Deletion mutants of *HD* and *P/R* genes were constructed and their ability to undergo sexual reproduction was evaluated, thus providing the first insights into the molecular determinants of *Phaffia* homothallic life cycle.

## Molecular Microbiology

### P276/F16

#### THE MtvR sRNA IS INVOLVED IN THE REGULATION OF Hfq

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Small non-coding regulatory RNAs (sRNAs) are key post-transcriptional regulators of gene expression in bacteria, affecting virulence in a number of bacterial pathogens (1). Trans-encoded sRNAs act by anti-sense base-pairing within a limited region of the 5'-untranslated region (5'-UTR) of their target mRNA, leading either to a negative or positive regulatory effect on the mRNA target. This interaction is often mediated by the Hfq RNA chaperone (2). Some trans-encoded sRNAs are known to regulate multiple targets, acting as global regulators, linking the stress responses to other cellular processes (3). About one half of the sequenced prokaryotic genomes contain one copy of an *hfq*-encoding gene (4). Bacteria of the *Burkholderia cepacia* complex (Bcc) are an exception, for being among the few prokaryotes harbouring two distinct genes encoding Hfq proteins in their genome (5,6).

This work describes the functional analysis and molecular characterization of the MtvR sRNA from the Bcc member *Burkholderia cenocepacia* J2315, with homologues restricted to the *Burkholderia* genus. The mRNA levels corresponding to 17 of 19 selected genes (from a total of 309 mRNAs putative targets predicted by bioinformatics) were found to be affected when MtvR was either overexpressed or silenced. Analysis of the interaction between MtvR and the *hfq* mRNA, one of its targets, showed that the sRNA binds exclusively to the 5'-UTR of the *hfq* mRNA. This interaction resulted in decreased protein synthesis, suggesting a negative regulatory effect of MtvR on the RNA chaperone Hfq. Bacterial strains with MtvR silenced or overexpressed exhibited pleiotropic phenotypes related to growth and survival to several stresses, swimming and swarming motilities, biofilm formation, resistance to antibiotics and ability to colonize and kill the nematode *Caenorhabditis elegans*.

The implication of the regulation of a global regulator by an sRNA will be discussed.

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## Molecular Microbiology

P277/F17

### DEVELOPMENT OF REAL-TIME PCR ASSAYS TARGETING THE FLAGELLIN GENE FOR THE IDENTIFICATION OF *BORRELIA BURGdorFERI* SENSU LATO GENOSPECIES

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Lyme Borreliosis (LB) is the most common arthropod-borne disease in North America and Europe, where the main vectors are ticks from *Ixodes* genus. LB is caused by a group of genetically diverse spirochetes belonging to *Borrelia burgdorferi* sensu lato complex (*B.b.s.l.*), currently comprising 19 named genospecies with diverse geographic distributions, hosts specificity and virulence. In Europe the four most prevalent *Borrelia* species are *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.) and *B. lusitaniae*, whose infection leads to a variety of clinical symptoms involving skin, nervous system, heart and joints.

The identification of *Borrelia* genospecies is essential to better understand the respective role in the pathological involvement on LB manifestations. Therefore, several PCR- and qPCR-based methods have been developed for the detection of these species, targeting genes such as *flaB*, 16S rRNA, *ospA* and 23S rRNA, where rapidity, sensitivity, specificity and the possibility of bacterial typing and quantification in samples are major advantages. In this context, the aim of this study was to develop and evaluate a TaqMan<sup>®</sup> multiplex real-time PCR (qPCR) assay targeting the *flagellin* gene for the detection and quantification of the most prevalent genospecies of *B.b.s.l.*

The *flagellin* gene sequences of the most prevalent *B.b.s.l* species in Europe were retrieved from GeneBank and analysed. Genus- and species-specific probes, and respective flanking primers, were designed targeting conserved and non-conserved regions of the gene, respectively. Probes were dual-labeled with different sets of fluorochromes and qPCR assays were optimized in a *Rotor-Gene 3000 thermocycler*. The analytical sensitivity and specificity of assays were evaluated.

Four sets of primers and probes targeting the genus *Borrelia*, and the species *B. afzelii*, *B. lusitaniae* and *B. burgdorferi* s.s. were evaluated and revealed to be 100% specific and highly sensitive, detecting the equivalent to one bacterial cell in the qPCR reaction mixture. Currently, these qPCR assays are being tested for the direct detection of *Borrelia* in patient's biological samples and in ticks vectors collected from animals and vegetation.

Although the preliminary nature of our results, the qPCR assays underdevelopment are promising for the identification and quantification of the most prevalent species of *Borrelia burgdorferi* s.l. in Europe, contributing to a faster and more efficient diagnosis of Lyme disease.

## Molecular Microbiology

P278/F34

## CERAMIDE METABOLISM MODULATES MITOCHONDRIAL OUTER MEMBRANE PERMEABILIZATION AND APOPTOSIS

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The yeast *Saccharomyces cerevisiae* undergoes a mitochondrial-dependent programmed cell death in response to different stimuli, exhibiting typical apoptotic markers of mammalian apoptosis, such as externalization of phosphatidylserine, DNA fragmentation, cytochrome *c* release from mitochondria and production of reactive oxygen species (ROS). However, the upstream signaling events in this process, including those leading to mitochondrial membrane permeabilization, are still poorly characterized. Changes in sphingolipid metabolism have been linked to modulation of apoptosis in both yeast and mammalian cells, and ceramides have been detected in mitochondria upon apoptotic stimuli.

In this study, we aimed to characterize the contribution of enzymes involved in ceramide metabolism to apoptotic cell death induced by acetic acid. We show that *isc1Δ* and *lag1Δ* mutants, lacking inositol phosphosphingolipid phospholipase C and ceramide synthase, respectively, exhibited a higher resistance to acetic acid that was associated with a decrease in some phytoceramide species. Additionally, combined exposure to C2-phytoceramide and acetic acid resulted in significantly greater sensitivity than individual exposure to each agent in both wild-type and mutant cells, suggesting that phytoceramide is sufficient to induce cell death and sensitize cells to acetic acid. Consistently, these mutant cells in comparison to wild-type strain, displayed lower levels of ROS production, reduced mitochondrial alterations, such as mitochondrial fragmentation and degradation, decreased activity of Pep4p, the yeast CatD orthologue, and less translocation of cytochrome *c* into the cytosol in response to acetic acid.

Our results suggest that ceramide production contributes to cell death induced by acetic acid, especially through hydrolysis of complex sphingolipids catalyzed by *Isc1p* and *de novo* synthesis catalyzed by *Lag1p*, and provide the first *in vivo* indication of its involvement in mitochondrial outer membrane permeabilization in yeast.

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## Session 5

# Bioprocess Engineering



# Plenary Lecture

## CONTINUOUS DOWNSTREAM PROCESSING, A CHALLENGE FOR THE BIOCHEMICAL ENGINEER

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Integrated continuous biomanufacturing is currently debated a lot and several companies have implemented fully continuous processes for production of biopharmaceuticals. Unstable products such as blood coagulation factors must be produced in a continuous way. In batch production the product would degrade in the culture broth. Perfusion culture has been connected with pseudo continuous capture and purification processes using membrane chromatography. Besides mastering products with a low stability continuous manufacturing has economic advantages. This is the reason why also industry is considering continuous manufacturing for stable products. The typical unit operation in downstream processing will be described and how they can be made continuous. In particular refolding, solid liquid separation, chromatography and precipitation will be addressed. Continuous reactors such as tubular reactor, CSTR, MSMPR and tanks in series will be described. The genealogy of continuous filtration will be delineated and examples are shown how these unit operations can be connected to a continuous process. For antibody purification process an economic analysis and comparison of batch and continuous production will be given. In addition the definition of a "production" batch is provided.

# Keynote Lecture

## KL05

# INTEGRATED PLATFORM FOR PRODUCTION AND PURIFICATION OF HUMAN PLURIPOTENT STEM CELL-DERIVED NEURAL PRECURSORS

**Margarida Diogo**

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Neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, can strongly benefit from the use of human pluripotent stem cell (hPSC) neural derivatives through their different biomedical applications, including Regenerative Medicine, drug screening and disease modeling. However, before these applications can be fully realized several technological hurdles must be overcome.

One of the most important challenges in this field is the development of scalable bioreactor culture platforms for tightly controlled expansion and neural induction of hPSCs under chemically-defined xeno-free conditions. In this context, a microcarrier-based spinner flask culture system was successfully developed for scaling-up expansion of human induced pluripotent stem cells (hiPSCs) in serum-free medium and using xeno-free synthetic beads based on a peptide-acrylate surface. Under these dynamic culture conditions hiPSC were able to maintain their pluripotency and tri-lineage differentiation potential. Importantly, the directed induction of the expanded hiPSCs into neural precursors was successfully achieved onto the xeno-free microcarriers by means of the dual-SMAD inhibition method based on the use of small molecules. Overall, we expect this technology to facilitate the standardized and automated scaling-up of hiPSCs expansion and integrated neural induction.

Another major hurdle related to the use of hPSC-derived neural cells is the presence of hPSC that are unable to differentiate since these cells have the potential to generate tumours after being transplanted and may interfere with the outcome of the *in vitro* differentiation protocol. In this context, a separation methodology was successfully developed for negative selection of hPSCs after neural induction through the use of magnetic activated cell sorting (MACS) based on the use of Tra-1-60 microbeads. Importantly, the rational integration of this separation methodology with the spinner flask culture system paves the way towards the establishment of an integrated bioprocess for the production and purification of human neural progenitors for further downstream applications including potential cell replacement therapies.

# Oral Presentations

#### OP4

### AQUEOUS TWO-PHASE SYSTEM IN A MICROFLUIDIC PLATFORM TO ACCELERATE BIOPROCESS DESIGN AND OPTIMIZATION OF MONOCLONAL ANTIBODIES

Daniel Filipe Camarneiro Silva<sup>1</sup>; Ana Azevedo<sup>2</sup>; Pedro Fernandes<sup>2</sup>; Virginia Chu<sup>1</sup>; João Pedro Conde<sup>1</sup>; Maria Raquel Aires-Barros<sup>2</sup>

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Monoclonal antibodies are, presently, one of the most important biological products and are widely used in medical treatments against a number of diseases, such as cancer, autoimmune diseases and neural disorders. Thus, there is a strong demand for reliable and cost-efficient techniques for the purification of antibodies.

Previous work has shown the potential of Aqueous Two Phase Systems (ATPS) to extract antibodies in macroscale. In this work, a microfluidic platform was designed for ATPS parallel microflow and was tested for monoclonal antibody separation, combining the mild environment and the low interfacial tension of ATPS with the high surface-to-volume ratios and the ease of scale-up of microfluidics.

Immunoglobulin G (IgG) tagged with fluorescein isothiocyanate (FITC) was used as a model system to test antibody partition in an ATPS of polyethyleneglycol (PEG)/phosphate buffer with NaCl in a PDMS microfluidic device. The ATPS was prepared and separated into PEG-rich and salt-rich phases. IgG was added to the salt-rich phase and then flowed into the middle inlet of a three inlet microchannel with the PEG-rich phase introduced in the side inlets. The test series included different structures with varying lengths (3.14 to 14.6 cm) and different flow rates for the salt-rich phase (1 to 2  $\mu\text{L}/\text{min}$ ) and the PEG-rich phase (0.2 to 0.5  $\mu\text{L}/\text{min}$ ). The microchannel had a height of 20  $\mu\text{m}$  and a width of 150  $\mu\text{m}$  in all tests. The laminar nature of microfluidics allowed the formation of two stable interfaces, resulting in the diffusion and partition of the IgG from the salt-rich phase to the PEG-rich phases. This phenomenon was measured by fluorescent microscopy and modeled using process simulation [1].

The results show that, under optimized conditions, the miniaturization of ATPS does not greatly affect the overall antibody extraction yield compared with macroscale results. Furthermore, the simulation developed successfully models the experimental results. Presently, a model system of antibody plus serum and cell extract is being used for a quantitative measurement of the overall yield and purification factor. In general, the miniaturization of ATPS-based antibody extraction shows a reduction of the operation time while maintaining an extraction yield similar to that obtained in macroscale, demonstrating the potential of this approach.

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## OP18

## PURIFICATION OF HUMAN PAPILLOMAVIRUS16 E6/E7 PLASMID DNA-BASED VACCINE USING AN ARGININE MODIFIED MONOLITHIC SUPPORT

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Human Papillomavirus Virus (HPV), a common sexually transmitted disease, is considered to be one of the main causes related to the development of many different kinds of tumors, mainly cervical cancer. This virus is responsible for the production of proteins E6 and E7, which can interfere with the cellular cycle and lead to uncontrolled proliferation, resulting in the growth of tumoral cells. Hence, it is of major importance to create a cancer therapy making use of these proteins. Recently, the development of DNA vaccines has been widely studied, representing a new and effective way to induce an immune response to prevent or treat a preexisting infection. Aiming to guarantee the purity of the DNA vaccine, several, chromatography methods have been explored. A new strategy to isolate the target molecule has been implemented by our research group, through the use of affinity chromatography based on interactions between amino acids and nucleic acids [1]. Monoliths are chromatographic supports whose popularity has increased in the last years since they present excellent mass transfer properties and high binding capacity for large molecules, overcoming many of the conventional support limitations [2]. Therefore, the main purpose of our work was based on the purification of the supercoiled plasmid HPV 16 E6/E7 by combining the specificity and selectivity of the arginine ligand with the versatility of monolithic supports. Quality control tests revealed a low level of impurities (proteins, endotoxins, genomic DNA and RNA), meeting the guidelines established by regulatory agencies. The monolith characterization unveiled a dynamic binding capacity of arginine–diol monolith twenty six fold higher than the conventional arginine–agarose matrix at 10% of breakthrough. Therefore it is possible to purify a DNA vaccine towards the HPV accordingly to the regulatory agencies, allowing us to proceed to *in vitro* and *in vivo* assays, in order to assure the efficiency and immunogenicity of the vaccine.

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## OP45

### PURIFICATION OF PHARMACEUTICAL GRADE PLASMID DNA BY HIC MEMBRANE CHROMATOGRAPHY – IMPACT OF PLASMID SIZE AND PROCESS THROUGHPUT

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Non-viral gene therapy and DNA vaccination target the treatment and prophylaxis of diseases. Both rely on plasmid DNA (pDNA) vectors to convey genetic information into the cells of patients. Plasmid DNA based pharmaceuticals are extremely versatile, able to target a display of diseases.

Hydrophobic interaction membrane chromatography (membrane-HIC) explores the interactions between nucleic acids and ligands on the surface of membrane. Membrane HIC separates the biologically active supercoiled pDNA isoform (SCpDNA) from the open circular (OCpDNA) counterpart and also from its more hydrophobic natural impurities (e.g. RNA, gDNA and lipopolysaccharides).

This work explores the interactions of three plasmids of different sizes (3.7 kbp to 10.4 kbp) with a Sartorius Nano 3mL Phenyl membrane cartridge, bearing hydrophobic phenyl moieties. The robustness of the process was addressed by considering changes in operating fluxes and sample loads, scalability, reproducibility after consecutive HIC challenges, and ultimately their influence in the pDNA formulation quality.

Process performance and product quality were assessed through agarose gel electrophoresis, HPLC, protein BCA assay, RT-PCR and transfection challenges in CHO cells using selected chromatographic fractions after suitable concentration and diafiltration.

A sequential stepwise elution profile strategy made evident the enhanced exposure of SCpDNA hydrophobic grooves in comparison to the OCpDNA isoform in the presence of high concentrations of a kosmotropic salt. After a slight drop of salt concentration all the OCpDNA of the studied plasmids readily eluted whereas the SCpDNA required further concentration drop in order to elute. This enabled the separation of SCpDNA from the least active OCpDNA isoform. Final plasmid solutions showed a removal of over 99% RNA contaminants for all plasmids with over 85% yields and resultant transfection efficiencies comparable to a conventional HIC process.



# Poster Presentations

## Bioprocess engineering

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### CELLULASE PRODUCTION BY THERMOPHILIC STRAIN *RHIZOMUCOR PUSILLUS* UNDER SOLID STATE FERMENTATION

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Actually the most cellulase procedures studied at industrial scale use *Trichoderma reesei* strains. These kinds of strains produce non-thermostable enzymes that reduce the process capacities. The employment of thermostable enzymes to carry out hydrolysis at higher temperatures is generally advantageous because it increases the speed of reaction and avoids microbial contamination contributing to increased technical and economical viability of the process. The aim of this work was to evaluate the cellulase production kinetic by the thermophilic strain *Rhizomucor pusillus* SOC-4A using corn cob as support-substrate, and the enzyme thermostability on the crude extract.

The extracellular cellulase production ability of *Rh. pusillus* SOC-4A (DIQ-UAdC collection) was analyzed quantitatively in solid state fermentation (SSF) using corn con (CC) as support-substrate in tray bioreactors. Total sugars content [1] was evaluated as substrate consumption. Biomass was estimated indirectly as soluble protein [2]. Endoglucanase activity was measured according to Cunha *et al.*, [3]. One unit of cellulase activity was defined as the amount of enzyme required to liberate 1  $\mu\text{mol}$  of glucose/mL under experimental conditions.

The fermentation for the quantitative analysis of the enzyme, show that the highest cellulase activity (1165 U/gds) was at 48 h. Cellulase optimum temperature range in 70–75 °C with a pH of 7.0. A mathematical model was applied to determine the different kinetic parameters related to SSF.

Production of enzymes such as cellulase by thermophilic fungi results in a proper option for the generation of thermostable enzymes, which are growing on demand at the industrial market. Besides, the use of agroindustrial wastes in the SSF allows generating sustainable processes.

## Bioprocess engineering

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### EFFECT OF TEMPERATURE VARIATION IN LIQUID FERMENTATION FOR THE PRODUCTION OF LIPASES BY *ASPERGILLUS NIGER*

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The lipases (EC 3.1.1.3) could be the most important enzyme in the group of biocatalysts for biotechnological applications (1). Its ability to develop very specific biotransformation has become very popular in the food industry, detergents, cosmetics, pharmaceutical and organic synthesis (2). The aim of this work, was to evaluate the production of lipases by *Aspergillus niger* GH1, considering the temperature as the main variation factor using an optimized liquid medium (previous work) at reactor level.

The strain used was *A. niger* GH1 (DIA–UAdeC collection). The lipase production was made in 1.5 L Bioreactor (New Brunswick BioFlo/CelliGen 115) during 216 h. Liquid medium consisting of inorganic salts and olive oil as carbon source. The factors monitored were dissolved oxygen, temperature, pH and mixing; with constant aeration (1 v/v/m). The enzymatic activity assay was done according to Bastida et al., (1998). One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1  $\mu\text{mol}$  of pNPP per minute under assay conditions. Biomass was calculated by dry weight at 80 °C for 24 h defined as (g/mL).

The highest lipase production was at 35 °C with 28.15 and 28.0 UE mL<sup>-1</sup> at 168 and 192 h respectively. These values are more than 3 times of that achieved at 20 °C, suggesting that the optimal lipase production should be around 35 °C for this strain. Literature mentions values about 29 UE mL<sup>-1</sup> by *Rhizopus* sp. (4) and 18 and 4 UE mL<sup>-1</sup> by *R. arrhizus* and *A. niger* respectively (2). The major amount of biomass obtained was 0.017 g/ml at 92 h and 0.0014 at 144 h in the fermentations at 30 y 35 °C respectively. The variation of pH was  $\pm 0.5$ ; the supply of O<sub>2</sub> and mixing kept constant and the lecture of O<sub>2</sub> dissolved in the medium has a decrement until 50 %.

The temperature determined for a liquid fermentation to get the highest lipase activity production under previously described conditions was at 35 °C. This value could help the process, reducing the time of it and getting the major volume of enzymatic extract; likewise, in this work we obtained important information to a scale–up of this process. Alike, we suggest analyze other factor as aeration and agitation altogether with obtained in this work to increase the production of lipases in a medium liquid.

## Bioprocess engineering

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### EVALUATION OF MUTS AND MUT+ *PICHIA PASTORIS* STRAINS FOR MEMBRANE-BOUND COMT BIOSYNTHESIS

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Catechol-O-methyltransferase (COMT, EC 2.1.1.6) is a magnesium-dependent enzyme that catalyzes the methylation of catechol substrates, playing an important role in the O-methylation of levodopa in Parkinson's disease patients treated with levodopa plus an aromatic amino acid decarboxylase inhibitor. While structural and functional studies of this membrane protein are still hampered by its low recombinant production, *Pichia pastoris* (*P. pastoris*) has been described as an attractive host for the production of correctly folded and inserted membrane proteins [1]. Hence, in this work we applied *P. pastoris* X33 and Km71H for the biosynthesis of the membrane-bound isoform of COMT.

Recombinant MBCOMT biosynthesis was achieved in shake-flasks and since it was under the control of the AOX promoter, after 16 hours, the carbon source was shifted from glycerol to methanol and the fermentation was carried out during 120 hours, being supplemented with 1% methanol every 24 hours. Then, by employing a simple lysis protocol using glass beads, immunologically and biologically active MBCOMT was recovered in the supernatant after a centrifugation at 500g. A further centrifugation at 16000g led to the preferential accumulation of highly biologically active MBCOMT in the pellet and the affinity values determined from both fractions were in agreement with previous results obtained by our research group. Furthermore, this bioprocess was monitored by measuring the cell density as well as the recombinant MBCOMT biological activity at every 24 hour of fermentation.

Finally, fermentations were carried out using mixed-feeds of glycerol/methanol or sorbitol/methanol for both *Pichia strains*. The methanol, glycerol and sorbitol levels were quantified using a HPLC coupled to a refractive index and were further correlated with the MBCOMT biological activity as well as the AOX activity. Overall, a new recombinant system intended to synthesize recombinant human MBCOMT was successfully developed. Also, this eukaryotic system seems to be a promising approach to deliver MBCOMT in high quantities from fermentor cultures, in which the tools needed to monitor *Pichia* fermentations implemented and validated here can play an important role in the scale-up of this bioprocess.

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## Bioprocess engineering

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### IN-SITU PREDICTION OF A RECOMBINANT ASPARTIC PROTEASE PRODUCTION IN BIOREACTOR WITH NEAR INFRARED SPECTROSCOPY AND PARTIAL LEAST SQUARES REGRESSION

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Near-Infrared Spectroscopy (NIR) is a fast and non-destructive vibrational spectroscopy based on molecular overtone and combination vibrations. It can be implemented during biotechnological development phases, for process monitoring and control, or in quality control laboratories for release and stability analysis. The cell culture process can be analyzed in-situ, with an IR-spectrometer coupled to a transfection sterilizable fiber optic (NIR probe) inserted into the bioreactor. NIR-spectroscopy requires chemometrics tools, such as partial least squares (PLS) regression, to extract quantitative information on the variables of interest from the spectral data acquired.

In this study NIR-spectroscopy was used to *in-situ* monitoring the growth of two distinctive *Saccharomyces cerevisiae* strains, both transformed with the same expression system derived from a pYEC plasmid and involved in an aspartic protease production. PLS calibration models on selected wavelength regions were developed for biomass, enzymatic activity, specific activity, carbon sources consumption (glucose and galactose) and for by-product production (acetic acid and ethanol), which are the critical variables involved in the growth control and recombinant aspartic protease production. The PLS model for biomass yielded a  $R^2$  of 0.98 and a RMSEP of 0.46, representing an error of 4% of the calibration range (0.5–13.2 g dcw/l). A  $R^2$  of 0.94 and a RMSEP of 167 was obtained for the enzymatic activity, whereas a  $R^2$  of 0.93 and RMSEP of 672 were obtained for the specific activity, corresponding to an error of 7% of the experimental data range (232–10,130 U/mg). For the carbon sources, a  $R^2$  of 0.99 and 0.96, a RMSECV of 0.52 and a RMSEP of 0.55 for glucose and galactose, showing high predictive capabilities within the range of 0–20 g/l and 11–20 g/l, respectively. For ethanol the PLS model was characterized by a  $R^2$ =0.92 and a RMSEP=0.06, which corresponds to a 0.3% error; for the acetate, a high accuracy PLS model with a  $R^2$  of 0.97 and a RMSEP of 1.08 was obtained, representing an error of 9% within the range of 0.41–1.15 g/l.

The present results show that it is possible to in-situ monitoring the critical variables involved in recombinant aspartic protease production in bioreactor, which will allow controlling recombinant *S. cerevisiae* cultures in real time. From the above, NIR-spectroscopy appears as a suitable analytical tool for in-situ monitoring, offering a fast, accurate and highly repeatable approach to monitor cultures of recombinant *S. cerevisiae*.

## Bioprocess engineering

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### IONIC LIQUIDS AS ADJUVANTS IN AQUEOUS TWO PHASE SYSTEMS FOR THE SELECTIVE EXTRACTION OF OVALBUMIN FROM EGG WHITE

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Chicken egg white is an abundant source of proteins with several applications in industry. Ovalbumin is a majority protein in egg white (53%) and it is used in the food industry due to its gelling features and in the pharmaceutical industry as an antihypertensive agent. The common methods used for the extraction and purification of proteins are mainly based on salt precipitation, isoelectric precipitation, ultrafiltration and liquid chromatography. However, these methods are still ineffective and have high operating costs when high purity levels are foreseen. Aqueous two-phase systems are liquid-liquid extraction approaches with diverse advantages over other and more common purification processes, such as their scale-up potentiality and high biocompatibility (water-rich environment) for the purification of different biological products, including proteins. In this study, we attempted to separate ovalbumin from the remaining egg white proteins using aqueous two-phase systems (ATPS) constituted by polyethylene glycol (PEG) and a citrate salt in buffered media using ionic liquids as adjuvants to tailor the phases' affinities. The obtained results indicate that the addition of small amounts of ionic liquids is crucial for the selective extraction of the target protein and proper compounds can lead to the complete purification of ovalbumin from egg white. The structure and stability of the protein was also addressed before and after the extraction procedure.

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## Bioprocess engineering

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### KINETIC COMPARISON OF TRAY AND COLUMN BIOREACTOR IN THE XYLANASE PRODUCTION BY *RHIZOMUCOR PUSILLUS* UNDER SOLID STATE FERMENTATION

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The corn cob (CC) utilization as a substrate in solid state fermentation (SSF) process provides an alternative pathway and added value for this residual; using it in xylanase microbial production. However, agroindustrial waste heterogeneity demands a better understanding of the bioprocess engineering aspects in SSF process, with the objective of make possible the optimization of such process. The aim of this work was to compare the microbial growth kinetic related to xylanase synthesis by *Rhizomucor pusillus* SOC-4A strain in both, tray and column bioreactor, using the CC as substrate-support in SSF process without forced aeration.

The xylanase production of *Rh. pusillus* SOC-4A (DIQ-UAdC collection) was assessed in solid state fermentation (SSF) using corn con (CC) as support-substrate in both tray and column bioreactors. Crude extract from process was used to quantify the substrate consumption [1] evaluated as total sugars content. Biomass was estimated indirectly as soluble protein [2]. Xylanase activity was measured according to Bailey *et al.*, [3]. One unit of xylanase activity was defined as the amount of enzyme required to liberate 1  $\mu\text{mol}$  of xylose per mL under experimental conditions. Several mathematical models were applied to determine the different kinetic parameters related to both tray and column bioreactors in SSF.

Growth specific rate and biomass/substrate yield were less during the column fermentation in comparison with the tray bioreactor, indicating that growth stimulation is present attributed to the air-contact surface; while the maintenance coefficient was higher in column than the presented in tray bioreactor. Regarding enzymatic activity, highest values (425 U/gds) were found in column bioreactor.

In conclusion, better surface in contact with air favor the mycelia formation, meanwhile a column shape bioreactor enhance the enzyme synthesis and with this a product enhance.

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## Bioprocess engineering

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### MODELLING THE SEPARATION OF PLASMID DNA AND RNA BY ULTRAFILTRATION

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In many industrial bioprocesses, membrane filtration techniques, namely ultrafiltration (UF) and nanofiltration (NF), are preferred to other separation techniques available for separating biomolecules for their higher throughput, lower labour costs and/or easier scale-up. Due to the extreme complexity of the solutions, the optimization of these operations is generally difficult to achieve without performing previous laboratory trials. The ability of predicting the extent of the permeation of different types of biomolecules is essential to reduce the time and effort required to select the best membranes to perform a certain separation and the best operating conditions.

The separation of plasmid DNA (pDNA) from RNA is of central importance in the industrial production process of pDNA for vaccine and gene therapy applications and can be carried out by different methods, including selective precipitation and adsorptive techniques [1]. The use of UF in this application is still highly unexplored. Besides this, the theoretical models currently available for estimating sieving coefficients of nucleic acids in ultrafiltration are still very simplified approaches and the permeation mechanisms are far from being understood.

Recently, in our research, a theoretical model was developed for the estimation of sieving coefficients of linear flexible molecules in ultrafiltration, including linear DNA and RNA [2]. The model was derived by simulating the permeation process of freely jointed chains through a porous surface, using a Monte-Carlo method. Then, the model was extended to the case of circular flexible molecules, like pDNA [3]. An analysis of the main different factors affecting the permeation of pDNA and RNA in ultrafiltration is made here, using the developed model, including the effects of permeate flux, the hydrodynamic conditions in the filtration process, the pore radius of the membrane and its distribution, the ionic strength of the solutions and the concentration of the macromolecules. Finally, the possibility of separating supercoiled pDNA from different types of RNA by ultrafiltration is analysed here. Results of ultrafiltration tests performed in our laboratory are compared with model predictions, showing that this separation is, in fact, possible to achieve.

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## Bioprocess engineering

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### NEAR-INFRARED (NIR) VERSUS MID-INFRARED (MIR) SPECTROSCOPY TO MONITOR THE BIOPRODUCTION OF HETEROLOGOUS PRODUCTS

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The interest in the use of near-infrared (NIR) and mid-infrared (MIR) spectroscopy to monitor bioprocesses have recently increased, since they enable a rapid and sensitive estimation of all critical variables of the bioprocess, namely, the host cell growth, the production of the heterologous product, the carbon sources consumption and by-products (e.g., acetate and ethanol) production and consumption. These techniques present specific characteristics, and therefore specific advantageous and limitations, that at the end may complement each other.

While MIR spectroscopy reflects the fundamental vibration of molecular bonds, NIR spectroscopy reflects overtones and combinations of vibrations, and consequently the MIR spectra presents more information concerning the samples' biomolecular composition, compared to NIR spectra. However, due to the high absorption of water in the MIR region, it is usually necessary to take the samples from the bioreactor and subsequently conduct the sample dehydration, which increases the risk of bioreactor contamination and inputs a time delay in the analysis. An advantageous is that it is possible to at-line conduct the MIR spectral acquisition in a high-throughput mode, using micro-plates (1), which is particularly important if hundreds of samples are to be analysed in a short period of time, as is the case of bioprocess optimization protocols. In spite of being less informative, NIR spectroscopy, combined with chemometrics allows the construction of calibration models for an accurate prediction of the critical variables of the bioprocess. Moreover, NIR fiber optic probes, that can be immersed directly in the culture broth and steam sterilised with it, enables the acquisition of information in-situ and in real time (2). Nevertheless, the use of this kind of probes in optimization protocols in microbioreactors may be impaired due to space constraints and low biomass concentrations.

In the present study, the high-throughput MIR spectral analysis and the in-situ analysis with a NIR fiber optic probe are compared to monitor the plasmid pVAX-LacZ bioproduction in recombinant E. coli DH5a cultures, conducted under different media conditions and on batch and fed-batch modes, and consequently producing different plasmid yields per biomass. Several partial least square (PLS) models were built for predicting the biomass growth and the plasmid production. The PLS models based on NIR spectra yielded a correlation coefficient  $R^2 \approx 0.96$  for biomass and plasmid productions and root mean squared error of predictions (RMSEP) of 0.4 and 9.7, respectively. For the models based on MIR spectra, a  $R^2 \approx 0.96$  for biomass and plasmid productions and a RMSEP of 0.4 and 9.8, respectively, were achieved. The results presented clearly indicate that both at-line high-throughput MIR and in-situ NIR spectroscopies can accurately predict the plasmid bioproduction in E. coli DH5a cultures, when different goals are to be met.

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## Bioprocess engineering

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### **POLYMER ELECTROSPUN NANOFIBROUS MATS FOR ENZYME IMMOBILIZATION AND BIOCATALYTIC APPLICATIONS**

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The structure and dimensions of the carrier material have great importance on the performance of immobilized enzymes. Among many types of carriers already investigated, nanostructured supports are believed to be able to retain the catalytic activity as well as to ensure immobilization efficiency and high catalytic performance of the immobilized enzymes, while allowing for low mass transfer limitations [1,2]. The electrospinning is a simple and inexpensive technique that allows the production of nanofibers from a wide range of polymers, with a small fiber diameter, variable and controllable pore size and high surface area. These characteristics make the electrospun polymeric nanofibers very attractive for use as carriers for filtration, enzyme immobilization, enzymatic membrane reactors, and sensors [3–4]. The encapsulation of the enzyme within the polymer matrix prior to electrospinning process provides great simplicity, which protects the active groups and improve enzyme loading efficacy [5].

The main objective of this study was to prepare polycaprolactone (PCL) nanofibers as supports for the immobilization of trypsin, taking into account the stability of the enzyme and the preservation of catalytic capacity. PCL nanofibers matrices loaded with trypsin were obtained by water/oil emulsion–electrospinning. The oil phase consisted of a PCL solution in chloroform/dimethylformamide, the water phase contained the enzyme in a phosphate buffer, and sorbitan monooleate as emulsifier. Improving the mechanical properties of the fiber mats was achieved by preparing polycaprolactone (PCL)/polylactic acid (PLA) blend nanofiber nonwovens.

Several properties of the polymer solution and process parameters have been adjusted in order to control the final fiber characteristics and improve the activity of immobilized trypsin. The selected conditions allowed obtaining well defined PCL nanofibers (average diameter of 150 nm) with reduced bead fibers, where the entrapped trypsin retained aprox. 35% activity, whereas in the PCL/PLA nanofiber mats (average diameter of 475 nm) the enzyme retained aprox. 55% activity, with small lost of activity during reuse and good stability during storage.

**Acknowledgements:** The present study was developed under the scope of the NanoBioCats project (PTDC/CTM–POL/112289/2009). Thanks are due to Fundação para a Ciência e a Tecnologia (FCT), European Union, QREN, FEDER and COMPETE for funding this project and also the QOPNA Research Unit (project PEst–C/UI0062/2013:FCOMP–01–0124–FEDER–037296).

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## Bioprocess engineering

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### SYNTHESIS OF THREE TARGET PEPTIDES FROM STEAP1 IN PROKARYOTIC SYSTEMS FOR ONCOLOGIC DIAGNOSE

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As we all know, antibodies are very specific to their antigens. However, the affinity properties of the binding between antibody and antigen are not fully clear. In the intent of study the affinity of an antibody to the six transmembrane epithelial antigen of prostate (STEAP), our research group produced three fragment of this protein in the form of peptides. To do that, three different DNA regions located in the extra, trans and intra membrane space were chosen, and further introduced into a plasmid. The transformed *Escherichia coli* strain XL1B was then grown in different media (LB Broth, Terrific Broth, Super Optimal Broth and a semi defined media) at 37°C, 250rpm for approximately 8h, followed by a freeze/thaw lysis that results in a convenient moderate expression. Possible due to their low molecular weights, and to their affinity properties, the products produced appear in the form of inclusion bodies (IBs), presenting itself as a major challenge to the performance of the purification step by an immobilized metal affinity chromatography (IMAC) even with the introduced His tag. To overcome this problem and maximize the recovery values different adjustments and combinations in the lysis, binding and elution buffers used in the process were made, and granted us with interesting and promising results.

## Bioprocess engineering

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### USE OF OREGANO (*ORIGANUM VULGARE*) WASTE AS A SUPPORT IN FUNGAL TANNASE PRODUCTION

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Tannin Acyl hydrolase (TAH) catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid. Extracts with tannase activity are actually used in the hydrolysable tannins monomer obtaining, such as gallic acid and ellagic acid, which are employed as powerful antioxidants in the pharmaceutical and food industries. Tannase (TAH) could be induced in the microorganism on the correct substrate. Oregano is a tannin rich material that could be employed as substrate for the tannase enzyme induction. The aim of this study was to evaluate the tannase production by *Aspergillus niger* LR using oregano (*Origanum vulgare*) waste as support and substrate in solid state fermentation (SSF).

The strain *A. niger* (DCTA-UAAAN collection) was used for the enzyme production. The oregano was recollected from the essential oils processing and previously treated as inert support [1]. SSF was realized with oregano waste, minimum Czapek-Dox media, molasses and tannic acid. Fermentation process was monitored every 24 h during 6 days. For the enzymatic extraction 40 mL sodium acetate 50 mM buffer solution was used (pH 6.0). Tannase activity determination was evaluated through Rhodanine method [2]. One unit of tannase activity was defined as the amount of enzyme required to release one  $\mu\text{mol}$  of gallic acid per minute, under assay conditions.

Higher tannase activity levels were at 120 h with an enzymatic concentration of 351 U/gds. Fermentation analysis show three phases were at 24–48 h appears the first high value of tannase activity (303 U/gds). The second stage is when the tannase activity decrease in the 72–96 h, probably due the complexing with proteins and precipitation. The third and last stage shows an increase in the enzyme levels to highest values, which could be related to the high biomass content.

Oregano waste could be a novel source for tannase and gallic acid production, due its polyphenols content, giving an added value to such residue.

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## Bioprocess engineering

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### USE OF *WITHANIA SOMNIFERA* EXTRACTS FOR GREEN SYNTHESIS OF SILVER NANOPARTICLES WITH ANTIMICROBIAL ACTIVITY

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In the present study, we report on green synthesis of silver nanoparticles (AgNPs) using *Withania somnifera* aqueous leaf extracts as reducing agent and their characterization. The formation, size and shape of green synthesized AgNPs were confirmed by physical-chemical techniques such as UV-Visible spectroscopy, laser Doppler anemometry, Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), X-ray diffraction (XRD) and X-ray energy dispersive spectroscopy (EDX). The SEM and AFM images confirmed that the size of synthesised AgNPs ranged between 70-110 nm, mainly spherical and some in hexagonal shape. AgNPs exhibited significantly higher antibacterial activity (up to 200x) against human as well as plant pathogens (*Escherichia coli*, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*) compared to AgNO<sub>3</sub> solution and *W. somnifera* leaf extract. The cellular interaction study coupled with SEM analysis revealed the effective uptake of AgNPs by the bacteria. Phytochemical studies demonstrate the relevance of *W. somnifera* phenolics and withanolides for the green synthesis of AgNPs.

## Bioprocess engineering

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### PURIFICATION OF IMMUNOGLOBULIN Y (IGY) USING AQUEOUS BIPHASIC SYSTEMS

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With the emergence of antibiotic-resistant microorganisms, diseases that are unresponsive to drug therapy, and the appearance of individuals that are unable to respond to conventional vaccines, the development of antigen-specific antibodies for use in passive immunotherapy is, nowadays, a major concern in human society. Despite the most focused mammal antibodies, antibodies obtained from egg yolk of immunized hens, immunoglobulin Y (IgY), are an alternative option that can be obtained in higher titres by non-stressful and non-invasive methods. This large amount of available antibodies opens the door for a new kind of cheaper biopharmaceuticals. Nevertheless, the production cost of high-quality and/or high-purity IgY still remains higher than other drug therapies due to the lack of an efficient purification method. Therefore, in this work, two types of aqueous biphasic systems (ABS) were studied constituted by polymers and Na<sub>2</sub>SO<sub>4</sub> and polymers, Na<sub>2</sub>SO<sub>4</sub> and ionic liquids (ILs) as adjuvants, as an alternative technique for the selective extraction, and thus purification, of IgY from egg yolk. According to the obtained results, a cost-effective platform using ATPS for the purification of the value-added IgY from egg yolk can be developed. The results obtained reveal that ATPS using ILs as adjuvants allow the selective extraction of  $\beta$ -livetin (the major contaminant) for one phase while retaining IgY in the opposite layer.

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## Downstream processing

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### BIORECOGNITION STUDY OF OLIGONUCLEOTIDES AND PLASMID DNA TO L-METHIONINE CHROMATOGRAPHIC SUPPORT

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Amino acid-based affinity chromatography appears as a promising approach to purify supercoiled (sc) plasmid DNA (pDNA) since it combines the selectivity of naturally occurring interactions with the simplicity of a small ligand. Therefore, the present study explores the effect of oligonucleotides composition on the mechanism of retention to L-methionine support by chromatography and saturation transfer difference (STD)-nuclear magnetic resonance (NMR) techniques. Surface plasmon resonance (SPR) experiments are also performed to evaluate the binding equilibrium of the oligonucleotides to L-methionine immobilized on the amine surface. The retention behaviour of linear pDNA with different sizes and base composition (2.7-kbp pUC19, 6.05-kbp pVAX1-LacZ, 7.4-kbp pVAX1-LacZgag and 14-kbp pcDNA-based plasmid) was also evaluated. The STD-NMR results show that thymine led to more contacts with the support. These results are according with binding profiles obtained by chromatography, since polyT had the highest retention time, followed by polyC, polyA and polyG. In general, the larger homo-oligonucleotides (poly30) are more retained to the L-methionine support. All chromatographic experiments were performed using 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, indicating the preferential involvement of hydrophobic interactions. From SPR-biosensor, cytosine is the base that shows highest affinity, followed by thymine and adenine, while the lowest affinity is found for guanine. The results show higher affinity with an increase in the number of nucleotides. The affinity order for hetero-oligonucleotides is CCCTTT > CCCAAA » AAATTT > GGGTTT, showing that oligonucleotides with cytosine have the highest affinity and the presence of guanine reduces the affinity with the L-methionine immobilized. Finally, comparing the retention time of linear pDNA, the smaller plasmid, pUC19, was more retained than the pVAX1-LacZ and the larger pcDNA-based plasmid, which in turn were more retained than the pVAX1-LacZgag. These results indicate that the underlying mechanism involves not only hydrophobic interactions, but also other biorecognition interactions between L-methionine ligands and pDNA molecules. Combined STD-NMR and SPR biosensor techniques allied with chromatography was successfully used to screen amino acid affinity support-nucleotide interactions, providing further insights into sc pDNA purification for therapeutic applications.

## Downstream processing

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### CENTRAL COMPOSITE DESIGN AS AN EFFICIENT TOOL IN THE COMPOSITION OF CHROMATOGRAPHIC MATRICES

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To improve the purification efficiency and in order to decrease the use of resources in chromatographic processes the development of new matrices is increasingly important. To accomplish this aim, different types of materials are used in the preparation of chromatographic matrices namely natural or synthetic polymers. Thus, gellan gum is a natural anionic polysaccharide with potential to be a chromatographic matrix, since it shares some properties with common chromatographic stationary phases. These properties are its porosity and hydrophilicity, and since this polymer is negatively charged it enables its interaction with specific biomolecules in anionic exchange conditions. So, with the present work it is intended to take advantage of these properties of gellan in order to prepare an anionic chromatographic gel. To find the best gel formulation to be applied in the chromatographic assays stability experiments were made. In order to facilitate the optimization of this process, experimental design tool was applied, allowing a better understanding also the relative importance of all the components of the matrix. Subsequently, to test the applicability of the matrix optimized before chromatographic assays with three model proteins were made (bovine serum albumin (BSA),  $\alpha$ -chymotrypsin and lysozyme). The results showed that the retention occurred in function of the net charge of each protein in buffer pH 6.2 and the elution was performed by increasing the ionic strength. Finally, to better characterize and to compare this matrix with commercial resins, its dynamic binding capacity was studied. The obtained values for the gellan stationary phase were 3.9 mg/mL and 17.4 mg/mL, at 10% and 50% of breakthrough, respectively. Concluding, this research work shows that gellan gum is a promissory chromatographic matrix, exploring ionic interactions, being applied in different purification strategies and getting the best benefit from its use at low cost, since this polymer may be produced bacterially.



## Downstream processing

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### INTEGRATION OF MEMBRANE TECHNOLOGY AND MONOLITHIC AFFINITY CHROMATOGRAPHY FOR SUPERCOILED PLASMID DNA PURIFICATION

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During the last years, research has been posted on the application of plasmid DNA (pDNA) on gene therapy. Therefore, the development of methods suitable for large scale preparation of highly pure pDNA is essential. Isopropanol and ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) precipitations are the most widely used techniques in the intermediate recovery of pDNA from alkaline lysates. However, some limitations related with high costs and environmental impact, have stimulated their replacement by other techniques. Over the last few decades, membrane technology has been investigated and used as a promising operation to isolate and clarify pDNA from alkaline lysates. Moreover, most of the industrial manufacturing employs at least a chromatographic step to recover a final product with the required purity degree. Monolithic chromatography results in an attractive option to conventional supports, due to the higher binding capacity and favorable hydrodynamic properties for large molecules, as pDNA. Thus, the purpose of this work intends to describe the integration of a membrane-based process with the monolithic chromatography to attain the sc pDNA isoform with high quality. With respect to the recovery step, filtration experiments were conducted using a hydrophilic nylon microfiltration membrane to achieve a solid-liquid separation of the lysate sample, as an alternative to centrifugation. The sample retrieved from membrane process was loaded onto a non-grafted monolithic disk of Carbonyldiimidazole to efficiently purify the pDNA. Chromatographic assays were carried out to select the best strategy to promote the separation of several nucleic acids. A stepwise gradient of ammonium sulfate was performed and the monolithic profile showed that open circular and genomic DNA (gDNA) were eluted at 2.70 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 50 mM phosphate buffer pH 8.0 and the sc pDNA isoform was isolated by decreasing the ionic strength to 1.71 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The RNA was eluted in a final washing step with 50 mM phosphate buffer. The chromatographic method demonstrated an efficient performance on supercoiled (sc) pDNA purity and recovery (100% and 84.44 %, respectively). The integrated process presents a great potential for industrial scale systems, aiming the sc plasmid purification as well as the reduction of RNA, gDNA and endotoxins levels, according to the limits recommended by the regulatory agencies.

## Downstream processing

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### MALTODEXTRINS AS ALTERNATIVE CONSTITUENTS TO FORM IONIC-LIQUID-BASED AQUEOUS BIPHASIC SYSTEMS

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Advances in biotechnology lead to a growing interest in biomolecules for application in a wide range of industrial fields. Due to its enhanced properties and functions, maltodextrin is applied in the paper, food and pharmaceutical industries. Nevertheless, it is in the food industry where the demand of maltodextrin is most notable. It is broadly used to improve the products stability by absorbing a large amount of oil or fats, but it can also be used as emulsifier, encapsulating agent and support for flavoring. Within this framework, it is important to study and develop new techniques to enable a cost-effective separation technique for maltodextrin, not only from its fermentation broth, but also considering the possibility of recovering it from industrial wastes. Ionic-liquid-based aqueous biphasic systems (IL-based ABS) have been the focus of intense research and development, revealing a great potential for the separation of biomolecules. [1]

This work investigates novel ABS composed of maltodextrin + IL + water, in which different phosphonium- and imidazolium-based compounds were studied. The influence of maltodextrin dextrose equivalent values was also evaluated. Ternary phase diagrams for each IL and maltodextrin combination were determined at 25°C. The obtained experimental data ascertained the potential to promote liquid-liquid demixing in aqueous medium by the combination of proper IL-maltodextrin pairs.

The use of carbohydrates to induce IL-based ABS can be regarded as a step towards profitable processes envisaging cleaner, cost-effective and more environment-friendly approaches. The systems investigated in this work can be seen as promising routes in the improvement of biotechnological processes which increasingly tend to be decisive in industry and require a more sustainable character.

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## Downstream processing

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### NEW INSIGHTS INTO RNA PURIFICATION: THE POTENTIAL OF AMINO ACIDS-BASED AFFINITY CHROMATOGRAPHY

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In recent years, RNA has been recognized as a central molecule in cellular processes with implications in many diseases, becoming one of the most exciting research areas. From basic to applied research, many procedures employ pure and intact RNA molecules. Moreover, RNA-based therapies involve RNA formulations that should fulfill rigorous quality criteria recommended by regulatory agencies. Therefore, there is a growing demand to develop new methodologies for RNA isolation.

Chromatography is undoubtedly one of the most diverse and potent methods in biotechnology due to its simplicity, robustness, versatility and high reproducibility. Affinity chromatography is recognized as a powerful technique with great applicability in purification of many biomolecules. The work that we have been developing considers new chromatographic strategies for RNA purification, exploiting affinity interactions between amino acids and nucleic acids. Histidine and arginine have been used as amino acid ligands, and their ability to isolate different RNA species proved the presence of specific interactions occurring between RNA and the amino acid matrices.

Hence, new approaches were accomplished allowing (i) obtaining RNA preparations from diverse sources with high integrity and purity: histidine matrix showed a specific recognition for 6S RNA, allowing its purification from a mixture of other *Escherichia coli* (*E.coli*) non-coding RNAs (ncRNA) [1]; in another strategy, the simultaneous isolation of ncRNA and ribosomal RNA from *E.coli* cell lysates, eliminating host DNA and proteins, was also attained by histidine chromatography [2]; and arginine matrix enabled the isolation of total RNA from impurities of an eukaryotic cell extract [3]; (ii) the analytical quantification and monitorization of RNA [4]. Accordingly, these methods demonstrate potential multipurpose applicability by aiding in molecular biology RNA-based analysis and RNA therapeutics.

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## Downstream processing

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### PRE-MIR-29 BIOSYNTHESIS AND PURIFICATION: POSSIBLE IMPLICATIONS IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is an incurable neuropathology that affects millions of people, posing a heavy economic and social burden. Recent studies demonstrated that the miR-29 is significantly decreased in AD patients displaying abnormally high levels of beta-amyloid precursor protein-converting enzyme 1 (BACE1). MicroRNA is arising as a new tool for gene silencing since it can act as powerful messenger RNA degrading molecules. Therefore, the requirement for the production of highly purified miRNAs and biologically active arises as one of the most important challenges in the development of therapeutic strategies. Thus, the strategy to produce recombinant pre-miR-29 starts by cloning the DNA sequence encoding for the pre-miR-29 into the pBHSR1-RM vector. Furthermore, the *Rhodovulum sulfidophilum* DSM 1374 bacterium was used as an alternative host for pre-miR-29 production. This host presents several advantages, such as differential secretion of nucleic acids during cell growth and absence of host RNases. Afterwards, a new affinity chromatographic method, by using an arginine-agarose support to specifically purify pre-miR-29 from a total RNA mixture, was developed by our research group. The purification was evaluated by using stepwise gradients of different sodium chloride and ammonium sulfate concentrations. The selectivity found for RNA molecules with this support suggests that the interaction is accomplished by a biologically-based recognition of the individual chemical structure. The successful isolation of pre-miR-29 by arginine affinity chromatography has a potential applicability for RNA structural and functional studies, which can provide nearly untapped opportunities on pharmaceutical applications. In the future, it will be assessed the efficiency of purified miRNA-29 in BACE1 knockdown, using *in vitro* neuronal cell lines and *in vivo* rat model of AD. Overall, the implementation of this cutting-edge approach provides the basis for the improvement of currently available methodologies of gene silencing as a putative genetic therapy for AD.

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## Downstream processing

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### SELECTIVE RECOVERY OF NATURAL RED COLORANTS FROM FERMENTED BROTH BY THE APPLICATION OF IONIC LIQUID-BASED AQUEOUS BIPHASIC SYSTEMS

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There is a growing demand for natural colorants. Natural colorants, widely used in the food industry, have been extracted from plants/animals, being nowadays its production by microorganisms considered a promising alternative route. This fact is motivating not only the optimization of the production step but also the search for more “environmentally friendly”, cost effective and efficient purification processes, representing higher recoveries, extraction yields and selectivity. This work studies the use of aqueous biphasic systems (ABS) based in ionic liquids as extraction systems for the recovery of red colorants from the fermented broth of *Penicillium purpurogenum* DPUA 1275. Several ABS based in quaternary ammonium and imidazolium were studied in this work aiming at separating the red colorants produced from the remaining colorants and contaminant proteins present in the fermented broth. The results suggest that the red colorants can be properly isolated by an appropriate manipulation of some of the process conditions, such as the use of quaternary ammonium with short alkyl chains, alkaline media and short tie-line lengths (extraction point systems with lower concentrations of ionic liquid). Thus, the ability of ammonium and imidazolium salts to purify the red colorant from the fermentation broth is demonstrated and improved selectivity parameters were achieved.

## Downstream processing

### P299

## UNDERSTANDING AFFINITY CHROMATOGRAPHY LIGANDS–NUCLEIC ACIDS INTERACTIONS

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Current processes of purification of plasmid DNA (pDNA) used affinity chromatography for their purification based on molecular recognition of pDNA and the ligand [1]. High selectivity makes this technique capable of achieving purification in a single process. The affinity purification mechanism employs a stationary immobilized amino acid ligand into a matrix and a mobile phase containing the target biomolecule that favors its specific binding to the ligand. Thereby the development of an affinity chromatography process is determined by the molecular interactions involved and the achievement of the experimental conditions [1].

Rapid and efficient screening methods have been proposed using surface plasmon resonance (SPR)–biosensor and saturation transfer difference–nuclear magnetic resonance (STD–NMR) spectroscopy to yield information about the specificity of the binding process between the amino acid affinity chromatography supports, used by our group, and nucleotide/pDNA sequences, manipulating experimental conditions like temperature, pH and salt [2–4].

For SPR, we present a strategy that immobilizes the amino acids (L–arginine, L–lysine and L–histidine) on a surface as model supports, and we analyze binding responses when nucleotides and pDNA isoforms (pGL101, pUC19 and pVAX1–LacZ, 2.39, 2.69 and 6.05 kbp respectively) are injected over the amino acid surface. The binding responses are detectable and reproducible, despite the small size of the immobilized amino acids. For example: to arginine surface, pGL101 shows the highest binding followed by pVAX1–LacZ, while pUC19 shows the lowest. For the three plasmid isoforms, the supercoiled ones have the higher binding affinity [4]. Different buffer environments affect the interaction strength with an increase in response for Tris–HCl and a marked decrease for high salt concentrations [4].

Using STD–NMR, we performed epitope mapping of the nucleotides bound to L–arginine–bisoxymethyl–Sepharose, L–lysine–Sepharose and L–histidine–Sepharose supports. Polynucleotide binding preferences differed; for example, polyC interacted preferentially through its backbone with the two supports, whereas polyT bound the supports through its thymine moiety [2,3].

Hence, the proposed methods are a novel and useful way to provide binding information about amino acid–nucleotide interactions and affinity parameters of this system.

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## Downstream processing

### P300

## VALORIZATION OF PHARMACEUTICAL WASTES THROUGH THE RECOVERY OF ADDED-VALUE DRUGS

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The environmental fate of active pharmaceutical ingredients has raised the society concern during the past decades. Having in consideration the principles of the Green Chemistry and Sustainability, this work aims at applying aqueous biphasic systems (ABS) to the recovery of valuable active pharmaceutical ingredients from the pharmaceutical wastes. The phase diagrams of ABS composed of six nontoxic and biocompatible quaternary ammonium halides ( $[N_{2,2,2,2}]Br$ ,  $[N_{2,2,2,2}]Cl$ ,  $[N_{3,3,3,3}]Br$ ,  $[N_{3,3,3,3}]Cl$ ,  $[N_{4,4,4,4}]Br$  and  $[N_{4,4,4,4}]Cl$ ) and three different salts (potassium citrate buffer, potassium carbonate and potassium phosphate buffer) were established at 298 ( $\pm 1$ ) K. These systems allow the investigation of the influence of the ammonium structure, the salting-out agent, and the pH of the aqueous medium on the ABS formation. The systems were then applied to the optimization study of the extraction of paracetamol, the model molecule adopted. Paracetamol shows an extensive partition towards the top (ammonium-rich) phase employing either the commercial pure compound or the solid state waste. Notably, extraction efficiencies, ranging from around 80 up to 100%, are achieved. This work reveals the potential capacity of ammonium-based ABS to extract an active compound from a pharmaceutical waste, opening a new window to the management, reduction and valorization of pharmaceutical wastes.

## Downstream processing

### P301/F22

## ENTHALPY CONTRIBUTIONS TO LYSOZYME ADSORPTION ONTO A CATION-EXCHANGE SUPPORT: EFFECT OF PH UNDER LINEAR AND OVERLOADED CONDITIONS

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The greatest challenge of any chromatographic technique, including ion-exchange chromatography (IEC), is predicting the adsorptive behaviour of biomolecules onto the chromatography resin. This investigation attempts to examine the complexity of protein adsorption onto an ion-exchanger and the role of non-specific effects in the establishment of the adsorptive process. It has been demonstrated that thermal events accompanying biomolecule adsorption can shed some light to the underlying mechanism. We have confirmed that flow microcalorimetry (FMC) can be used to dynamically measure the heat of biomolecule adsorption, providing invaluable insights to the biomolecule adsorption process. So, flow microcalorimetry and adsorption isotherms measurements were used to illustrate lysozyme adsorption mechanism on carboxymethyl cellulose (CMC) in absence and presence of salt (NaCl 50mM) at both pH 5 and 8.

FMC results show that under all the studied conditions the adsorptive process is, as expected in ion exchange, enthalpy driven. Direct correlation between microcalorimetry data and isotherm measurements is observed. Furthermore, FMC data show different signal profile for each pH suggesting a different lysozyme adsorption mechanism. Adsorption behaviour under linear and overloaded conditions was also analysed.

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## Downstream processing

### P302/F02

## IONIC-LIQUID-BASED AQUEOUS BIPHASIC SYSTEMS: AN ALTERNATIVE APPROACH FOR THE SEPARATION OF EXTRACELLULAR POLYSACCHARIDES

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Aqueous biphasic systems (ABS) have been intensively studied in the context of finding more benign and efficient techniques for the separation and purification of a wide variety of biomolecules. In the past few years, the interest in ionic-liquid-based ABS has exponentially raised due to their advantages over the traditional ABS composed of polymer-polymer or polymer-salt. Previous studies have shown that ionic liquids combined with aqueous solutions of inorganic/organic salts, saccharides, amino acids and polymers were able to form ABS [1]. These systems are of low viscosity and allow a proper tailoring of the phases' polarities for the extraction of a target biomolecule [1].

The present work is focused on the search of novel ionic-liquid-based ABS to separate extracellular polysaccharides (EPS) by more efficient, cost effective and environmental friendly approaches. EPS are produced in fermentation broths and their separation from this complex matrix is a main challenge aiming at decreasing their commercial cost due to the increasing demands by pharmaceutical and food industries. Thus, novel ABS composed of ionic liquids and dextran are here proposed. The ternary phase diagrams for several ionic liquids and dextran of different molecular weights were determined at 25°C. The effect of the ionic liquids molecular structure was investigated towards their ability to form ABS and to separate and concentrate dextran in a second liquid phase. Moreover, more complex matrices were evaluated and the selective separation of dextran in the presence of sucrose, the initial carbon source, was also addressed.

The novel systems here presented should be regarded as alternative routes for sustainable biotechnological processes which tend to become of high industrial relevance.

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## Renewables, biofuels and bioenergy

P303

### BACTERIAL TRIACYLGLYCERIDES FOR BIODIESEL PRODUCTION

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The concern about the dependence on petroleum supplies and the negative environmental consequences of the current energetic model has stimulated the development of renewable alternatives to fossil-fuels. Biodiesel, an ideal substitute for petroleum-based fuel consisting of fatty acid methyl esters (FAMES) and fatty acid ethyl esters (FAEEs) can be mixed at any ratio with pure diesel to run compression engines (Peralta-Yahya et al., 2010). Biodiesel can be obtained by chemical transesterification of triacylglycerides (TAGs) with methanol or ethanol. Nonetheless the limited supply of bioresources to obtain TAGs represents a great bottleneck for its production (Duan et al., 2011). First generation biofuels are usually made from vegetable oil feedstocks that are also food sources, which is both an ethical and economically controversial issue. As a solution for that inconvenience the second generation biofuels have emerged. Oleogenic microorganisms (capable of accumulating more than 70% of the total cellular dry weight as TAGs, as Wältermann reported in 2005) might be employed to obtain single cell oil (SCO). However, its high generation time and difficulty to be genetically engineered lead to research in other microorganisms as *Escherichia coli* as industrial host for large-scale TAGs production. In the work presented here, a codon-optimized DNA sequence from the oleogenic actinomicete *Thermonospora curvata* codifying for an enzyme responsible for the creation and accumulation of TAGs in prokaryotes, the wax ester synthase/triacylglycerol:acilCoA acyltransferase (WS/DGAT), was introduced in an *Escherichia coli* strain. Thin Layer Chromatography (TLC) analysis of the lipid fraction from the resulting strain showed a significant amount of TAG accumulation. This phenotypic characteristic was directly related to the induction of the heterologous expression of the protein, as seen in SDS polyacrylamide gel electrophoresis. The lipid droplets accumulated in the cells were observed under a fluorescence microscope by dying with Red Nile. Ultra High Pressure Liquid Chromatography/ Mass Spectrometry (UPLC/MS) and Gas Chromatography (GC) analysis confirmed that the TAGs accumulated were constituted of the main free fatty acids present in the wild type *E.coli* strain. The transformation of a microorganism widely used in industry into a producer of value-added oil means a crucial step toward the production of biodiesel in a near future.

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## Renewables, biofuels and bioenergy

## P304

## BIOETHANOL PRODUCTION FROM HARDWOOD SPENT SULPHITE LIQUOR (HSSL) USING SCHEFFERSOMYCES STIPITIS – DEALING WITH HSSL TOXICITY PROBLEMS

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Presently, the production of added-value products from cheap resources, such as industrial by-products and wastes, is attracting attention and industrial interest. Hardwood Spent Sulphite Liquor (HSSL) is the by-product of the acidic sulphite pulping of hardwood. Despite its great potential, this by-product from paper manufacture industry is normally burned for chemical and energy recovery. HSSL is rich in sugars, mainly xylose. *Scheffersomyces stipitis*, native xylose-metabolizing yeast, can convert such pentose and other carbohydrates to ethanol. Nevertheless, HSSL contains toxic compounds (e.g. acetic acid and phenolics), which can inhibit yeast growth. Adaptation of microorganisms to inhibitory compounds is a possible approach for dealing with toxicity problems.

Therefore, the main objective of the present work was to investigate the influence of two different parameters, (1) yeast adaptation to HSSL and (2) yeast pre-cultivation on HSSL, on the fermentation performance of *S. stipitis* using HSSL.

A continuous reactor with increasing HSSL concentrations (20–60 % (v/v)) was run for 68 days, and the final population of *S. stipitis* (adapted population) was collected. Yeast pre-cultivation was carried out on either 60 % HSSL or 100 % chemically-defined media (CDM). To study the influence of the above referred parameters, three batch reactor assays with 60 % HSSL were performed under different conditions. Along each assay, biomass and metabolite concentrations were analyzed.

During the fermentation process with the adapted population pre-cultivated on HSSL (control), a maximum ethanol concentration of 2.92 g L<sup>-1</sup> was obtained with an uptake rate of xylose of 0.27 g L<sup>-1</sup> h<sup>-1</sup>. Lower xylose consumption was attained (0.14 g L<sup>-1</sup> h<sup>-1</sup>) using the non-adapted population pre-cultivated on HSSL. Higher maximum ethanol concentration (6.93 g L<sup>-1</sup>) was achieved with the adapted population pre-cultivated on CDM.

Experimental results suggest that adaptation and pre-cultivation on HSSL may improve xylose consumption and ethanol production, respectively. In short, these are good strategies to improve the fermentation performance of *S. stipitis* using HSSL.

## Renewables, biofuels and bioenergy

## P305

BIOLOGICAL TREATMENT OF HSSL (HARDWOOD SPENT SULPHITE LIQUOR) WITH THE FUNGUS *PAECILOMYCES VARIOTII*T.M. Henriques<sup>1</sup>; S.R. Pereira<sup>1</sup>; L.S. Serafim<sup>1</sup>; A.M.R.B. Xavier<sup>1</sup><sup>1</sup>CICECO, University of Aveiro, Chemistry Department, Aveiro, Portugal

Hardwood Spent Sulphite Liquor (HSSL) is a by-product from acidic sulphite pulping of wood, rich in pentoses, which can be fermented by the yeast *Scheffersomyces stipitis* to produce second generation bioethanol. Unlike *Saccharomyces cerevisiae*, *S. stipitis* had the advantage of fermenting both hexoses and pentoses. However, *S. stipitis* is quite sensitive to inhibitors present in HSSL, namely acetic acid. For this reason, before sugars fermentation to ethanol a prior step of HSSL detoxification is required. This detoxification can be performed by *Paecilomyces variotii*, a filamentous fungus able to use some of the toxic compounds found in HSSL as carbon source. Furthermore, the biomass of *P. variotii* can be also used in animal feed as single cell protein. This work aimed to investigate the ability of ethanol production by yeast *S. stipitis* in defined medium containing HSSL with and without detoxification by *P. variotii*. Firstly, HSSL was subjected to chemical pre-treatment and then detoxified with *P. variotii*. The detoxification step was performed in a sequential batch reactor with three cycles. Two assays were performed, one with an inoculum of 70 mg of biomass/L and another one with an inoculum of 110 mg of biomass/L. Then, bioethanol production by *S. stipitis* was studied in four assays: one with defined Verduyn medium; another one with HSSL without detoxification; another one with detoxified HSSL with sugar supplementation; and one other with detoxified HSSL without sugar supplementation. *P. variotii* proved to be able to detoxify HSSL with 17 g/L of acetic acid without consuming xylose. It was also observed that although it was possible to speed up the uptake of acetic acid by increasing the initial concentration of inocula, the increase in the acetic acid uptake rate was not very significant. As regards to the *S. stipitis* assays while in the assay with Verduyn medium the high fermentative efficiency of this yeast was shown, in assay with HSSL without detoxification the presence of inhibitory compounds was demonstrated. Finally, in the assays with detoxified HSSL with and without sugar supplementation yields of ethanol/substrate of 0.30 and 0.31 g/g were obtained, respectively. This showed that the initial sugar concentration did not affect the yield. The ethanol production success indicates that the *P. variotii* has efficiently consumed the inhibitory compounds of the HSSL.

## Renewables, biofuels and bioenergy

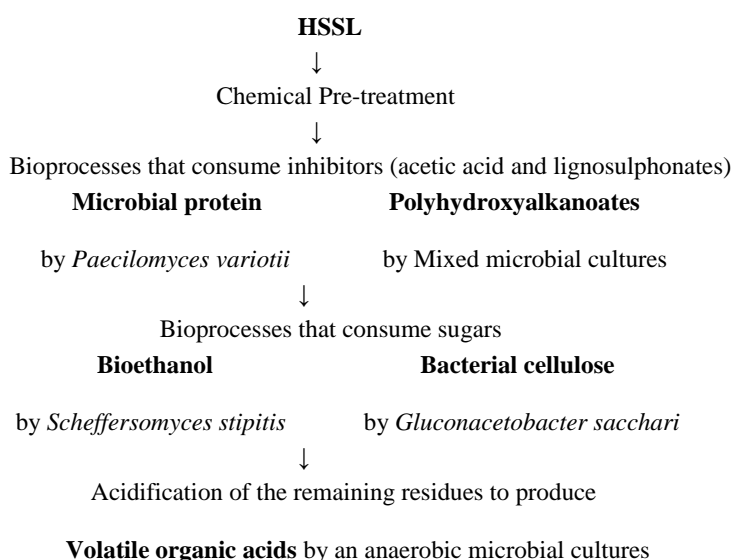
P306

### CELLULOSIC BASED BIOREFINERY FOR PULP AND PAPER INDUSTRY VALORIZATION

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A biorefinery concept which derived from traditional petroleum refineries is defined as a complex which integrates various biomass processing units by biological or chemical techniques to obtain different products that include chemicals, fuels or materials [1]. Hardwood Spent Sulphite Liquor (HSSL) is a by-product from the acidic sulphite pulping processes from Pulp and Paper Industry, rich in monomeric sugars and produced in high volumes that can be converted to high value products [2]. The integration of extractive, thermochemical and biochemical processes in a biorefinery should contribute to increase the specific productivity, flexibility and efficiency of industrial processes. This work proposes the use of a byproduct of the paper industry, HSSL, as a substrate for the integration of different biological processes according to the biorefinery concept. The processes described in this work, in addition to ethanol [3] and microbial protein, also allow to obtain biopolymers as polyhydroxyalkanoates [4] or bacterial cellulose [5] and volatile organic acids [6].



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## Renewables, biofuels and bioenergy

P307

EFFECT OF CONCENTRATION OF CARBON SOURCE IN BIOETHANOL PRODUCTION BY *SCHEFFERSOMYCES STIPITIS*Maria Dolores D. R. F. Moreira<sup>1</sup>; Ana Jorge Tróia<sup>2</sup>; Susana R. Pereira<sup>2</sup>; Inês Portugal<sup>2</sup>; Luísa S.Serafim<sup>2</sup>; Ana M. R. B. Xavier<sup>2</sup><sup>1</sup>*Instituto Superior de Engenharia de Coimbra, Instituto Politécnico de Coimbra;*<sup>2</sup>*Department of Chemistry & CICECO, University of Aveiro, 3810-193 Aveiro, Portugal*

Hardwood Spent Sulphite Liquor (HSSL) is a by-product from pulp and paper industry. It is rich in monosacharides being a suitable raw material for the production of 2nd generation bioethanol. However, it also has compounds which may have an inhibitory effect on some microorganisms. In this way, the aim of this work was to study the concentration of the carbon source in the production of bioethanol using the yeast *Scheffersomyces stipitis*.

In order to study the influence of substrate composition on the production of bioethanol, eight fermentations were initially performed. The xylose and glucose concentrations varied between 10, 20, 30 and 40 g/L, and 0 and 5 g/L, respectively. The acetic acid concentration was maintained at 4 g/L. The assays with 40 g/L of xylose were the ones with the higher fermentation parameters: ethanol concentration of 17,3 g/L, maximum ethanol productivity of 0,477 g/L.h and the ethanol yield of 0,390 g/g. The highest specific growth rate was obtained in a 30 g/L xylose assay, 0,124h<sup>-1</sup>.

In the second part of this work three fermentations took place with 30 g/L of xylose, 5 g/L of glucose, and the acetic acid concentration varied between 6, 8 and 10 g/L. It was found that increased concentration of this compound has a negative effect in the specific growth rate, in ethanol productivity, ethanol yield and concentration and mostly in the lag phase period. In these assays the highest ethanol concentration was 10,6 g/L, with a maximum ethanol productivity of 0,196 g/L.h and an ethanol yield of 0,259 g/g.

Finally, two fermentations were performed. The first one with 60% not detoxified HSSL and the second one with synthetic solutions in order to maintain the xylose (34g/L), glucose (5g/L), and acetic acid (16g/L) concentrations in HSSL. In both assays it was observed a drastic decline in all studied parameters. The maximum ethanol concentration was 5,52 g/L in the synthetic medium assay and 3,32 g/L in the 60% HSSL assay. Besides *Scheffersomyces stipitis* being able to grow in the 60% not detoxified HSSL assay, it has shown that the direct fermentation of non-detoxified HSSL did not provide satisfactory results, urging its detoxification to an acetic acid concentration below 6 g/L.

## Renewables, biofuels and bioenergy

P308

### ENGINEERING YEAST TOLERANCE TO INHIBITORY LIGNOCELLULOSIC BIOMASS

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In recent years the necessity for biotechnological manufacturing based on lignocellulosic feedstocks has become evident. However, the pre-treatment step in the production of lignocellulosic bioethanol leads to the accumulation of inhibitory byproducts. Robust second generation bioethanol processes require microorganisms able to ferment these inhibitory lignocellulosic hydrolysates. Significant progress has been made in the understanding of the determinants of yeast tolerance to lignocellulose biomass-derived inhibitors, however further knowledge at the genetic level is of essential importance for the improvement of lignocellulose conversion technology. Based on genome-wide results previously obtained [1], two key genes, *PRS3* and *RPB4*, were found to contribute to the maintenance of cell viability in wheat straw hydrolysate and to the maximal fermentation rate of this substrate. Here we describe the outcome, in bioethanol productivity, of fermentations in *Eucalyptus globulus* wood hydrolysate, using recombinant *Saccharomyces cerevisiae* BY4741 overexpressing these genes. Furthermore, we studied their expression in an industrial strain isolated from a Brazilian bioethanol production plant, which was previously demonstrated to have very robust characteristics with outstanding fermentation performances [2]. This expression evaluation was performed during a fermentation mimicking industrial conditions, under the absence and presence of inhibitory compounds, and provides insights into the roles of *PRS3* and *RPB4* in the adaptation to toxic biomass hydrolysates. This study expands our understanding of the underlying molecular mechanisms involved in yeast response to the multiple stresses occurring during lignocellulose fermentations under industrially relevant conditions.

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## Renewables, biofuels and bioenergy

### P309

#### ENZYMATIC CONVERSION OF ANIMAL FAT INTO BIODIESEL

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Biodiesel is receiving increasing attention as a clean and renewable energy. It is commonly produced by methanolysis of edible vegetable oils via base-catalyzed transesterification creating a competition with food supply. Also, the current price of biodiesel is still high due to the high cost of the feedstocks. However, raw-materials with high free fatty acid (FFA) content such as waste grease from cattle are discarded in large amounts, being therefore an attractive feedstock to envisage for biodiesel production. Their use could decrease the production cost and simultaneously allow avoiding a disposal problem.

Nevertheless, the traditional alkaline catalysis of high FFA raw-materials promotes the formation of soaps thus reducing the process yield and increasing technical difficulties. To solve this problem, fat may be pre-treated via acid esterification of the FFA, followed by base transesterification of the glycerides. As homogeneous acid catalysts' use present chemical and environmental problems, heterogeneous catalysts may be a better and greener alternative. The choice in this work was the use of an immobilized enzyme able to catalyze both esterification and transesterification reactions and produce fatty acid methyl esters (FAME) from high FFA content fat.

The purpose was then to evaluate the efficiency of Lipozyme TL IM in producing FAME from different samples of cattle fat, poultry fat and olive pomace oil with acid values ranging from 5–65mg<sub>KOH</sub>/g. The process was optimized through a Central Composite Design studying the effects of the amount of catalyst, water content and methanol to fat molar ratio for 15 h at 30°C and using poultry fat. The optimal conditions for the conversion were molar ratio=5.2, Lipozyme amount=7.3% (m/m) and water amount=15.4% (m/m). These reaction conditions applied to other raw-materials allowed verifying that this enzyme works better for feedstocks with lower FFA content (Table 1).

Table 1 – Initial acid value of raw materials and esters content after enzymatic assays.

	t(h)	Poultry fat	Olive Pomace oil	Cattle fat	
				1	2
Acid Value (mg <sub>KOH</sub> /g)	–	15.4	14.3	5.2	65.2
FAME (% m/m)	15	80.6	86.0	82.1	53.6
	20	90.3	83.4	92.5	58.2

With poultry fat, the direct reutilization of Lipozyme resulted in an activity reduction of 5% in the 2<sup>nd</sup> cycle and 40% in the third.

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## Renewables, biofuels and bioenergy

## P310

MATURATION OF TWO DIFFERENT NI-Fe HYDROGENASES IN THE *CYANOBACTERIUM ANABAENA* SP. PCC 7120: A MULTIDISCIPLINARY STUDY

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The genome of *Anabaena* sp. PCC 7120 contains information coding for structural subunits of two types of Ni-Fe hydrogenases: an uptake (HupSL) and a bidirectional (HoxEFUYH) hydrogenase. Extensive physiological data has been collected over the years demonstrating the presence and activity of both enzymes. According to the *Escherichia coli* model of Ni-Fe hydrogenase maturation and assembly, several accessory proteins are necessary to convert the hydrogenase apoprotein into an active enzyme. During this process, the Ni-Fe active center and respective ligands are properly inserted into the hydrogenase large subunit. The final maturation step consists of a proteolytic cleavage at the C-terminus of the hydrogenase large subunit, elicited by an endopeptidase that has the ability to bind to nickel. This step can be considered a maturation checkpoint, where only hydrogenases possessing nickel and iron atoms in their active centers will be recognized by the endopeptidase and further cleaved, securing the proper assembly and functionality of the hydrogenase; thus, hydrogenase apoproteins lacking nickel will not be cleaved. Based on sequence homology, two putative endopeptidases have been suggested to be involved in the maturation of HupSL and HoxEFUYH in *Anabaena* sp. PCC 7120, namely HupW and HoxW. Recent data suggests that HupW specifically cleaves HupL [1]. However, some questions remain unanswered: is HoxH specifically cleaved by HoxW, similarly to what is described in *Synechocystis* sp. PCC 6803 [2], or can its maturation be completed by another peptidase? Does the transcription of hydrogenase structural genes follow the same expression pattern as the putative endopeptidases, and are their relative expression levels comparable? Is the transcription localized or is it equally spread throughout the filament? Can HupW and HoxW have additional targets? In order to clarify these questions and gain a better insight into the hydrogen metabolism of *Anabaena* sp. PCC 7120 we have started by constructing mutants lacking *hoxW* and *hupW/hoxW*. Their phenotypes are being evaluated in relation to the wild-type as well as to a strain lacking *hypF* (generated in our laboratory), which is involved in earlier stages of hydrogenase maturation. Quantitative gene expression is currently being performed by RT-qPCR using RNA extracted from cells grown under different conditions, assessing expression not only of hydrogenase putative proteases genes, but also of hydrogenase structural genes and a number of transcription factors. Localization of gene expression is being studied using promoter-GFP fusions. Western blot analyses, making use of newly developed antibodies raised against both hydrogenases, will aid evaluating the specificity of each endopeptidase. Our most recent results will thus be presented and discussed.

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## Renewables, biofuels and bioenergy

### P311

#### OPENING NEW PERSPECTIVES FOR BIOREMEDIATION AND BIOFUEL PRODUCTION: FUNCTIONAL CHARACTERIZATION OF PCCH A KEY CYTOCHROME FROM *G. SULFURREDUCTENS*

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The bacterium *Geobacter sulfurreducens* (*Gs*) is capable of anaerobic oxidation of a large variety of compounds and transfer electrons directly to metal surfaces even in the absence of soluble mediators. *Gs* cells can also reduce toxic and/or radioactive metals and grow on electrode surfaces converting renewable biomass into electricity [1]. The bacteria can also accept electrons from electrodes, in current-consuming biofilms [2]. In this process, the reducing power provided by an electrode, maintained at a sufficient negative electrochemical potential, can be used by the cells to synthesize valuable organic compounds, thus opening new perspectives in the field of bioremediation and biofuel production. To develop these applications, it is essential to understand the mechanisms by which microorganisms can accept electrons from electrodes. Recent gene knockout studies have demonstrated that deletion of the gene coding for cytochrome PccH completely inhibited electron transfer from electrodes in current-consuming fumarate-reducing biofilms [3]. In this work, the *pccH* gene was cloned and the protein heterologously expressed in *Escherichia coli*. Several techniques including CD, UV-visible and NMR spectroscopy were used to characterize PccH. This protein is a mono-heme *c*-type cytochrome containing a low-spin heme group with His-Met axial coordination. The redox potential values of PccH, obtained by visible spectroscopy in the physiological pH range for *Gs* growth, are unusually low compared to other cytochromes with His-Met axial coordination. Such unusual features might explain its natural selection as a crucial protein in current-consuming biofilms and can be explored to optimize biotechnological applications in the bioremediation and biofuel production fields.

Acknowledgments: PTDC/BBB-BEP/0753/2012 and SFRH/BD/89701/2012 from FCT (Portugal).

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## Renewables, biofuels and bioenergy

### P312

#### OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF DILUTE-ACID PRETREATED CARDOON FOR BIOETHANOL PRODUCTION

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European Union strongly incentives research focusing biotechnological solutions for energy and chemical demands from renewable resources, such as, forestry wastes and agricultural biomass residues. Second generation bioethanol can be produced by conversion of lignocellulosic biomass (LCB), such as residues of cardoon (*Cyanara cardunculus*) widely grown in Alentejo. Before sugars fermentation polysaccharides have to be converted in monosaccharides by hydrolytic processes. Enzymatic hydrolysis (EH) is a commonly used process for saccharification of LCB, since it ensures cellulose and hemicellulose selective breakdown without producing fermentation inhibitors. In order to reduce bioethanol production costs, strategies to minimize the amount of enzymes used in EH need to be developed. In the present work, experimental design and response surface methodologies were applied to maximize the glucose concentration obtained by commercial enzymes hydrolyses (EH) of cardoon, pre-treated with 3.5% sulphuric acid at a liquid-to-solid ratio of 10.

A central composite design with 17 runs was performed, where 3 factors – solid loadings (SL: 2–8% dry biomass), cellulase (10–50 FPU/g dry biomass) and cellobiase (0–50 CBU/g dry biomass) doses were assessed by a 2<sup>3</sup> factorial design, three replicates at the central point and 8 axial points. The response surface method (RSM) was used to approximate the response function at the various factors levels, fitting a second order equation to the experimental data, which allowed the prediction of a maximum glucose concentration at the optimal conditions.

SL was the most important factor in the process, invoking the largest variation in glucose concentrations. The second most important factor was cellulase concentration and the least important cellobiase concentration. At low SL (2%) a negative effect with high enzyme loadings on EH was observed. This could result from the nonspecific adsorption of the enzymes to the substrate. The maximum glucose concentration predicted by RSM was 31.7±1.0 g/L for 8% of solid loading, 44.6 FPU and 51.9 CBU of cellulase and cellobiase concentration, respectively.

The highest experimental glucose concentration obtained in the experimental design was 31 g/L, with all factors at their highest level, which corresponded to 57% of sugars yield. Although a higher SL should provide better saccharification results, the load of solids to values superior than 8% was not allowed due to the absorption of the liquid fraction by the insoluble residue.

## Renewables, biofuels and bioenergy

P313

### OPTIMIZATION OF HIGH-SOLID ENZYMATIC HYDROLYSIS OF EXTRACTED OLIVE POMACE

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In order to achieve an economical feasible second generation ethanol production the availability of high glucose concentrations are mandatory so that high ethanol titers can be obtained in the end of the fermentation stage. This is not an easy task as high biomass loadings are difficult to achieve, due to many mechanical, biological and physic-chemical hindrances. Several of these can be overcome by using biomass pretreatments.

Among all the pretreatments currently available, the dilute acid hydrolysis, is still one of the most studied and widely used regardless of its potential drawbacks. Some of its advantages are the efficient hydrolysis of hemiceluloses into monosaccharide and also its high impact on cellulose digestibility. In this work, dilute acid pretreated Extracted Olive Pomace (EOP), an agro-industrial byproduct from olive oil industry, is used as a model feedstock to study the impact of biomass loading (5 – 35%) and the effect of enzyme dose (2 – 48 FPU per g dry weight) using a Doehlert statistic experimental design on cellulose saccharification obtained by enzymatic hydrolysis during a period of three days with sample analysis at 24, 48 and 72 h.

After 48 hours of saccharification no significant change was observed for both glucose and xylose concentrations and yields, in all tested conditions. For the same enzyme dosage, it was observed that both the concentration of glucose and xylose increased with the increase of biomass concentration. According to Doehlert experimental design the optimum saccharification conditions, were 35% biomass and 25.82 FPU. Under these conditions an estimated glucose production of 29.9 g/L was expected and was confirmed experimentally. Additional increment of enzyme dosage, tested with an extra experiment with solid and enzyme loading with maximum values (35% and 44.92 FPU) produced the same results as the optimum conditions, thus further validating the optimal operational values obtained. This is the highest solid loading to be effectively hydrolyzed to be reported in literature for enzymatic saccharification studies of olive oil industry by-products.

## Renewables, biofuels and bioenergy

### P314

#### VALORIZATION OF CANNED SARDINE AND MACKEREL RESIDUES THROUGH EXTRACTION OF BIOACTIVE COMPOUNDS

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Healthful and valuable compounds can be recovered from the fish canning residues and employed in high-priority fields such as medicine and food, or in other areas such as agrochemical and animal feedings. Proteins, lipids, biopolymers, amino acids and enzymes can be recovered either from wastewaters or from solid residues (head, viscera, skin, tails and flesh) generated along the canning process of sardine and mackerel, throughout the salting, cooking and filleting stages.

Sardine and mackerel scales were processed for the recovery of collagen and its hydrolysed derivatives, either enzymatically, such as collagen peptides, or thermally, such as gelatine. The hydroxyapatite and calcium phosphate-based materials were extracted from bones throughout calcination, while anti-hypertensive peptides were recovered from flesh or cooking wastewater through enzymatic hydrolysis of muscle proteins. Peptides obtained from hydrolysis showed bioactivity namely high anti-hypertensive property.

Collagen and collagen peptides could be employed in cosmetics and biomedicine, while gelatine could be used in low-fat food formulations, due to its fat-like melting properties which can contribute to a smooth and creamy mouth-feel.

Hydroxyapatite and calcium phosphate could be used for the development of biocompatible bone cement for craniofacial, oral-maxillofacial and orthopaedic defect repair, and coating for femoral components; the ion-exchange properties of hydroxyapatite could make it also suitable for wastewater treatment (heavy metals removal). Finally, anti-hypertensive peptides from flesh residues, as well as collagen peptides (<3000 Da) could be employed in the development of functional foods and drinks formulations.

This research shows the opportunities for the valorisation of bioactive compounds from sardine and mackerel canning residues. These are amongst the most consumed fish in the Mediterranean area; moreover, canning is one of the most important and applied methods of preservation. The large quantities of by-products generated have great potentials of valorisation and the extraction of bioactive compounds will also contribute to reduce their impact on the environment.

## Renewables, biofuels and bioenergy

P315/F01

### BIOETHANOL PRODUCTION FROM EXTRACTED OLIVE POMACE

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Extracted olive pomace (EOP) is a by-product from the olive oil extraction industry that is produced in huge amounts in the Alentejo region. Its composition has a significant cellulose content that can be potentially converted into soluble sugars and subsequently fermented to ethanol. One of the major obstacles for the recovery of sugars is the high recalcitrance of EOP to enzymatic hydrolysis, that can potentially be overcome by biomass pretreatments [1].

In this work, we revise and compare the results gathered within the RefinOlea project regarding two different strategies for biomass pretreatment, –dilute acid hydrolysis (DAH) and autohydrolysis (ATH)–, and their impact on cellulose enzymatic digestibility and fermentation either using Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF).

Both pretreatments were highly selective for hemicellulose, whose removal is crucial to increase the efficiency of the subsequent enzymatic treatments for the depolymerization of cellulose. On the other hand, glucan and Klason lignin content increased after pretreatments, showing that these fractions were almost not affected. Cellulose digestibility increased for both pretreatments from 4 to 22 for DAH and 89 % for the best condition (ATH).

The results for SHF and SSF fermentation processes, obtained with 15% solid loading, 20 FPU and 14 CBU per g of dry pretreated biomass showed that the residues obtained after ATH are more prone to enzymatic hydrolysis, producing higher ethanol concentration than the residues obtained after DAH. SSF process was more efficient than SHF process for both pretreated EOP. Enzymatic hydrolysis efficiency reached 90% and subsequent ethanol fermentation 94% of the theoretical yield, producing 27 g/L of ethanol, which is, to the best of our knowledge, the highest value reported in literature from olive oil by-products.

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## Session 6

# Cellular Microbiology and Pathogenesis

# Plenary Lecture



## STUDYING MALARIA INFECTIONS: UNDERSTANDING TO INTERVENE

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Despite renewed eradication efforts from the international community, malaria still exerts an enormous disease burden, with nearly half the planet's population at risk of infection. Within the human host, the disease-causing Plasmodium parasites pass through two distinct lifecycle stages, each in a different cellular environment. During the liver stage, a single Plasmodium sporozoite will invade a hepatocyte, and while sheltered there, supposedly undetected by the host, gives rise to thousands of new parasites, which will go on to initiate the subsequent blood stage of infection. While only 10–20 new parasites will be generated inside an erythrocyte, consecutive cycles of cell lysis and reinfection causing a potent host response, as well as the symptoms of malaria. The host contribution to infection outcome, on both the cellular and organismal levels has recently moved to center stage. We have identified hepatocyte molecules that modulate the success of liver stage infection, and showed that distinct host factors, not just the parasite itself, drive the onset and severity of diverse malaria syndromes. Our ongoing work indicates that the web of host–Plasmodium interactions is densely woven, with liver stage-mediated innate immune system activation, host nutritional status, and an antagonistic relationship between the two parasite stages themselves all working to modulate the balance between parasite replication and human health.

# Keynote Lecture

## KL07

# POST-TRANSCRIPTION REGULATION IN BACTERIAL PATHOGENESIS: ROLES PLAYED BY RNA CHAPERONES AND SRNAS

Jorge H. Leitão

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Post-transcriptional regulation of gene expression is a powerful means used by bacterial pathogens to rapidly adapt to the challenges faced when infecting their hosts. This fast adaptation results from the fact that translation occurs from the existing transcripts rather than relying on the adjustment of the transcript levels by *de novo* synthesis. Mechanisms of post-transcription regulation include all the interactions of a transcript with molecules that affect its normal translation. The best known mechanisms of post-transcription regulation in bacterial pathogens involve regulatory RNA-binding proteins and small non-coding RNAs (sRNAs), which can interact with mRNAs, affecting their stability and/or availability to bind to the ribosome. The protein Hfq, present in about half of the prokaryotic genomes and with homology to the Sm metazoan proteins, emerged as a central component of a global post-transcriptional regulatory network in bacterial pathogens, where it facilitates short and imperfect interactions of sRNAs with their mRNA targets [1].

Over the last years, we have focused our research on the biology of pathogens of the *Burkholderia cepacia* complex (Bcc), a group of bacterial species that emerged in the 1980s as important pathogens to cystic fibrosis patients, and more recently to hospitalized patients [2]. Contrasting with other bacteria, Bcc organisms encode in their genomes 2 distinct and functional Hfq-like proteins, Hfq and Hfq2 [3, 4]. Results will be presented and discussed on our work on both the functional characterization of Hfq and Hfq2 and their exploitation to experimentally identify sRNAs [5], and on the functional characterization of the sRNAs h2cR [6] and MtvR [7].

Acknowledgements:

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# Oral Presentations

## OP13

## CANDIDA ALBICANS CUG MISTRANSLATION IS A MECHANISM TO CREATE CELL SURFACE VARIATION

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Interactions with the surrounding environment represent a continuous challenge for microorganisms. Until now, the mechanisms regulating cell-surface changes in the most common human fungal pathogen, *Candida albicans*, remain unclear. In *C. albicans*, the CUG codon is naturally mistranslated as two biochemically distinct residues, serine and leucine. The variable incorporation of these two biochemically distinct amino acids has the potential to generate a family of proteins with different structures and functions. Interestingly, genes encoding adhesins such as the *ALS* family are particularly enriched in CUG codons, suggesting that CUG mistranslation may influence the interactions of the microorganism with the host. In order to evaluate the role of CUG mistranslation in *C. albicans* adhesion, we compared the behavior of two strains: a wild-type strain and a highly CUG mistranslation strain, with 3% and 28% of CUG codons mistranslated as leucine, respectively. We found that the increase in leucine misincorporation enhances cell wall hydrophobicity and the capacity of yeast cells to adhere to biomaterials and extracellular matrix proteins. This adhesion phenotype was further confirmed in *Saccharomyces cerevisiae*, through heterologous expression of *C. albicans ALS3* gene in its two isoforms: Als3p-Leu and Als3p-Ser. Als3p-Leu yeasts prompted a strong adhesion phenotype, resulting in flocculation in liquid media and two to five fold more adhesion than Als3p-Ser. The cell wall variability generated by CUG mistranslation may not be confined to adhesion and could be relevant for immune system evasion. Indeed, highly CUG mistranslating cells were less susceptible to phagocytosis by murine macrophages than wild-type, probably due to reduced exposure of cell surface  $\alpha$ -glucans. We propose that CUG mistranslation was maintained during *C. albicans* evolution due to its potential to generate cell surface variability.

## OP33

STAPHYLOCOCCUS EPIDERMIDIS BIOFILM LIFECYCLE AND ITS VIRULENCE:  
FROM PLANKTONIC GROWTH, TO BIOFILM STRUCTURE AND SYSTEMIC  
DISSEMINATION

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*Staphylococcus epidermidis* has been recognized as a leading cause of several clinically relevant infections, with particular association with the use of medical devices. This is related with its ability to colonize the surface of these devices and form biofilms. The major clinical implications of biofilm formation are the high resistance to antimicrobials and the host immune system, resulting in the development of chronic infections. *S. epidermidis* biofilm lifecycle is divided into 3 stages: initial adhesion, accumulation and maturation, and biofilm disassembly. Despite its impact in the development of acute infections, biofilm disassembly is the less studied of all stages, and little is known about the phenotype and the interaction of the released cells with the host immune system.

To uncover the mechanisms by which biofilms evade the host immune system and cause chronic infections, a transcriptomic analysis of *S. epidermidis* biofilms exposed to human blood was performed. Our results revealed an extensive remodelling of the transcriptome suggesting a quick adaptation to the new environment. Genes involved in amino acids biosynthesis, as well as iron uptake were strongly affected, indicating these mechanisms as important factors in bacterium survival and virulence.

Furthermore, to understand the particularities and virulence associated with biofilm disassembly and the development of acute infections, biofilm-released cells (Brc) were characterized by the following parameters: total protein and gene expression profiles, antimicrobial susceptibility, initial adhesion, CLSM analysis of surface biomarkers, opsonophagocytic killing assays, and finally, the interaction of brc with the host immune system was assessed using a murine infection model. Our results revealed that *S. epidermidis* Brc are unique in their phenotype and virulence potential, sharing some features with planktonic cells, such as expression of *psmβ*, but simultaneously displaying features similar to biofilms, such as high antibiotic tolerance. The phenotypic differences were translated to differences in the immune response. Brc elicited higher amounts of IL-6 and KC cytokines, and lower amounts of MCP-1 than biofilm cells showing, concomitantly, higher colonization of the spleen and liver. Thus, targeting of particular properties of the Brc could present new opportunities to effectively prevent the pathologic events associated with dissemination of cells from a biofilm to more distant sites.

## OP34

SATP, A SUCCINATE–ACETATE TRANSPORTER PROTEIN IN *ESCHERICHIA COLI*

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Members of the YaaH family are found in archaea, eukaryotes and bacteria, with some members experimentally demonstrated as acetate transporters such as Ady2 in the yeast *Saccharomyces cerevisiae* (1) and AcpA in the filamentous fungus *Aspergillus nidulans* (2). In this work we describe a new carboxylic acid transporter in *Escherichia coli* encoded by the gene *yaaH* (3). In contrast to what had been described for other YaaH family members, the *E. coli* member is highly specific for acetic acid (a monocarboxylate) and for succinic acid (a dicarboxylate), presenting the following affinity constants at pH 6.0,  $1.69 \pm 0.45$  mM acetic acid and  $1.20 \pm 0.22$  mM succinic acid. In glucose grown-cells the *yaaH* mutant is compromised for the uptake of both labelled acetic and succinic acids. YaaH, together with ActP, previously described as an acetate transporter associated with the acid assimilation (4), have to be knocked out simultaneously to abolish acetate transport. Both YaaH and ActP affect the use of acetic acid as sole carbon and energy source as determined by growth and analysis of expression by qPCR. The uptake of acetate and succinate was restored when *yaaH* was expressed in *trans* in  $\Delta yaaH \Delta actP$  cells. The amino acid residues Leu131 and Ala164 when mutated to Val and Gly, respectively, increase the ability to transport lactate in YaaH. The assignment of this protein as an acetate–succinate transporter leads to its new nomenclature as SatP: the Succinate–Acetate Transporter Protein.

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# Poster Presentations



## Host-Pathogen Interactions

### P316

#### ANALYSIS OF THE ROLE OF EUKARYOTIC SMALL G PROTEINS OF THE ARL FAMILY IN INFECTION OF HOST CELLS BY CHLAMYDIA TRACHOMATIS

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*Chlamydia trachomatis* are obligate intracellular bacteria that reside and replicate within a large membrane-bound vacuole, an inclusion. *Chlamydia* are characterized by a developmental cycle involving the inter-conversion between infectious elementary bodies and non-infection reticulate bodies. During its developmental cycle, *C. trachomatis* interfere with a wide diversity of host cell processes through the delivery of bacterial effector proteins into the host cytosol and membranes. Our knowledge of the mechanisms underlying *C. trachomatis* virulence is limited, largely because genetic manipulation of *Chlamydiae* remains challenging. In this work, we analyzed if host ADP-ribosylation factor (Arf)-like (Arl) proteins could be involved in infection of host cells by *C. trachomatis*. Arls are a family of poorly characterized small G proteins, but known to regulate cytoskeleton dynamics and intracellular membrane transport. We constructed a library of plasmids encoding for fusions of individual Arls to the N-terminus of EGFP that we used to individually transfect HeLa cells and compare by immunofluorescence microscopy the subcellular localization of Arl-EGFP proteins in uninfected cells and in cells infected by *C. trachomatis*. This revealed that Arl5a, 5b, 8b, 10, 17 and Arfrp1, appeared to accumulate around the inclusion. However, after disrupting the Golgi apparatus with brefeldin A or the microtubules with nocodazole, only Arl5a and Arl10 remain significantly associated with the inclusion. In uninfected cells, Arl5a-EGFP co-localized with a Golgi protein marker (GMAP210) and Arl10-EGFP localized in the mitochondria. However, analysis of untransfected cells infected by *C. trachomatis* did not reveal a significant association of GMAP210 or of the mitochondria with the inclusion. By comparison to cells expressing EGFP alone, expression of EGFP fusions containing dominant negative Arl5a, or wild-type, constitutively active and dominant negative Arl10, significantly inhibited the developmental cycle of *C. trachomatis*. Therefore, the function of Arl5a and Arl10 could be important for *C. trachomatis* infection. To analyse this further, we are currently investigating the consequences of siRNA-mediated depletion of Arl5a or of Arl10 in *C. trachomatis* development.

## Host-Pathogen Interactions

P317

### ANTI-*CRYPTOCOCCUS NEOFORMANS* ACTIVITY OF SYNTHETIC CHALCONE IN A MURINE MODEL

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Cryptococcosis is a systemic and opportunistic mycosis mainly caused by the *Cryptococcus* spp yeast, specially the *C. neoformans* species. The most important clinical manifestations are meningitis and meningoencephalitis, which present difficult treatment. The drugs of choice for the treatment of cryptococcosis are amphotericin B (AMB) and fluconazole, a long-term treatment with severe adverse reactions. Thus, the search for new anti-*Cryptococcus* substances is the target of several studies. In our laboratory, the research by antifungal activity of chalcones showed the high potential of this compound in the treatment of cryptococcosis. The minimum inhibitory concentration data showed potent activity of chalcones at low concentration, and no reported high toxicity in human cells. The objective of this study was to evaluate the efficiency of chalcone as cryptococcosis treatment in a murine model. The activity of chalcone was evaluated by testing fungal burden. Mice BALB / C, were intratracheal infected with  $1 \times 10^6$  CFU / mL of *Cryptococcus neoformans* ATCC 90012. Seven days after this infection, the treatment was started in these groups. We used AMB and PBS as controls of the test. The animals were weighed every two days of treatment. After seven and fourteen days of treatment, the animals were killed and the organ (heart, liver, kidney, lung and brain) was macerated, diluted and plated on Sabouraud agar with chloramphenicol. After 48 hours we performed the UFC counting by organ. A group of uninfected animals were treated and subjected to the same conditions. Our results showed that the chalcone significantly reduced the number of CFU per organ. The chalcone treatment showed reduction the fungal burden in the heart, liver and kidney to chalcone, but not as much as AMB. In the lungs, it was observed an equivalence in reduction of fungal load, between chalcone and AMB. In the brain, treatment with chalcone showed significant reduction in fungal load when compared with AMB. The physiological parameter of corporeal weight loss during the antifungal treatments, showed that the groups not infected and treated, presented no body weight loss. The treatment with chalcone in the infected group showed that treaties reduced the weight corporeal, however not as much as those who received no treatment. Our results showed the high efficiency of antifungal treatment with chalcone for the treatment of infection by *C. neoformans* in murine animal model.

CAPES, FAPESP; FCFAR

## Host-Pathogen Interactions

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### BACTERIOPHAGES WITH POTENTIAL TO TREAT FURUNCULOSIS IN MARINE FISH: SURVIVAL, HOST SPECIFICITY AND HOST PHAGE DYNAMICS.

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The increasing problem of antibiotic resistance in common pathogenic bacteria and the concern about the spreading of antibiotics in the environment, enhance the need to find new methods to control fish pathogens. Phage therapy appears to represent a useful and flexible tool for the inactivation of bacterial pathogens in aquaculture. Two previously isolated phages AS-2 and AS-3 produced on *Aeromonas salmonicida*, causative agent of furunculosis, were used to examine the host specificity, survival in marine water and host-phage dynamics. The host specificity was evaluated by cross infection tests. The survival was determined in marine water through quantification by the soft agar overlay technique. The host-phage dynamics was characterized in tryptic soy broth, through quantification of phages by the soft agar overlay technique and host quantification in TSA medium. Phage AS-2 infected *V. anguillarum* and *V. parahaemolyticus* with efficiencies of 57 and 91 %, respectively. Phage AS-3 infected *V. anguillarum* and *V. parahaemolyticus* with efficiencies of 76 and 97%, respectively. None of the two phages were effective against *Aeromonas hydrophilla*, *Photobacterium damsela* subsp. *damsela*, *Vibrio fischeri*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas gingeri* or *Pseudomonas segetis*. The pattern of phage survival in aquaculture water was different for the both. The concentration of AS-2 decreased by two orders of magnitude in the first 105 days. The concentration of AS-3 decreased by one order of magnitude in the first 20 days and reached a plateau until 77 days. Afterwards, the phage titer decreased by three orders of magnitude until 156 days. The results show that the growth of the *A. salmonicida* was inhibited by the two phages, resulting in a decrease of  $\approx 3$  log after 4-10 h of incubation. Phages AS-2 and AS-3 showed good survival in marine water, a high specificity for the causative agent of furunculosis and an efficient inactivation of *A. salmonicida*, being potential candidates as agents for the biological control of furunculosis in aquaculture systems.

## Host–Pathogen Interactions

### P319

#### COMPARISON OF *DIPLODIA CORTICOLA* SECRETOME IN AN INFECTION-INDUCED ENVIRONMENT

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Cork oak (*Quercus suber*) it is an evergreen tree characteristic of Mediterranean forests that plays a remarkable ecological role, not only as a biodiversity hotspot, but also as a regulator of water balance and soil conservation. Moreover, the numerous industrial applications of its renewable bark stress its socio-economic importance, particularly in Portugal, the current worldwide leader of cork production, transformation and exportation.

Unfortunately, since 1980s the cork oak mortality has been notoriously growing. This decline results from the conjugation of adverse environmental factors that weaken the tree's health, becoming thus more susceptible to opportunistic pathogens. Among these, *Diplodia corticola* is considered the most aggressive pathogen associated with cork oak decline in the Iberian Peninsula, affecting mainly *Q. suber* and *Q. ilex*, and also species like *Vitis vinifera*. This endophytic fungus exacerbates the disease symptoms, unbalancing as well the tree's metabolic processes, and its action commonly culminates in plant death.

To develop efficient disease management strategies it is necessary to understand the hitherto unknown molecular mechanism of this fungal infection. The characterization of *D. corticola* secretome may therefore contribute to disclose the proteins involved in its pathogenicity and virulence. The lack of genome information of this organism forced us to implement methodologies, such as 2-D PAGE and *de novo* sequencing, to separate and identify *D. corticola* extracellular proteins.

This work enabled, for the first time, to identify several extracellular proteins of *D. corticola* that may have an active role on its phytopathogenic lifestyle, highlighting key protein targets for diagnosis of infection and cork oak protection. These include the hydrolases glucoamylase, glycoside hydrolase 71, neuraminidase and glucan- $\beta$ -glucosidase and, in less extent, the proteases carboxypeptidase S1, serine protease and M35 deuterolysin.

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## Host-Pathogen Interactions

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### EXTRACELLULAR PROTEOME OF DIPLODIA CORTICOLA STRAINS

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*Diplodia corticola* is a phytopathogen that causes dieback, canker and vascular necrosis in oak trees. This fungus, which is considered the most aggressive pathogen associated with cork oak in the Iberian Peninsula, is able to infect *Quercus* species. As a consequence, it has been notorious a progressive deterioration of cork oak forests that culminates in death of trees. *Quercus suber* is a typical evergreen tree found in Mediterranean forests of increasing importance due to its renewable bark, cork. This natural product has numerous industrial applications, with emphasis to cork stoppers, of which Portugal is currently the production, processing and export leading country, producing about 40 million corks stoppers per day. Portugal represents about 50% of exports of cork in the world and there are six hundred companies related to cork. In addition to the high economic value, cork oak plays important roles with ecological and social impacts. It is therefore fundamental to unravel the underlying extracellular enzymatic activity and the differential expression to further develop efficient disease management strategies.

We have investigated the production of extracellular enzymes (gelatinases, caseinases, amylases, xylanases and endoglucanases) by 10 isolates of *Diplodia corticola* in the presence of host tissue. Data showed that total extracellular enzymatic activity of *D. corticola* is dependent on the presence of host tissue. Gel-based was performed for compare the secretome of *D. corticola* in an infection-similar environment with cells grown in standard media. Proteins were identified by Mass Spectrometry.

The presence of stem increased the enzymatic activity, which may be related with the infection mechanism of *D. corticola*. In the absence of host tissue, xylanase activity was not detected. Gelatinases and caseinases were also differentially expressed by the presence of host tissue.

Concluding, these enzymes are potentially associated to the infection mechanism of *D. corticola* as demonstrated by a different pattern of enzymatic activity in the presence of host tissue.

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## Host-Pathogen Interactions

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### HISTOPLASMA CAPSULATUM INFECTION PRESENTING NEW NUCLEAR EFFECTS IN ALVEOLAR MACROPHAGES

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*Histoplasma capsulatum* var. *capsulatum* is a dimorphic fungus that causes histoplasmosis, mainly respiratory and systemic disease. *H. capsulatum* is primarily acquired via aerosol exposure with the inhalation of microconidia or hyphal fragments. The evolution of respiratory disease depends on the ability of *Histoplasma* yeast to survive and to replicate within alveolar macrophages. Besides the lung infection, the yeast can disseminate to other body's organs, including the spleen, liver, and heart, causing the most lethal form of histoplasmosis disease. Thus, it is clear that the interaction between macrophages and *H. capsulatum* is a decisive step for the occurrence of spread of pathogenic yeasts. In this sense, nuclear damage in host cells can be characterized as a cellular effect able to contribute to the pathogenesis of histoplasmosis. Therefore, this study was designed to investigate the nuclear effect as well as the behavior of the nuclear envelope proteins during infection, since the degradation of cell nucleus is associated with the nuclear envelope breakdown. *H. capsulatum* strains, EH 315 and 60I, were used. To evaluate the infection pattern of yeasts in macrophages, we used the AMJ<sub>2</sub> - C11 cell line, infected with 5x10<sup>6</sup> cells/ml. Images of infection were acquired by the *IN Cell Analyzer 2000 System light microscopy*. The potential of nuclear fragmentation by *H. capsulatum* in macrophages was measured by the percentage of fragmented DNA using the comet assay and TUNEL technique. Then, the nuclear envelope proteins Sun2, Nesprin2 and Emerin were labeled in infected and non-infected cells. The comet assay and TUNEL technique shows that for the two strains tested, 60I and EH-315, DNA damage occurs, significant differences were observed when compared to the negative control. The labeling of nuclear envelope proteins showed that the yeast does not reach the cell nucleus after 5 hours of infection and that in this period it's observed the formation of large phagosomes within macrophages. The present study revealed the occurrence of new events during infection of macrophages by *H. capsulatum*, and they may be involved in the survival and persistence of the pathogen in the intra-phagosomal environment, as well as contribute to the pathogenesis of local tissue.

CNPq; FAPESP; CAPES; FCFAR.

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## Host-Pathogen Interactions

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### IDENTIFICATION OF AN EXTRACELLULAR INFECTION-INDUCED GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF THE PHYTOPATHOGENIC PROTEOBACTERIUM *PSEUDOMONAS SYRIN*

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According to molecular biology, genomic and proteomic data, the phytopathogenic gammaproteobacterium *Pseudomonas syringae* pv. tomato DC3000 produces a number of proteins that may promote infection and draw nutrients from plants. Remarkably, *P. syringae* DC3000 strain possess three paralogous gap genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes with different predicted molecular sizes and metabolic functions. As GAPDH was shown to be a virulence factor in other microbial pathogens, in the current study we analyzed by real-time PCR the expression levels of each paralogous gap gene to understand the actual impact of their protein products on *P. syringae* virulence. We found that all of them were strongly induced during the infection process. Nevertheless, proteomic analysis of culture supernatants revealed that only the class I GAPDH1 encoded by the gap1 gene was identified as an extracellular protein in infective cells. These results strongly suggest that this GAPDH should play a role in the infective process, besides its well-know enzymatic function in the glycolytic metabolic pathway.

## Host–Pathogen Interactions

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### INACTIVATION OF AQUACULTURE *VIBRIO PARAHEMOLYTICUS* BY THE USE OF PHAGE COCKTAILS

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Aquaculture is one of the fastest growing sectors in the world, having a very important role in the economy. However, the financial losses associated with bacterial infections leads to huge economic costs. Vibriosis is a main disease of marine and estuarine fishes, both in natural and commercial production systems throughout the world. The regular use of artificial food supplemented with antibiotics in intensive and semi-intensive aquaculture systems, to prevent the spread of diseases and their massive use to control infections, has resulted in the development of resistant strains, which have contributed to the inefficacy of antibiotic treatments. To reduce the risk of development and spreading of microbial resistances and to control the fish diseases in aquaculture, alternative strategies must be developed to allow the use of reasonably cheap and more environmentally friendly methods. Phage therapy is an alternative eco-friendly approach to prevent and control pathogenic bacteria in aquaculture. The aim of this study was to evaluate the efficacy of cocktails of two and three phages of *Vibrio parahaemolyticus* (VP-1, VP-2 and VP-3 phages) to control *Vibrio parahaemolyticus* infection in aquaculture systems. The host–phage dynamics was characterized in tryptic soy broth, through quantification of phages by the soft agar overlay technique and host quantification in TSA medium. The phages were tested alone and as a cocktail, at a MOI of 100. All the three phages were effective against *Vibrio parahaemolyticus* (reductions of about 2.8 log), but the cocktails with the three phages and the cocktails with only two of them were considerably more effective (reductions between 3.5–3.9 log). In conclusion, under the evaluated conditions, the use of phage cocktails appears to be an effective approach to treat vibriosis in aquaculture.



## Host–Pathogen Interactions

### P324

#### INNOVATIVE ANTI-MICROBIAL STRATEGIES AGAINST BURULI ULCER: BACTERIOPHAGE THERAPY

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Buruli Ulcer (BU), caused by *Mycobacterium ulcerans*, is an emerging necrotizing skin disease. Currently, antibiotherapy with rifampicin and streptomycin is recommended by the WHO, but in extensive ulcerative lesions, surgical resection of the infected skin is still necessary. Bacteriophages and their lysins (Lys) are a class of antimicrobial agents that have been regarded as an alternative method to control bacterial infections. The overall goal of this study is to evaluate the efficacy of mycophages and Lys in the control of *M. ulcerans*, as a novel therapeutic approach against BU.

After establishing the antimycobacterial activity of mycobacteriophage D29 *in vitro*, we further tested its efficacy *in vivo*. For that mice were injected s.c. with a single dose of mycophage D29 in infected footpads, at an advanced stage of infection. The efficacy of phage treatment was evaluated by footpad swelling and viable *M. ulcerans* growth. We show that a single injection of mycophage D29 can effectively decrease *M. ulcerans* proliferation and prevent footpad ulceration.

Additionally, we wanted to determine whether mycophage Lys were effective in controlling *M. ulcerans* proliferation. For that, Lys were expressed in an *E. coli* BL21(DE3)(pET) *system* and purified by an affinity chromatography. In order to evaluate the antimicrobial activity of Lys, a lysoplate assay was performed with *M. ulcerans* isolates from endemic BU areas. Our results show that Lys has lytic activity *in vitro* against *M. ulcerans* isolates, in a dose dependent manner as demonstrated by induced spots in bacterial lawns.

Our next step was to evaluate the bioavailability and cytotoxicity of Lys *in vivo*. At different time points after s.c. Lys injection in the footpad, the presence of protein in tissue supernatant and in the serum of mice was detected by western blotting. Histological analysis was performed on footpad tissue sections stained with hematoxylin and eosin. We observed no significant alterations in the footpad of treated mice, showing that Lys is not cytotoxic *in vivo*. Importantly, our results show an enzymatic activity associated with the detection of Lys in footpads of mice, at least 6h after s.c. injection.

After these promising results our research is now focused on therapeutic studies to test Lys activity *in vivo* against *M. ulcerans*. One possible approach to improve Lys bioavailability could be the association of Lys with a drug delivery system with topical application.

## Host–Pathogen Interactions

### P325

#### THE *CHLAMYDIA TRACHOMATIS* TYPE III SECRETION SUBSTRATES CT142 AND CT143 MAY BE PART OF A PROTEIN COMPLEX IN THE LUMEN OF THE BACTERIAL VACUOLE

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*Chlamydia trachomatis* causes ocular and genital infections in humans and only multiplies within host cells in a large vacuole, an inclusion. *C. trachomatis* encodes for a type III secretion (T3S) system, which enables the bacterium to inject effector proteins into the cytosol or membranes of host cells. We have screened for new *C. trachomatis* T3S substrates and, among others, identified CT142, CT143, and CT144 as putative effectors. The genetic manipulation of *C. trachomatis* is difficult and CT142, CT143 and CT144 do not display significant amino acid similarity to characterized proteins. However, their encoding genes are localized adjacently in the chromosome, suggesting that they could have a related function. In this work, we showed that *ct142*, *ct143* and *ct144* are organized in an operon and found a transcription start site upstream from the start codon of *ct142*. This suggested that expression of *ct142–ct143–ct144* is driven by  $\sigma^{66}$ , the *C. trachomatis* homolog of the *Escherichia coli* main  $\sigma$  factor. We raised antibodies against CT142 and CT143 to determine the subcellular localization and expression of the proteins during infection. It has been previously shown that expression of *ct142*, *ct143*, and *ct144* is transcriptionally regulated by the *Chlamydia*–virulence plasmid, which is present in the vast majority of isolates. Therefore, we infected HeLa cells with *C. trachomatis* L2/434 or with *C. trachomatis* L2/25667R, a rare plasmidless isolate. In cells infected by strain L2/434, both proteins could be detected by immunoblotting from 20–30 h post–infection. Immunofluorescence (IF) microscopy revealed that CT142 and CT143 appeared mostly as intra–inclusion globular structures that at least in the case of CT143 did not overly co–localized with the IF signal from the bacteria. In contrast, in cells infected by strain L2/25667R, expression of either of the proteins could not be detected by immunoblotting and we observed a significant decrease in the intensity of their IF signal. We tested for possible interactions between CT142, CT143, and CT144 by immunoprecipitation after their expression in *Yersinia*. This suggested that CT142 could self–interact and that CT143 may bind CT142. Thus, CT142 and CT143 may form a multi–protein complex in the lumen of the inclusion. Experiments are in progress to understand the function of this putative complex.

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## Host–Pathogen Interactions

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### THE SALMONELLA EFFECTOR STEA IS A PHOSPHATIDYLINOSITOL-4-PHOSPHATE-BINDING PROTEIN

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*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is an intracellular pathogen, which causes gastroenteritis in humans and replicates within host cells in a membrane-bound compartment, the *Salmonella*-containing vacuole (SCV). Virulence of *S. Typhimurium* is associated to type III secretion systems that mediate translocation of ~40 bacterial effector proteins into host cells. We have shown that SteA is a *Salmonella* effector that controls membrane dynamics of the SCV. Here, we have analyzed determinants of its intracellular localization. In infected cells, SteA localized in the SCV membrane and in *Salmonella*-induced membrane tubules extending from the vacuole. In addition, ectopically expressed SteA localized at the plasma membrane (PM) and in the Golgi region in mammalian cells, and at the PM and vacuole in yeast *Saccharomyces cerevisiae*. As SteA does not possess an obvious membrane localization domain, we tested if it could bind lipids found in eukaryotic membranes. We used Echelon lipid strips to show that GST–SteA binds specifically to phosphatidylinositol-4-phosphate [PI(4)P], normally found at the PM and in the Golgi. In *S. cerevisiae*, the PM localization of SteA was lost in cells lacking Stt4, a kinase that synthesizes the PM pools of PI(4)P, but not in cells lacking Pik1, which synthesizes PI(4)P at the Golgi. Moreover, the PM localization of SteA in yeast was unaffected by depletion of PM-localized phosphatidylinositol-4,5-bisphosphate. To understand how SteA binds PI(4)P, we noticed two clusters of basic residues within the primary structure of SteA, one in its N-terminal half (K26, K33 and K36) and the other in its C-terminal half (K154, K156, R159 and R161). Alanine-replacements of these residues in the N-terminal, but not in the C-terminal, half of SteA, impaired its binding to PI(4)P *in vitro* and its PM localization after ectopic expression in eukaryotic cells. In infected cells, the localization of SteA<sup>K36A</sup> was also affected. Furthermore, we used a fusion to EGFP of the well-characterized PI(4)P binding region of the *Legionella* effector SidC as a probe to show that PI(4)P is present in the SCV membrane and associated tubules. Therefore, binding of SteA to PI(4)P seems important for its intracellular localization. We are currently investigating how binding of SteA to PI(4)P may be required for its effector functions.

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## Host-Pathogen Interactions

### P327

## THE SALMONELLA TYPE III SECRETION EFFECTOR STEA PARTICIPATES IN THE CONTROL OF THE MEMBRANE DYNAMICS OF SALMONELLA-CONTAINING VACUOLES

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*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is a facultative intracellular bacterium that injects several effector proteins into host cells using type III secretion systems (T3SSs). SteA is an effector secreted by both the *Salmonella* Pathogenicity Island-1-encoded T3SS (T3SS-1) and the T3SS-2 that is required for *Salmonella* virulence in mice models of systemic or persistent infection. Initial studies revealed that a *S. Typhimurium steA* mutant had no noticeable defects in the capacity of *Salmonella* to invade non-phagocytic cells (T3SS-1-dependent) or to grow intracellularly within macrophage-like RAW267.4 murine cells (T3SS-2 dependent). We next analyzed in more detail well-characterized T3SS-2 phenotypes associated with *Salmonella* infection of epithelial-like HeLa cells. Compared to cells infected by wild-type *S. Typhimurium*, cells infected by *steA* mutant bacteria displayed less *Salmonella*-induced membrane tubules enriched in late endocytic markers such as LAMP1, an increased clustering of the *Salmonella*-containing vacuoles (SCVs), and morphologically abnormal SCVs characterized by an aberrant accumulation of LAMP1 membranes and containing more than one bacteria per vacuole. These defects are likely related to an altered membrane dynamics or movement of the SCVs, which have been shown to depend on manipulation of host cell molecular motors (dynein and kinesins). *Salmonella* possesses T3SS-2 effectors that bind kinesin-1 (PipB2) or are thought to recruit dynein (SseF and SseG). Studies with *S. Typhimurium steA sseF*, *steA sseG* and *pipB2 sseF* double mutants revealed that deleting the genes encoding SseF or SseG in a *steA*-null mutant background significantly reduced the percentage of infected epithelial cells with *steA*-related defects in bacterial clustering and in SCV membrane partitioning, whereas introducing the *pipB2* mutation to the same background was neutral. This result indicated that SteA is functionally linked to SseF and SseG. Moreover, inhibition of either the centripetal motor dynein or the centrifugal motor kinesin-1 in cells infected by *steA* mutant *S. Typhimurium*, suppressed the characterized defects in SCV clustering and morphology. All together, our observations indicate that SteA participates in the control of SCV membrane dynamics, possibly by contributing to the manipulation of the function of both dynein and kinesin-1.

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## Host-Pathogen Interactions

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### VIRULENCE OF PARACOCCIDIOIDES SPECIES AND EFFICACY OF AMPHOTERICIN B AGAINST *P. LUTZII* IN THE NON-MAMMALIAN HOST *GALLERIA MELLONELLA*

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The *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* species belong to a group of the thermo-dimorphic fungus and cause the paracoccidioidomycosis, which is one of the most important and systemic endemic fungal diseases in South and Central America, mainly affecting rural workers and immunodeficient individuals. The study of the virulence of fungi in the host *G. mellonella* has been reported because the insect is easy to manipulate, presents low rearing costs and immune response structural and functional similarities to the innate immune response of mammals. Moreover, *G. mellonella* has also been utilized to determine the efficacy of antifungals. This study aimed to evaluate the virulence of *Paracoccidioides* species in *G. mellonella* as well as efficacy of amphotericin B in the *P. lutzii*-*G. mellonella* model. The suspension of the fungus *P. brasiliensis* phylogenetic specie S1 isolate 18 (São Paulo) and *P. lutzii* strain 01 (ATCC MYA-826/Goiânia) was prepared in phosphate-buffered saline (PBS) with ampicillin (20 µg/ml). *G. mellonella* larvae (0.1-0.3 g) were inoculated with 10 µl of  $5 \times 10^5$ ,  $10^6$  and  $5 \times 10^6$  yeast cells. The larvae were incubated at 37°C and were monitored daily for 7 days by lack of movement to physical stimulation. A group of non-infected larvae and a group of larvae inoculated with PBS were used as controls. Infected larvae were treated with amphotericin B (0.5; 1 and 2 mg/Kg) and another group of larvae were treated with antifungal alone to test toxicity. Survival curves were analyzed by Log-rank (Mantel-Cox) in the Graph Pad Prisma 5. The fungi killed the *G. mellonella* in a dose manner dependent, which the most of the larvae inoculated with  $5 \times 10^6$  yeast cells killed at the end of 7 days, while with other doses, most of larvae remained alive in the same period. The survival of the larvae infected with *P. lutzii* increased 62,5% with the dose of 0.5 and 1 mg/Kg of amphotericin B and 75% with the dose of 2 mg/Kg compared to control. Moreover, in the tested doses, the antifungal was not toxic for the larvae. The experiment is in the process of standardization, so it will be repeated and results more representative will be presented. These results showed that *G. mellonella* is susceptible to *P. brasiliensis* and *P. lutzii* infection and the efficacy of amphotericin B was observed with all the doses against *P. lutzii* in model. This opens possibility to screen new antifungal compounds in this infection model.

RENAMA-CNPq; FAPESP; CAPES; FCFAR

## Host–Pathogen Interactions

P329/F39

## WHEN FUNGAL PROTEOMICS MEETS CORK OAK DECLINE

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Cork oak (*Quercus suber*) it is an evergreen tree characteristic of Mediterranean forests that plays a remarkable ecological role, not only as a biodiversity hotspot, but also as a regulator of water balance and soil conservation. Moreover, the numerous industrial applications of its renewable bark stress its socio–economic importance, particularly in Portugal, the current worldwide leader of cork production, transformation and exportation.

Unfortunately, since 1980s the cork oak mortality has been notoriously growing. This decline results from the conjugation of adverse environmental factors that weaken the tree's health, becoming thus more susceptible to opportunistic pathogens. Among these, *Diplodia corticola* is considered the most aggressive pathogen associated with cork oak decline in the Iberian Peninsula, affecting mainly *Q. suber* and *Q. ilex*, and also species like *Vitis vinifera*. This endophytic fungus exacerbates the disease symptoms, unbalancing as well the tree's metabolic processes, and its action commonly culminates in plant death.

To develop efficient disease management strategies it is necessary to understand the hitherto unknown molecular mechanism of this fungal infection. The characterization of *D. corticola* secretome may therefore contribute to disclose the proteins involved in its pathogenicity and virulence. The lack of genome information of this organism forced us to implement methodologies, such as 2–D PAGE and *de novo* sequencing, to separate and identify *D. corticola* extracellular proteins.

This work enabled, for the first time, to identify several extracellular proteins of *D. corticola* that may have an active role on its phytopathogenic lifestyle, highlighting key protein targets for diagnosis of infection and cork oak protection. These include the hydrolases glucoamylase, glycoside hydrolase 71, neuraminidase and glucan– $\beta$ –glucosidase and, in less extent, the proteases carboxypeptidase S1, serine protease and M35 deuterolysin.

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## Host–Pathogen Interactions

### P330/F32

## DIVERSITY OF BOTRYOSPHAERIACEAE SPECIES FROM *EUCALYPTUS* SPP. IN PORTUGAL

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Species of the fungal family Botryosphaeriaceae occur as endophytes or latent pathogens on a diverse array of woody hosts. Several species have been associated with *Eucalyptus* spp. wherever they are grown.

*Eucalyptus* spp. are one of the most widely planted forest trees because of their economic importance. In Portugal, these plants (mostly *E. globulus*) represent currently 26% of the total forest area, being the most abundant forest tree in the country. Despite its economic importance, there are no studies related to the occurrence of Botryosphaeriaceae species associated with eucalypts in Portugal.

The objective of this work was to study the diversity of Botryosphaeriaceae species found on healthy and diseased *Eucalyptus* spp. in Portugal. For that, we obtained pure cultures from symptomatic and asymptomatic plant material. In a first approach all isolates were separated in 6 clusters based on BOX–PCR fingerprinting. The identification of the isolates was based on cultural characteristics, spore morphology and DNA sequences of the ITS region.

A total of 203 isolates belonging to 3 different genera (*Neofusicoccum*, *Diplodia* and *Botryosphaeria*) were obtained, with *Neofusicoccum* being clearly dominant (95% of the isolates). Six species were identified within the isolates, namely *N. australe* (52%), *N. eucalyptorum* (41%), *N. parvum* (2%), *D. seriata* (2%), *D. corticola* (2%) and *B. dothidea* (1%). Most of the isolates (77%) were derived from symptomatic tissues. All species were found both in symptomatic and asymptomatic material except *B. dothidea* and *D. corticola* that were present only on asymptomatic and symptomatic material, respectively.

*Diplodia seriata* and *D. corticola* were found associated with *Eucalyptus* spp. for the first time worldwide. On the other hand, *N. eucalyptorum* represents a new report of the species in Portugal.

This study allowed the identification of a diversity of species of Botryosphaeriaceae associated with eucalypts in Portugal. Pathogenicity tests should be done in the future to determine which species are more aggressive pathogens of eucalypts and may represent a threat to plantations.

This study was supported by FEDER funding through COMPETE program and by national funding through FCT within the research project PANDORA (PTDC/AGR-FOR/3807/2012 – FCOMP-01-0124-FEDER-027979) and grants to Alan J.L. Phillips (PEst-OE/BIA/UI0457/2011) and Carla Barradas (SFRH/BD/77939/2011). Artur Alves was financed by ESF (EU) and POPH funds (Programa Ciência 2008).

## Host–Pathogen Interactions

### P331/P31

## BLACK ROT DISEASE IN PORTUGAL: A DIVERGENT PATHOSYSTEM FOR *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* AND GALICIAN – PORTUGUESE CABBAGES

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Black rot disease (BRD), caused by the Gram–negative bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) is the most important bacterial disease of Brassicaceae vegetable crops worldwide. In Portugal, Xcc affects mainly the most consumed *B. oleracea* varieties. In this study, a collection of 33 Portuguese isolates collected previously identified as *X. campestris* was characterized, combining phenotypic and genomic data. In addition to commonly used biological assays for race determination and aiming to assess the aggressiveness of Portuguese strains, quantification of symptoms was performed using three hosts: Savoy cabbage, Galega kale and Portuguese Tronchuda cabbage. Isolates were screened for the presence of eight selected virulence genes (*adk*, *dsbB*, *hpaR*, *hrpF*, *katG*, *mip-like*, *ppsA* and *zur*) and a phylogenetic analysis was conducted by sequencing GyrB gene and by Multi Locus Sequence Typing (MLST) using 4 loci (*dnaK*, *fyuA*, *gyrB* and *rpoD*). Biological assays for race determination allowed the identification of 27 Xcc isolates causing BRD symptoms. Of those, 11 isolates belong to race 6 and eight to race 7. The remaining eight isolates corresponded to five previously undescribed race profiles. 3 isolates causing leaf spots typical of *X.c.* pv. *raphani* were recorded, as well as 3 nonpathogenic isolates. Assessment of symptoms, by measuring the average infected leaf area, showed that CPBF 278 was the most aggressive isolate, while CPBF 213 was the least aggressive. For all isolates, the most resistant host was Galega kale, while Savoy and Portuguese cabbages were similarly susceptible. Galega kale responded differently to races 6 and 7 in later stages of infection, suggesting this host's usefulness to distinguish between the two. Screening of virulence genes and *gyrB*–based phylogeny did not provide enough resolution to distinguish pathovars or races. The MLST–based phylogeny allowed the clear differentiation of Xcc from other pathovars and grouped Xcc isolates in three distinct clusters. This work allowed concluding that: 1) aggressiveness of the tested isolates varies significantly; 2) the current race structure cannot be fully applied to Portuguese isolates and continues to lack genomic support; 3) MLST was the only genomic–based approach able to distinguish between Xc pathovars. The polyphasic strategy implemented disclosed novel insights on the race structure of Portuguese Xcc isolates, as well as the underlying phylogenetic relationships.



## Host-Pathogen Interactions

## P332/F30

## TLR9 ACTIVATION DAMPENS THE EARLY INFLAMMATORY RESPONSE TO PARACOCCIDIOIDES BRASILIENSIS, IMPACTING HOST SURVIVAL

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*Paracoccidioides brasiliensis* causes paracoccidioidomycosis, one of the most prevalent systemic mycosis in Latin America. Thus, understanding the characteristics of the protective immune response to *P. brasiliensis* is of interest as it may reveal targets for disease control. The initiation of the immune response relies on the activation of pattern recognition receptors, among which are TLRs. Both TLR2 and TLR4 have been implicated in the recognition of *P. brasiliensis* and regulation of the immune response. However, the role of TLR9 during the infection by this fungus remains unclear. We used *in vitro* and *in vivo* models of infection by *P. brasiliensis*, comparing wild type and TLR9 deficient (-/-) mice, to assess the contribution of TLR9 on cytokine induction, phagocytosis and outcome of infection. We show that TLR9 recognizes either the yeast form or DNA from *P. brasiliensis* by stimulating the expression/production of pro-inflammatory cytokines by bone marrow derived macrophages, also increasing their phagocytic ability. We further show that TLR9 plays a protective role early after intravenous infection with *P. brasiliensis*, as infected TLR9-/- mice died at higher rate during the first 48 hours post infection than wild type mice. Moreover, TLR9-/- mice presented tissue damage and increased expression of several cytokines, such as TNF- $\alpha$  and IL-6. The increased pattern of cytokine expression was also observed during intraperitoneal infection of TLR9-/- mice, with enhanced recruitment of neutrophils. The phenotype of TLR9-/- hosts observed during the early stages of *P. brasiliensis* infection was reverted upon a transient, 48 hours post-infection, neutrophil depletion. Our results suggest that TLR9 activation plays an early protective role against *P. brasiliensis*, by avoiding a deregulated type of inflammatory response associated to neutrophils that may lead to tissue damage. Thus modulation of TLR9 may be of interest to potentiate the host response against this pathogen.

## Microbial Pathogenesis

P333

### A NOVEL MODEL OF IBD THROUGH VACCINATION WITH NORMAL GI FLORA

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Current investigations have focused on mechanisms of inflammation in inflammatory bowel disease (IBD). The gut bacteria play an important role in the pathogenesis of IBD, however due to the complexity of gut microflora some skepticisms still remain. Most of currently animal models of colitis are mainly based on bowel mucosal damage which has some limitation in terms of dissimilarity to human IBD. In the present study, a new model based on defined bacterial infections has been developed and tested.

Seven groups of six rats were involved; normal, positive control which received trinitrobenzenesulfonic acid enema, Adjuvant + Ethanol 30% enema, Adjuvant + Ethanol 20% enema, Adjuvant + Ethanol 10% enema, Ethanol 30% enema and Adjuvant + Ethanol 30% enema which was treated with 5/mg/kg/day infliximab. We administered two courses of Freund's complete adjuvant mixed with inactivated total enteric bacteria, then various percentages of ethanol enema used as a barrier breaker to expose the host immune system to its own flora. Colonic status was investigated two weeks after enemas. Macroscopic, histological and biochemical analyses were performed on samples.

Ethanol 30% enema in vaccinated rats caused histological damage and resulted in a significant rise of TNF- $\alpha$ , IL-1, IL-17, myeloperoxidase activity, and oxidative stress biomarkers in comparison to Sham.

This model is an immunogenic and reliable model, which demonstrates microscopic and macroscopic characteristics more similar to human IBD. These findings introduce a novel experimental IBD model and shed light on disease pathogenesis.

## Microbial Pathogenesis

P334

### *ESCHERICHIA COLI* PHYLOGENETIC GROUP DETERMINATION AND ANTIBIOTIC RESISTANCE PROFILE ISOLATED FROM WATER SAMPLES COLLECTED IN AN AQUACULTURE EXPERIMENTA

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The expansion of aquaculture in recent years using intensive cultivation methods including growth promoters and antibiotics under sub-therapeutic conditions, may lead to the appearance of antibiotic resistant bacteria in water, therefore becoming a potential public health problem. Thus, we evaluated the microbiological quality of water samples from different tanks of an aquaculture *experimental* installation, searching for *Escherichia coli* as an “indicator bacteria”. Sixty-eight isolates were obtained and identified through morphophysiological methods and their putative virulence were evaluated by multiplex PCR allowing their classification in different phylogenetic groups as described by Clermont *et al.*, 2000. Also, the susceptibility to different antibiotic groups were determined in all strains. The determined phylogenetic groups revealed, that the majority of the strains belong to phylogenetic group A while only four strains were classified in group B1 generally considered as commensals. All isolates were resistant to erythromycin and showed lower frequency of resistance to streptomycin (25%), amoxicillin (19%) and tetracycline, piperacillin, piperacillin/tazobactam and cephalothin (13%).

## Microbial Pathogenesis

P335

### EVALUATION OF THE PATHOGENIC POTENTIAL OF *AEROMONAS* SPP. USING IN VITRO METHODOLOGIES

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*Aeromonas* spp. have been isolated from virtually every environmental niche known to men and are also present in many raw/semi-processed foods. Ubiquitous in nature, but being considered minor pathogens, these bacteria can be responsible for a wide range of intestinal and extraintestinal diseases. *Aeromonas* infections have been significantly underestimated however, the increasing number of immunocompromised/malnourished individuals, should raise concern regarding aeromonas–water and foodborne outbreaks.

Studies on other enteropathogens indicate that in order to cause gastrointestinal disease they must be able to attach to host target cells, via either toxin production or host cell invasion, or both. Thus, with the aim of evaluating the pathogenicity potential of environmental aeromonads, the present work consisted in testing 24 strains obtained from different sources/countries (e.g. water, food, food processing surfaces and clinical samples), for mammal cell adherence (including adhesion pattern), invasion and cytotoxicity. Cytotoxicity evaluation involved the assessment of cell viability post bacterial–cell contact and post contact with the bacterial culture media.

Results identified 19 (79%) and 13 (54%) strains with the ability to adhere and invade differentiated Caco–2 cells, respectively; while 22 (92%) and 11 (46%) aeromonads revealed the ability to adhere and invade undifferentiated cells. These results indicate that most *Aeromonas* interact optimally with cultured human intestinal cells at cellular sites expressed in the brush border early in the differentiation process of Caco–2 cells. Moreover, in 13 (54%) strains an aggregative adhesion pattern was observed, similarly to those detected in other enteropathogens. Results also showed that 6 (25%) aeromonads express both adherence and extracellular cytotoxicity, while 9 (45%) express mostly cell–contact–dependent cytotoxicity, as evidenced by induced lysis of host epithelial cells.

Overall, *Aeromonas* isolated from water, food and food processing surfaces have adhesive, invasive and cytotoxic patterns, similar or larger than clinical strains. Hence, suggesting that environmental *Aeromonas* spp. have the potential to cause human illness and that food and water sources may act as dissemination vehicles of this human pathogen, with implications in public health.

## Microbial Pathogenesis

P336

### THE EFFECT OF TEMPERATURE ON THE SECRETOME OF *LASIODIPLODIA THEOBROMAE*

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There is evidence supporting the hypothesis that environmental changes modulate the way microorganisms interact with other organisms: the stress imposed by such changes determines alteration of the phenotype expressed by the microorganism.

Factors such as temperature, which can be crucial in the relationship among microorganisms and their hosts, can modulate significant phenotypic characteristics such as virulence.

The fungus *Lasiodiplodia theobromae* is typical from tropical and subtropical regions, being responsible by several pathologies in plants. Occasionally, it acts as an opportunistic pathogen in humans, revealing a large adaptability. Thus, it is crucial to understand which conditions maximize the expression of virulence mechanisms.

The aim of this investigation was to understand how temperature affects two isolates of *L. theobromae* – an environmental isolate, CAA019, and a clinical isolate, CBS339.90.

Extracellular gelatinolytic, xylanolytic, amylolytic and cellulolytic activities at 25°C and 37°C were characterized by zymography. For most conditions, we showed that temperature induced an alteration on the enzymatic profiles of both isolates.

Cytotoxic effect against mammalian cells was quantified in both isolates. The effect of temperature seems to be different depending on the isolates. For the environmental isolate a cytotoxic effect was detected mainly at 25°C while for the clinical one maximum cytotoxic effect was detected at 30°C and 37°C.

The secretome, at 25 °C, 30 °C and 37 °C, was characterized by GeLC MS/MS. Four proteins of the environmental isolate and six proteins of the clinical isolate were identified. Most of the identified proteins are glycoside hydrolases.

We showed that temperature modulates the behavior of *L. theobromae*: of note is the increased expression of a protein involved in cell adhesion by isolate CAA019 at 37°C. We have also detected an apparent adaptation of the clinical isolate to higher temperatures in opposition to the environmental isolate. This modulation may be associated with host-specificity requirements or metabolic activity.

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## Microbial Pathogenesis

### P337/F10

#### IDENTIFICATION OF TYPE III SECRETION SUBSTRATES OF *CHLAMYDIA TRACHOMATIS* USING *YERSINIA ENTEROCOLITICA* AS A HETEROLOGOUS SYSTEM

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*Chlamydia trachomatis* is an obligate intracellular bacterial pathogen that causes ocular and urogenital infections that are a significant clinical and public health concern. *C. trachomatis* uses a type III secretion (T3S) system to manipulate host cells, through the delivery of effector proteins into their cytosol and membranes. In this work, we aimed to identify *C. trachomatis* T3S substrates. We first analysed the genome of *C. trachomatis* L2/434 strain for genes encoding uncharacterized proteins that do not appear to possess a signal of the general secretory pathway and that have not been previously shown to be T3S substrates. We selected 40 genes with these characteristics and analysed T3S of the encoding proteins using *Yersinia enterocolitica* as a heterologous system. We identified 23 *C. trachomatis* proteins whose first 20 amino acids were sufficient to drive T3S of the mature form of  $\beta$ -lactamase TEM-1 by *Y. enterocolitica*. Furthermore, we found that 10 of these 23 proteins were also type III secreted in their full-length versions by *Y. enterocolitica*, providing additional support that they are T3S substrates. Finally, real-time quantitative PCR analysis of expression of genes encoding these 10 proteins showed that 9 of them were significantly expressed during *C. trachomatis* infection of host cells. In summary, we identified 9 *C. trachomatis* T3S substrates (CT053, CT105, CT142, CT143, CT144, CT338, CT429, CT656, and CT849) whose encoding genes were expressed during infection and which therefore could be effectors subverting host cell processes.

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## Microbial Pathogenesis

P338/F09

### DETECTION OF PREMATURE STOP CODONS LEADING TO TRUNCATED INTERNALIN A AMONG FOOD AND CLINICAL STRAINS OF *LISTERIA MONOCYTOGENES*

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*Listeria monocytogenes* is a food-borne pathogen capable of causing listeriosis, a severe invasive disease and responsible for sporadic outbreaks. Internalin A protein (InIA) encoded by *inIA* has a key role in the infection mechanism, being necessary for *L. monocytogenes* to cross the intestinal barrier and to establish a systemic infection. Studies on *inIA* in *L. monocytogenes* have shown that mutations leading to premature stop codons (PMSC) occur naturally and are associated with attenuated virulence of this pathogen. The aim of this study was to evaluate the prevalence of PMSCs in *inIA* of eleven food and eleven clinical strains of *L. monocytogenes* from Portugal. The characterization was accomplished genetically by *inIA* sequencing to find mutations carrying PMSC leading to production of a truncated and secreted InIA. The PMSC were detected in three strains recovered from different food products (namely, from raw chicken, non-fermented sausage, and cheese), one strain recovered from a food-processing environment sample, and in one strain from a human clinical case. These strains belonged to different genoserogroups, including: 1/2a-3a, 1/2b-3b-7 and 1/2c-3c. The mutations found lead to a predicted truncated InIA with 492, 576 and 685 amino acids, have been previously reported by other authors. The remaining seventeen strains tested did not show PMSC in *inIA*. Invasion efficiency of strains with PMSC in the human colorectal adenocarcinoma epithelial cell line (Caco-2 cells) was further evaluated. The results show a lower invasiveness efficiency of strains with PMSC in *inIA* compared to the positive control strain. InIA protein is necessary to cross the intestinal barrier, thus being a critical virulence factor promoting *L. monocytogenes* infection. When *inIA* shows PMSC leading to a truncated form of InIA, the isolate has reduced invasion efficiency. Understanding the importance of attenuated-virulence strains with PMSC in *inIA*, occurring mainly in foods, could be explored to prevent risk of human disease. Despite the important results obtained by Caco-2 invasion assays, it is necessary to study other in vitro and animal models to understand the pathophysiology of listeriosis in vivo.

## Session 7

# Genomics and Systems Biology



# Plenary Lecture

## AN OVERVIEW OF TRANSMISSION PATTERNS AND POPULATION STRUCTURE OF *STAPHYLOCOCCUS AUREUS* INFERRED FROM NEXT-GENERATION SEQUENCING

Edward Feil

*University of Bath, UK*

Next-generation sequence (NGS) data is revealing unprecedented detail as to the molecular evolutionary processes affecting clones of pathogenic bacteria over short time scales. Moreover, a closer synthesis is now becoming possible between changes affecting the genome and spatial and temporal patterns of spread. I will present an overview of what next-generation sequencing revealed concerning clonality and patterns of spread in *Staphylococcus aureus*, and will discuss the opportunities and challenges for the future.

# Keynote Lecture

## KL08

# FUNGAL STATISTICAL PROTEOMES: CAN THEY SHAKE CURRENT CONCEPTS OF THE GENE?

Manuel Santos

*University of Aveiro*

The concept of statistical proteins was proposed in 1965 by Carl Woese in his theory of the origin of the genetic code and the translational machinery (Woese, 1965). Woese defined statistical proteins as mixtures of polypeptides whose primary structures are related to some theoretical average primary structure. In extant organisms they are synthesized through low level mistranslations of mRNA arising from tRNA misacylation, ribosome misreading, RNA editing and RNA modification. Canonical gene translation produces homogeneous mixtures of polypeptides, but statistical proteins are heterogeneous arrays of polypeptides whose cellular functions may also be heterogeneous, complicating the already complex relationship between genes and phenotypes. We have discovered that the main human fungal pathogen *Candida albicans* has a statistical proteome (Gomes et al, 2007). Its genome encodes 6202 protein genes – a similar number of genes to other fungi –, but ambiguous gene translation by the ribosome results in millions of different proteins that are not degraded by the *C. albicans* protein quality control machinery. In this fungus, there is no correlation between gene number and protein number despite the lack of alternative splicing. I will illustrate in my talk that statistical proteins are produced through global re-programming of the genetic code and have specific functions. They increase dramatically phenotypic and genetic diversity, expand adaptation capacity in changing ecological landscapes and influence virulence, biofilm formation and drug resistance. That is, statistical proteins allow *C. albicans* to produce phenotypic diversity in the absence of alternative splicing and without increasing gene number.

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# Oral Presentations

OP09

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF A NEW SOLVENT-CAST FILM  
BASED ON A FUNCTIONALIZED ELASTIN-LIKE POLYMER

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The spread of antimicrobials resistant microorganisms and the lack of new antibiotic discoveries in last years have triggered the search for new advanced antimicrobial materials for many fields of application, especially for medical devices. These advanced materials need to have the ability to kill microorganisms without damaging human tissues. Recent advances in material fabrication technologies and characterization, and especially through the use of synthetic biology approaches, it is now possible to reengineer novel functionalities and structures of protein-based materials, by taking advantage of their extreme versatility and applicability.

The combination of antimicrobial peptides (AMPs) with recombinant protein-based polymers can be explored for the development of advanced medical devices, to overcome biofilm formation and nosocomial infections. AMPs are normally cationic, small molecules that occur as part of the innate immune system in many organisms, and present even in microbes and virus. Here we describe the design, biological production and processing of a protein-based-polymer containing a functional domain based on a synthetic cationic AMP, ABP-CM4, fused in frame with an elastin-like-polymer consisting of 200 repeats of the structural unit VPAVG (A200). The functionalized protein-based-polymer was produced in *Escherichia coli* and further purified by exploring the thermoresponsiveness property of poly-VPAVG.

Free standing films, were obtained by solvent-cast especially thinking in future downstream processing for its application in coating of medical devices. The antibacterial and antifungal activities of the cast films were confirmed against different bacterial and fungal species, by *in vitro* and *ex vivo* assays. The recombinant CM4::A200 biopolymer displayed high growth inhibition against a wide range of bacterial species, both gram negative and positive, and yeast species. The antimicrobial activity was time dependent and remarkably, in some bacterial species, almost 100% of cell death was detected after 30 minutes of cell suspension in contact with cast films.

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## OP15

## PHAGEDUCTION: A NEW TECHNOLOGY FOR CELL-TARGETED DNA DELIVERY

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Technologies for delivering genes into cells have been, still are and will continue to be on the basis of many biotechnological and medical applications. Essentially, gene delivery strategies can be classified in viral and non-viral, depending on whether viruses (or their modified forms) or designed molecules are used as delivering vehicles. The most successful technologies currently available still fail to completely fulfill the requirements of an ideal system, that is, one that allows efficient and stable gene delivery, in a cell-specific and non-toxic manner, independently of the desired target cell and at reasonable costs (1). This work is a prospect for the development of a new cell-targeted DNA delivering technology, here designated as PhageDuction, where we will change the natural tropism of a virus so that it can deliver its DNA cargo to new target cells. PhageDuction builds on the extensive knowledge that our lab and collaborators have been accumulating on the system bacteriophage SPP1 and its cellular receptor, the *Bacillus subtilis* membrane protein YueB. We have shown that there are only three essential requirements for virus SPP1 DNA ejection into host cells: the presence of a YueB receptor ectodomain at the *B. subtilis* surface, an energized cytoplasmic membrane and a calcium gradient between the extra- and intracellular milieu. Since the vast majority of cells growing under physiologic conditions are known to maintain a membrane potential and a calcium gradient, then SPP1 should be able to deliver its genome, and any cloned foreign DNA to heterologous cells that had been decorated with the phage receptor. To accomplish this we have envisaged that specific ligands fused to the YueB ectodomain would target the receptor to specific cell surface epitopes. In this communication we will present a proof of concept of PhageDuction by showing that SPP1 is able to deliver its DNA content to *B. subtilis* and other bacterial species lacking YueB, after decorating their surface with a YueB-LysM fusion. The LysM motifs are widespread among proteins designed to target peptidoglycan, a general component of the bacterial cell wall (2). Other YueB fusions are currently under study that aim the targeting of other cells, including mammalian cells.

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## OP16

### DEVELOPMENT OF A MINICIRCLE PRODUCTION SYSTEM BASED ON PARARESOLVASE-MEDIATED *IN VIVO* RECOMBINATION

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Minicircles are small supercoiled DNA molecules devoid of bacterial backbone that exhibit increased transfection efficacy and transgene expression when compared to their parental plasmid molecules. The use of minicircles instead of plasmids in transient gene expression in mammalian cells, DNA vaccination and gene therapy is hindered by the lack of cost-effective manufacturing processes able to produce high amounts of minicircle molecules. Minicircles are produced *in vivo* by intra-molecular site-specific excision of parental plasmid molecule into two supercoiled pDNA molecules: i) a minicircle (MC) that carries the transcription unit required for therapeutic gene expression and ii) a replicative miniplasmid (MP) that contains the unwanted bacterial elements. In this work, minicircle production system relies on the enzymatic activity of ParA resolvase, a recombinase that is expressed under the transcription control of the arabinose inducible expression system  $p_{BAD}/AraC$ , and on *E. coli* strain improved for arabinose uptake. Tight control of recombination is crucial to maximize minicircle yields and purity. Undesired recombination already after 4 hours of incubation in Luria-Bertani broth at 37°C was observed due to the leaky expression from  $p_{BAD}/AraC$ . While addition of glucose to the growth media repressed this leaky expression, it triggered a pH drop to 4.5 during exponential phase in shake flasks, which suppressed growth and plasmid production. Hence, to ensure the stability of minicircle-producing system, seed cultures should be grown at 30°C with glucose overnight whereas cells for minicircle production should be grown at 37°C without glucose up to early stationary phase when the recombination is induced by addition of arabinose.

For final protocol, we wanted to express ParA resolvase under the regulation of the  $P_{BAD}/araC$  promoter and with an optimised ribosome binding site (RBS) from a single copy of the gene in the chromosome. The engineered strain BW2P containing optimised RBS exhibited faster and more effective parental plasmid excision reaching near complete recombination after induction of late exponential cells grown in shake flasks with 0.01% (w/v) arabinose. At high density cell cultivation in bioreactors, the recombination efficiency varied from 75–100% depending on induction cell phase: Volumetric productivities between 47.0–58.4 mg total pDNA per liter were obtained that are at least 10 fold higher than those reported in the literature.



## OP17

## THE MAJOR FACILITATOR SUPERFAMILY MULTIDRUG RESISTANCE TRANSPORTERS (MFS-MDR) IN HEMIASCOMYCETOUS YEASTS: PHYLOGENETIC AND SYNTENIC ANALYSES

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The simultaneous acquisition of resistance to a wide range of structurally and functionally unrelated cytotoxic chemicals (multidrug resistance, MDR) may result from the action of drug-efflux pumps, such as those belonging to the Major Facilitator Superfamily (MFS) [1]. In the hemiascomycetous yeasts, the MFS-MDR transporters belong to the 12-spanner drug:H<sup>+</sup> antiporter family 1 (DHA1) and the 14-spanner drug:H<sup>+</sup> antiporter family 2 (DHA2). The translated ORFs of 31 yeast strains of the hemiascomycete sub-phyla were compared using a local sequence similarity algorithm and a network was built representing this pairwise similarity data [2, 3]. The MFS-MDR proteins were identified by constraining and traversing this network. Tree building methods and gene neighbourhood analysis were combined to allow the reconstruction of the evolutionary history of the encoding genes

The 20 clusters' classification of the DHA1 protein family proposed [2] expands considerably a previous classification. Sixteen DHA1 gene lineages were formed during the evolution of the Hemiascomycetes. The chromosome environments where DHA1 genes reside is remarkably conserved in the *Saccharomyces* and CTG complex species, with the exception of the lineage containing the *FLR1/MDR1* homologs. The evolutionary history of genes associated with the development of fluconazole resistance in *Candida* clinical strains was detailed, in particular the evolutionary history of *CaMDR1*, *CaFLU1*, *CdMDR1*, *CtMDR1* and *CpMDR1*.

The analysis of the 14-spanner MFS-MDR amino acid sequences revealed that the ARN siderophore transporters and the GEX glutathione:H<sup>+</sup> antiporters were jointly gathered with the DHA2 proteins [3]. The combined tree building/gene neighbourhood strategy adopted identified 7 DHA2 gene lineages, 5 ARN gene lineages, and 1 GEX gene lineage. The hypothesis that these transporters share a common evolutionary root is the explanation that better fits the results of this phylogenetic study and we propose a new family (DAG) to accommodate the DHA2, ARN and GEX proteins, spanning these three phylogenetic subfamilies of 14-spanner MFS transporters.

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[2] Dias PJ et al. (2010). OMICS. 14: 701–710.

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# Poster Presentations

## Bioinformatics

P339

COMPUTATIONAL MODELS REVEAL GENOTYPE–PHENOTYPE ASSOCIATIONS IN *SACCHAROMYCES CEREVISIAE*Ricardo Franco–Duarte<sup>1</sup>; Inês Mendes<sup>1</sup>; Lan Umek<sup>2</sup>; João Drumonde–Neves<sup>1</sup>; Blaz Zupan<sup>2</sup>; Dorit Schuller<sup>1</sup><sup>1</sup>CBMA (Centre of Molecular and Environmental Biology) / Department of Biology / University of Minho, Braga, Portugal<sup>2</sup>Faculty of Computer and Information Science, University of Ljubljana, Ljubljana, Slovenia

Genome sequencing is essential to understand individual variation and to study the relationship between genotype and phenotype. Recently, large–scale sequencing projects of *Saccharomyces cerevisiae* revealed the existence of a few well defined lineages and some mosaics of that lineages, and suggested the occurrence of two domestication events during the history of association to human activities, one for sake strains and one for wine yeasts.

The objective of the present work was to undertake high–throughput approaches for a genetic evaluation of 172 *S. cerevisiae* strains from different geographical origins and technological uses, and computationally relate the results with 30 phenotypic tests that were previously obtained. Genetic characterization was performed using eleven polymorphic *S. cerevisiae* specific microsatellite loci, and revealed 280 alleles from 11 loci, with microsatellite ScaAT1 contributing the most to intra–strain variability, together with the alleles 20, 9 and 16, from the microsatellites ScaAT4, ScaAT5 and ScaAT6. These microsatellite allelic profiles are characteristic both for the phenotype and the origin of yeast strains. We confirm the strength of these associations by construction and cross–validation of computational models that can predict the technological application and origin of a strain from the microsatellite allelic profile. The phenotypes associated with higher number of alleles were the reduced production of hydrogen sulphide and the high resistance to cycloheximide. Microsatellites C11 and ScYPL009c, and alleles ScaAT1–49 and ScaAT2–19 were strongly associated with phenotypes, while for microsatellites ScaAT3 and ScaAT4 the association was weak. We have also observed a tendency between alleles with higher repeat numbers and a larger number of phenotypic associations, mainly for loci ScaAT1, ScaAT2 and C11, indicating the importance of microsatellite allelic size variations as genomic characteristics that shape the phenotypic landscape of *S. cerevisiae* strains.

Our study demonstrates the usefulness of the developed computational models to estimate a strain technological group and phenotype from microsatellite allelic combinations. Our findings can contribute to the development of new tools for yeast strain selection and improvement.

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## Bioinformatics

### P340

#### INFERRING MICROBIAL SYNERGIES USING INTER-SPECIES PROTEIN INTERACTION PREDICTION DATA

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There have been great efforts in the Microbiology research area to identify synergies between different microbial species. Still, since this identification is usually performed using *in vitro* experimental methods (*i.e.* microbial cultures), it takes a considerable amount of time and resources to successfully identify these relationships.

Symbiotic relationships are generally defined as the living together of apart organisms. While said definition is deterrent to only two generic organisms, it is common knowledge that there is a great number of microbial species living within us human beings. Eventually, different microorganism species inhabiting the same region will develop a community. This implies several degrees of cooperation, such as the exchange of substrates, inter-species protein-protein interactions, and the creation of nutritional and atmospheric gradients.

Considering for instance the oral cavity, early colonizers will adhere to the dental surface and begin to proliferate. These are usually aerobic bacteria, capable of surviving oxygen-rich environments. Secondary colonizers attach to the bacteria present in the newly formed dental plaque, creating numerous microbial layers. Such sequential layer placement greatly reduces oxygen levels, forming an atmospheric gradient and, therefore, allowing colonization by anaerobic organisms. Even though being indirect, this is an example of microbial synergism.

Currently, the Human Microbiome Project (HMP) Data Analysis and Coordination Center (DACC) comprises 14 different microbiome categories, with the oral cavity being just one of those. Knowing this, the relevance of developing a methodology to predict microbial synergies in a fast and affordable manner becomes clear. In this work we propose a computational approach to predict synergies between different microbial species.

To do this, we use data from predicted inter-species protein interactions within the oral cavity from a previous work - OralCard (available at <http://bioinformatics.ua.pt/OralCard/>). We then identify all the unique organism-organism pairs and the respective numbers of interactions between each organism pair. Then, we calculate the Maximal Information Coefficient for each organism pair in order to identify candidate synergies. Additionally, we expand the predictive protein interaction network obtained in our previous work with metabolic pathways data using the Cytoscape open-source network visualization software. Finally, case-studies will be performed in order to validate the proposed approach.

We strongly believe this methodology can produce a series of high-quality synergy predictions, which can be subsequently used for better understanding disease-onset mechanisms and potentially for the preliminary screening of infectious diseases.

## Bioinformatics

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### PGP: PROKARYOTE GENE PREDICTION SOFTWARE

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Correct gene prediction and annotation of bacterial genomes is essential to apply sequence data in many (bio)medical research topics such as microbiology, immunology and infectious diseases. Even though there are several methods for bacterial gene prediction like Glimmer [1] GenemarkHMM [2] and Prodigal [3] and complete pipelines such as ISGA [4], xBASE [5] and Consensus Prediction [6], we believe that gene predictions can be improved, combining *ab initio* and homology methods.

We developed PGP, a pipeline for bacterial gene prediction using *ab initio* and homology methods. The *ab initio* software Prodigal was used as starting point for PGP, due to better performance over Glimmer and GenemarkHMM. Next, we performed homology searches with BlastP with the predicted proteins against the NCBI database. PGP analysed alignment results and determined the need to shorten or extend genes, and introduced the required corrections. To test PGP, we used 8 bacterial genomes, *Escherichia coli* K12 MG1655, *Bacillus subtilis* 168, *Lactobacillus plantarum* WCFS1, *Lactococcus lactis* KF147, *Streptococcus pneumoniae* TIGR4, *Salmonella enteric* subsp. Enteric serovar Typhi str. Ty2, *Neisseria meningitidis* MC58 and *Haemophilus influenzae* Rd KW20 from NCBI.

PGP final result was compared to the respective reference genome, and the error rate and sensibility were calculated [6]. Furthermore, PGP average error rate and sensibility were compared to those of ISGA, xBASE and Consensus Prediction for the 8 genomes. The average error rate and sensibility for PGP, ISGA and xBASE, and Consensus Prediction were: 15% and 96%; 21% and 96%; 19% and 91%, respectively.

PGP improved correct gene prediction in 6% comparing with ISGA and xBASE, and 4% compared to Consensus Prediction, while maintaining a 96% of sensibility. The preliminary results here described are quite promising and further developments will create an improved tool for bacterial gene prediction.

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## Bioinformatics

### P342

## PHENO-METABOLOMICS CHARACTERIZATION OF A *SACCHAROMYCES CEREVISIAE* STRAIN COLLECTION BY INTEGRATIVE DATA ANALYSIS APPROACHES

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The objective of the present work was to characterize the pheno-metabolomic diversity of a *Saccharomyces cerevisiae* wine strain collection, by combining genetic, phenotypic and metabolomic data using bioinformatic approaches.

A strain collection was constituted comprising 172 strains with different geographical origins and technological uses. Phenotypic screening (30 physiological tests) and genetic characterization (11 microsatellite loci) was previously performed [1], and revealed strain variability. Must fermentations were performed with each strain and from the combined data (fiber optics spectroscopy, physiological and molecular characterization) a sub-group of 24 strains were chosen for further approaches.

HPLC analysis revealed variable results, with glucose, fructose and acetic acid contributing most for inter-strain variability. Relevant metabolites related to aromatic profiles were determined by GC-MS. PCA showed significant variance between strains with a clear separation between the amounts of alcohols and esters produced, according to the technological group. Hexyl acetate, ethyl octanoate and decanoic acid showed the highest weight in strain variability, according to the PCA plot. Partial least squares discriminant analysis (PLS-DA) was then used in a pairwise approach to predict the metabolic profiles of the strains, using phenotypic and genetic data. For 15 (70%) of the 24 metabolites, the PLS model found associations (coefficient of correlation above 0.80) with phenotypic or genetic data.

A more holistic matrix factorization approach [2] was then used to project data onto a common system of coordinates, in which the most related variables are weighted together and placed apart from the axis origin. The search for multi-dimensional modules is currently underway, to obtain a group of most-correlated features.

This work was financially supported by the program PTDC (AGR-ALI/103392/2008).

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## Bioinformatics

### P343

#### THE GENE POOL OF SEQUENCED PLASMIDS – WHAT CAN WE LEARN?

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Adaptation of prokaryotes is a consequence of the diversity and plasticity of their genomes. Massive sequencing projects have revealed that genomes are not rigid and finite structures, and up to 20% of their gene content is considered to have origins in other microbial organisms. The flow of genes across prokaryotes is carried out by mobile genetic elements that have shown to foster the mobilization and rearrangement of genes.

Albeit the sequence information is accumulating in databases, the *in silico* analysis of this gene flow has been lagging behind. To address this, we looked at 4156 complete plasmid sequences available at NCBI database. Using a text-mining approach we investigated the distribution of genes coding for transposases, integrases, conjugative proteins, virulence and antibiotic resistance factors in prokaryotes. Plasmids from Archaea represent only 3.1% of the total sequences whereas plasmids from Bacteria represent more than 95% of the plasmids. More than 30% of plasmids are bigger than 50 Kb and nearly 10% of them are linear. The presence of transposases and/or integrases correlates with the presence of peaks of 1–2 Mb in the size of plasmids, presumably due to the presence of large transposons. The total number of potentially protein-encoding sequences contained in plasmids ascends to 318074, 50% of which are hypothetical proteins. Among Proteobacteria, this trend is maintained across different genera. The proportion of genes coding for virulence factors and antibiotic resistance is higher in the  $\beta$ -proteobacteria, followed by the  $\alpha$ -,  $\gamma$ - and lastly the  $\epsilon$ -proteobacteria. Generally, there is an overlap between genes coding for resistance and virulence and conjugative elements, the only exception are the  $\beta$ -proteobacteria in which resistance genes and virulence factors seem to be located on mobilizable or non-mobilizable plasmids rather than conjugative plasmids.

The high number of putative proteins carried by plasmids opens new horizons of research and the biological significance of their presence in autonomously replicating genomic elements. This *in silico* analysis constitutes a preliminary basis to design strategies that could facilitate risk assessment studies and understand how genes are shuffled across different species.

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## Comparative Genomics and Evolution

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### GENETIC VARIATION AND SPECIES BOUNDARIES IN THE BASIDIOMYCETOUS YEASTS *CRYPTOCOCCUS VICTORIAE* AND *C. CARNESCENS* (TREMELLALES) – A MULTIGENE APPROACH

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*Cryptococcus victoriae* and *C. carnescens* are phenotypically indistinguishable sister species that may be mistaken for *C. laurentii* based on phenotype. Phylogenetic separation between *C. victoriae* and *C. carnescens* was based on LSU and ITS sequence analyses, but very little is known on their intraspecific genetic variability or population structure. In the present study we examined 100 strains of the two species from different substrates and geographic locations, and used an MLST approach to assess genetic variation and re-examine species boundaries. The following six loci were chosen for sequencing: the LSU rRNA gene (D1/D2 domains), the ITS region, the IGS1 spacer, and fragments of the genes encoding the largest subunit of RNA polymerase II (*RPB1*), second largest subunit of RNA polymerase II (*RPB2*) and translation elongation factor 1 alpha (*TEF1*). We performed separate phylogenetic analyses to determine the discriminatory power of each locus. Amplification of IGS1 was inconsistent and the few sequences obtained were apparently not species-specific, while *TEF1* was highly variable and intron number and position varied among strains of the same species. However, *TEF1* coding sequences were too conserved and not informative. IGS1 and *TEF1* were therefore not included in the MLST analysis. The *RPB1* gene fragment showed the highest inter- and intraspecific variability (56 and 31 alleles in *C. victoriae* and *C. carnescens*, respectively), while LSU showed the highest conservation (5 alleles for each species). Phylogenetic network analyses of the concatenated sequences of four loci confirmed the genetic separation of the two species and revealed two additional related undescribed species. Our analyses also showed a high degree of genetic heterogeneity within *C. victoriae*, but the relationship between the different lineages was not clearly resolved and further analyses are required to define the limits of this species. In contrast, *C. carnescens* was more homogenous and a group of two strains formed a clearly separate lineage that may represent an undescribed species. Geographic origin and substrate of isolation did not reflect the phylogenetic relationships of strains from either species. Our results emphasize the importance of studying large numbers of strains and argue against using single barcode markers to determine species boundaries, which should rely on multigene approaches.

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## Comparative Genomics and Evolution

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## GENOMIC DIVERSITY OF DRUG-RESISTANT MYCOBACTERIUM TUBERCULOSIS ISOLATES IN LISBON PORTUGAL: TOWARDS TUBERCULOSIS GENOMIC EPIDEMIOLOGY

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Multidrug- (MDR) and extensively drug resistant (XDR) tuberculosis (TB) present a challenge to disease control and elimination goals. Lisbon, Portugal, has a high TB incidence rate and, unusual and successful XDR-TB strains that are found in circulation for almost two decades.

In the present study, 56 *Mycobacterium tuberculosis* isolates, mostly recovered in Lisbon, were genotyped by 24-*loci* Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeats (MIRU-VNTR) and the genomes sequenced using a next generation sequencing platform – Illumina HiSeq 2000. The genotyping data revealed three major clusters associated with MDR-TB (Lisboa3-A, Lisboa3-B and Q1), two of which associated with XDR-TB (Lisboa3-B and Q1). Whilst the genomic data contributed to elucidate the phylogenetic positioning of circulating MDR-TB strains, showing a high predominance of a single SNP cluster group 5. Furthermore, a genome-wide phylogeny analysis from these strains, together with 19 publicly available genomes of *Mycobacterium tuberculosis* clinical isolates, revealed two major clades responsible for M/XDR-TB in the region: Lisboa3 and Q1. On the overall, 9419 different SNPs were identified, ranging between 488 – 1465 per isolate (mean: 928 SNPs/isolate).

The data presented by this study contributes to the expanding knowledge of *Mycobacterium tuberculosis* genomic diversity yielding insights on microevolution and identification of novel compensatory mutations associated with rifampicin resistance in *rpoB* and *rpoC*. The screening for other structural variations revealed putative clade-defining variants. One deletion in PPE41, found among Lisboa3 isolates, is proposed to contribute to immune evasion and as a selective advantage. Insertion sequence (IS) mapping has also demonstrated the role of IS6110 as a major driver in mycobacterial evolution by affecting gene integrity and regulation. A total of 251 candidate insertion sites were detected, of which 105 were intergenic and 64 were predicted to have a putative upregulatory effect.

Additionally, the analysis of non-synonymous/synonymous ratios revealed heterogeneities across the chromosome, genotype and Clusters of Orthologous Groups, highlighting possible and different evolution strategies. Globally, our data supports the notion of a growing genomic diversity facing both setting and host adaptation.

## Comparative Genomics and Evolution

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### HINTS ON THE EVOLUTION OF MATING SYSTEMS IN BASIDIOMYCETES COMING FROM THE ORDER LEUCOSPORIDIALES

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In Basidiomycetes, sexual reproduction systems rely on the conjugation of two cells carrying genetic information to specify a distinct sexual identity/mating type. Two classes of proteins define mating type in this phylum: lipopeptide pheromone and plasma membrane pheromone receptors (P/R) mediate recognition of a compatible mating partner, while homeodomain transcription factors (HD) control post-mating behavior. The regions encoding these proteins are called MAT loci and, depending on their arrangement in the genome and number of different allelic forms, establish a determined mating system. When P/R and HD regions are in linkage they form a bipolar system, which is typically biallelic and therefore specifies two mating types. In turn, if the MAT loci are unlinked, they create a tetrapolar system, which generates four mating types out of each mating cross, and is usually multiallelic for at least the P/R or the HD genes. Yet a third mating system has been described in Sporidiobolales: the pseudobipolar system, which is intermediate between the other two, as it is not strictly bipolar, but rather allows rare events of independent segregation of the P/R and HD genes and, thereby, the generation of more than two mating types.

Within the subphylum Pucciniomycotina, which contains bipolar and pseudobipolar species, *Leucosporidium scottii* appears as the only saprophytic yeast proposed to have a tetrapolar mating system. The present work aims at providing molecular evidence for this tetrapolarity, through 1) the search for a genomic signature of MAT loci in tetrapolar systems in the genome assemblies we generated; 2) the analysis of molecular mating types' diversity in a collection including mainly wild isolates; finally, we intend to 3) identify the genomic localization of the P/R and HD alleles in this species, presumably present in two different chromosomes, by chromoblot inspection.

We have found that gene composition and even gene order within *L. scottii* MAT loci are quite similar to the ones from members of the neighbor order Sporidiobolales. In addition, sequence analysis of HD genes from 26 strains from *L. scottii* revealed the presence of thirteen HD alleles, a diversity commonly found in tetrapolar species. However, all the strains possessing the same HD allele also possessed the same P/R locus, except for the case of two strains, a finding that strongly argues against the possibility that these loci are genetically unlinked. Together, these observations suggest *L. scottii* may have a pseudobipolar mating system and that, therefore this intermediate MAT loci configuration may be quite disseminated in the early diverged Pucciniomycotina lineage of Basidiomycetes.

## Comparative Genomics and Evolution

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### MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *LIGNICOLOUS LULWORTHIALES* FROM PORTUGUESE WATERS

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Marine fungi occur mainly in coastal marine environments where lignicolous substrates are abundant. They are the major decomposers in marine and estuarine conditions, being vital to the recycling of nutrients. Since two decades ago, Portuguese marine fungal communities have been studied and characterized based on morphological features. *Lulworthia* spp. have been frequently detected on a wide range of substrates such as *Spartina maritima* plants and baits [1, 2, 3], woody and herbaceous drift substrates [4, 5] and *Fagus sylvatica* and *Pinus pinaster* baits [6, 7]. Considering the availability of a high number of isolates of Lulworthiales, most of them not identified to species level, from a survey involving two Portuguese marinas [6, 7] this study intended their morphological and molecular characterization, aiming identification down to species level.

Procedures related to detection and morphological characterization and identification followed the current guidelines of classical taxonomy. The molecular characterization was based on the amplification and sequencing of rDNA regions, initially the LSU locus. Consensus sequences of 40 isolates selected for this study were aligned with sequences retrieved from GenBank and subjected to phylogenetic analysis.

Attending to their morphological features, 2 isolates were identified as *Zalerion maritima* and 38 isolates were identified as *Lulworthia* sp.. The phylogenetic analysis placed our isolates into 3 clades already defined for the Lulworthiales [8]. The isolates identified as *Z. maritima* were placed in the *Lulwoana uniseptata/Zalerion maritima* clade and the 38 isolates of *Lulworthia* genus were distributed into two clades, both strongly supported by high bootstrap values: *Lulworthia* sp. clade (32 isolates) and *Lulworthia purpurea* clade (6 isolates).

Considering the need for a more extensive molecular data, 10 isolates of *Lulworthia* sp. were selected for a multiloci analysis involving the SSU and ITS rDNA regions. Results from these analyses will be presented and evaluated.

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4– Figueira et al. 2007. *Mycologia* 99: 20–23

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6– Azevedo et al. 2010. *Nova Hedwigia* 90: 521–531

7– Azevedo et al. 2011. *Animal Biodiversity* 34: 205–215

8– Campbell et al. 2005. *Mycol. Res.* 109: 556–568

## Comparative Genomics and Evolution

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### EVOLUTION OF FUNGAL MATING SYSTEMS: INSIGHTS FROM RED YEASTS GENOMES

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A key evolutionary aspect common to all organisms is the way by which species exchange and transfer genetic material, a process better known as reproductive or mating system. In Fungi, but also in other groups of organisms, deviations and transitions in mating systems may have been crucial to the emergence of different lifestyles since these changes usually influence the genetic variation in populations.

Within Fungi, basidiomycetes have developed a complex and fascinating mating system involving two sets of genes: (i) lipopeptide pheromones and their cognate receptors (P/R) that coordinate cell-cell recognition leading to cell fusion, and (ii) compatible homeodomain transcription factors (HD) that regulate the progression through the sexual cycle. When both *MAT* genes are determining mating type, their genomic organization defines the mating system as bipolar (if P/R and HD are located the same chromosome and genetically linked) or tetrapolar (if the genes are unlinked, e.g. located in different chromosomes). In addition, we proposed a third mating configuration referred to as pseudo-bipolar in the saprobic red-pigmented yeasts *Sporidiobolus salmonicolor* and *Rhodospidium babjevae* [1]. This system was suggested based on two main observations: firstly, we found multiple HD alleles in natural isolates of these species, but each allele was always associated with only one of the two P/R alleles; and secondly, we found that recombination may occur (albeit rarely) between P/R and HD genes as assessed in the progeny of a laboratory cross between *S. salmonicolor* compatible strains. This deviation from the bipolar-tetrapolar mating paradigm formed the basis of our current research that aims to expand the characterization of the pseudo-bipolar system. Therefore, we have obtained whole-genome sequence data from two different mating types of *S. salmonicolor* and *R. babjevae*, which is currently allowing us to exactly determine the extension and genetic structure of the *MAT* locus, while providing new clues towards the elucidation of the mechanisms contributing to suppression of recombination within this region. Finally, and to complement this, we are also determining the chromosomal localization of the P/R and HD genes, which involved setting up a protocol for separation of chromosomes by PFGE for these species, followed by chromoblot using *MAT* gene-specific probes.

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## Functional Genomics

## P349

BUILDING AN ATLAS OF HFQ-DEPENDENT SMALL NON-CODING RNAs FROM THE OPPORTUNISTIC HUMAN PATHOGEN *BURKHOLDERIA CENOCEPACIA* J2315

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Small non-coding regulatory RNAs (sRNAs) play important roles in regulating gene expression at the post-transcriptional level and often require the RNA chaperone Hfq. The human opportunistic pathogen *Burkholderia cenocepacia* J2315 encodes two distinct RNA chaperones, Hfq and Hfq2. The present work describes the experimental identification and validation of 24 Hfq-dependent and 38 Hfq2-dependent sRNAs from *B. cenocepacia* J2315, based on the co-purification of sRNAs with the bacterium Hfq or Hfq2 protein, followed by conversion into cDNA, cloning, computational analysis of sequences and validation by Northern blot analysis. These sRNAs are unevenly distributed within the genome: 21 in the larger chromosome1, 35 in chromosome 2, and 4 in chromosome 3. The sRNAs here reported escaped identification by previous studies based on transcriptomics or bioinformatic analyses. Nonetheless, using a custom-made microarray, a total of 105 putative sRNA-encoding genes were validated based on the detection of the corresponding transcripts in the microarrays. However, only 19 and 76 sRNAs were found as differentially expressed in the  $\Delta hfq$  and  $\Delta hfq2$  mutants, respectively. Results presented indicate that 3 Hfq-binding sRNAs are exclusive to bacteria of the *Burkholderia cepacia* complex and have no homologues in other bacteria, while the other 21 share homology, at different extents, to sRNAs of other bacterial species.

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## Functional Genomics

### P350

## IDENTIFICATION OF SMALL NON-CODING RNAs AND THEIR ROLES ON THE BIOLOGY OF OPPORTUNISTIC PATHOGENS OF THE BURKHOLDERIA CEPACIA COMPLEX

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Small non-coding regulatory RNAs (sRNAs) play important roles in regulating gene expression at the post-transcriptional level, often requiring the RNA chaperone Hfq to interact with their mRNA targets. Hfq is a RNA chaperone that plays a major role in riboregulation, promoting the interaction of RNAs [1]. Burkholderia cepacia complex are among the few prokaryotes that encode two distinct and functional Hfq-like proteins in their genome [2,3]. Our research group has initiated an experimental strategy envisaging the identification of sRNAs from B. cenocepacia J2315 based on co-precipitation of total RNA with the two Hfq-like proteins, Hfq and Hfq2 [3,4].

A total of 40 sRNAs were identified and validated based on an experimental based on co-precipitation experiments of total RNA from B. cenocepacia J2315 with Hfq and Hfq2, followed by conversion into cDNA, cloning, computational analysis of sequences and validation by Northern blot analysis. Results indicate that some sRNAs are exclusive to bacteria of the Burkholderia cepacia complex and some have no homologues in other bacteria, while the others share homology, at different extents, to sRNAs from other bacterial species. Ongoing work involves the use of bioinformatics to orientate the functional analysis of sRNAs identified.

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## Functional Genomics

## P351

***P. BRASILIENSIS* VIRULENCE IS AFFECTED BY SCONC, THE NEGATIVE REGULATOR OF INORGANIC SULFUR ASSIMILATION**

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Conidia/mycelium-to-yeast transition of *Paracoccidioides brasiliensis* is a critical step for the establishment of paracoccidioidomycosis, a systemic mycosis endemic in Latin America. Thus, knowledge of the factors that mediate this transition is of major importance for the design of intervention strategies. So far, the only known pre-requisites for the accomplishment of the morphological transition are the temperature shift to 37°C and the availability of organic sulfur compounds. In this study, we investigated the auxotrophic nature to organic sulfur of the yeast phase of *Paracoccidioides*, with special attention to *P. brasiliensis* species. For this, we addressed the role of SconCp, the negative regulator of the inorganic sulfur assimilation pathway, in the dimorphism and virulence of this pathogen. We show that down-regulation of *SCONC* allows initial steps of mycelium-to-yeast transition in the absence of organic sulfur compounds, contrarily to the wild-type fungus that cannot undergo mycelium-to-yeast transition under such conditions. However, *SCONC* down-regulated transformants were unable to sustain yeast growth using inorganic sulfur compounds only. Moreover, pulses with inorganic sulfur in *SCONC* down-regulated transformants triggered an increase of the inorganic sulfur metabolism, which culminated in a drastic reduction of the ATP and NADPH cellular levels and in higher oxidative stress. Importantly, the down-regulation of *SCONC* resulted in a decreased virulence of *P. brasiliensis*, as validated in an in vivo model of infection. Overall, our findings shed light on the inability of *P. brasiliensis* yeast to rely on inorganic sulfur compounds, correlating its metabolism with cellular energy and redox imbalances. Furthermore, the data herein presented reveal SconCp as a novel virulence determinant of *P. brasiliensis*.

## Functional Genomics

## P352

SCREENING OF A *ZYGOSACCHAROMYCES BAILII* GENOMIC LIBRARY TO SEARCH FOR GENES RESPONSIBLE FOR ACETIC ACID RESISTANCE

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*Zygosaccharomyces bailii* is considered the most problematic food spoilage yeast, particularly in acidic foods and drinks due to its exceptional capacity to resist to high concentrations of weak acids widely used as fungistatic preservatives at low pH. However, the mechanisms underlying its intrinsic high resistance to acetic acid remain poorly characterised. A *Z. bailii* ISA1307 genomic library previously prepared [1] was screened for genes responsible for acetic acid resistance by transformation of the acetic acid highly susceptible *Saccharomyces cerevisiae* BY4741\_Δ*haa1* deletion mutant. The expression of 31 different *Z. bailii* DNA inserts, ranging from 1 to 6 kb, from the *Z. bailii* genomic library plasmids was found to significantly increase Δ*haa1* resistance to acetic acid. The identification and *in silico* analysis of these DNA inserts were based on the genome sequence of *Z. bailii* ISA1307 strain recently available (Abstract submitted to Microbiotec 2013). A total of 32 complete ORFs and 33 truncated ORFs were identified as putative determinants of resistance to acetic acid. A putative *S. cerevisiae* gene homologue was found for most of the identified ORFs, clustered into functional classes according to their biological function. The most representative classes found in the dataset are “Transcription”, “Cell cycle and DNA processing”, “Cellular transport, transport facilities and transport routes”, “Protein synthesis”, “Protein fate” and “Lipid, fatty acid and isoprenoid metabolism”. Approximately 15% of these yeast ORFs were already found to be involved in acetic acid resistance in *S. cerevisiae* [2]. *Z. bailii* genomic library plasmids containing a sole ORF were expressed in the haploid *S. cerevisiae* deletion mutants lacking the homologous gene. Based on phenotype complementation results obtained by comparing growth susceptibility in the presence of acetic acid, we propose *GYP8* (encoding a GTPase-activating protein involved in the regulation of ER to Golgi vesicle transport) and *TIF3* (encoding a translation initiation factor) as determinants of acetic acid resistance in *Z. bailii*.

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## Synthetic Biology

P353

### A SYNTHETIC BIOLOGY APPROACH TO ENGINEER "THERAPEUTIC" BACTERIA

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The high incidence and mortality of solid tumors like breast cancer makes the development of novel therapeutic agents a high priority. Curcumin, a natural substance from the rhizome of *Curcuma longa*, has captured the attention of the scientific community. Pre-clinical trials and extensive research has demonstrated its ability to prevent cancer. Indeed, curcumin has been shown to target critical genes involved in angiogenesis, apoptosis, cell cycle and metastasis, and consequently to inhibit cell growth. Currently, the clinical use of curcumin is mainly limited by its poor bioavailability which implies repetitive oral doses in order to achieve the therapeutic concentrations inside the cell. The idea of the present work is to design a strategy that could link the common technique used to treat solid tumors (ultrasound) with the therapeutic effects of curcumin. The plan is to use the temperature increase (consequence of ultrasound treatment) to trigger the *in situ* expression of curcumin by engineered bacteria. *Escherichia coli* was chosen as the model organism in which the genes involved in the curcumin pathway will be cloned.

Those genes (4-coumarate: CoA ligase, diketide-CoA synthase and curcumin synthase) were successfully cloned under the control of a temperature sensitive promoter (dnaK). The proof-of-concept that the dnaK promoter can be induced by a temperature increase, leading to the expression of the 3 necessary genes, is currently being tested, using several biochemical assays.

Moreover, several knockouts (KO) of specific genes from the *E. coli* K-12 MG1655 genome were performed in order to maximize the production of curcumin. The deletion strategy, as well as the definition of the non-essential genes to be KO, was determined *in silico*. This strategy included one single KO (*gnd* gene) and the multiple KO of five non-essential genes for aerobic growth (*fumA*, *fumB*, *fumC*, *ccmA* and *argO*) and *serA* gene for anaerobic growth. After optimizing the genes expression under the control of the temperature inducible promoter, the several KO will be transformed with this construction to confirm the improvement of curcumin production.

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## Synthetic Biology

## P354

THE EFFECT OF RPIA OVEREXPRESSION ON PLASMID BIOPHARMACEUTICAL PRODUCTION BY *ESCHERICHIA COLI* GALG20, A PGI-GENE KNOCKOUT STRAIN

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The use of plasmid DNA (pDNA) as a biopharmaceutical increased due to its attractive potential application in gene therapy and with the approval of the first DNA vaccines. Currently, there are few products licensed for veterinary applications and numerous plasmids in clinical trials for use in humans. Most *Escherichia coli* host strains used to produce pDNA were originally mutated to facilitate cloning and production of recombinant proteins and thus may not be optimal for pDNA production. Recently, new discoveries in the field of strain engineering for pDNA production demonstrate the importance of strain genetic background in the creation of new *E. coli* strains for pDNA production. The *E. coli* host strain GALG20 was specifically designed for pDNA production by knocking out the *endA*, *recA* and *pgi* genes in the wild-type strain MG1655. As a consequence of the *pgi*-mutation, the GALG20 strain preferentially uses the pentose phosphate (PP) pathway as the main via for glucose consumption. This innovative mutation leads to a production of 25-fold more pDNA (19.1 mg/g DCW) than the parental strain MG1655 $\Delta$ *endA* $\Delta$ *recA* (0.8 mg/g DCW), in glucose, as well as to a reduction of acetate synthesis during fermentation [1]. Nevertheless, it is not known whether other enzymes in the PP pathway are limiting extra synthesis of pDNA by GALG20. In this work, the impact of overexpression of the *rpiA* gene in the novel strain GALG20 will be analyzed. The *rpiA* gene codes for ribose-5-phosphate isomerase A, and is responsible for nucleotide synthesis in the PP pathway. Previous work demonstrated that overexpression of *rpiA* increased pDNA yields, using a plasmid expression system in *E. coli* BL21 [2]. Thus, the replacement of *rpiA* promoter, directly in the genome of GALG20 strain, could potentially enhance nucleotide synthesis and consequently increase pDNA production. This replacement is currently under evaluation using batch and fed-batch fermentations.

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## Synthetic Biology

### P355

#### TURNING *CORYNEBACTERIUM GLUTAMICUM* INTO A CELL FACTORY FOR THE PRODUCTION OF 2,3-BUTANEDIOL

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*Corynebacterium glutamicum* is an important industrial bacterium, used worldwide in aerobic production of amino acids such as L–glutamate and L–lysine. Recently, major metabolic engineering efforts have improved this organism as a cell factory for production of industrial relevant compounds, such as D–lactic acid and isobutanol (1,2). Herein we described the engineering of *C. glutamicum* towards the production of 2,3–butanediol, an important chemical feed–stock with a wide range of applications. Our strategy involved the over–expression of the *Lactococcus lactis* biosynthetic pathway responsible for the conversion of 2 molecules of pyruvate into 1 molecule of 2,3–butanediol (3). As a host strain we used *C. glutamicum* lacking the genes involved in the conversion of pyruvate into lactate and acetate. High yields of 2,3–butanediol were achieved in a two–stage fermentation (aerobic growth followed by anaerobic production phase). Under anaerobic conditions a yield of 0.4 mol 2,3–butanediol/mol glucose was achieved while under microaerophilic conditions this yield was improved 1.7–fold (0.67 mol/mol glucose). The engineered strain had a lower glucose consumption rate when compared to the wild–type strain; therefore, further efforts will be invested to increase 2,3–butanediol productivity.

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## Systems Biology

P356

### AN ECOSYSTEM WITHIN OUR MOUTH

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The oral cavity is an ecosystem in which hundreds of microorganisms establish themselves mainly as biofilm communities. The different oral biofilms have been studied for a while and the amount of information on these communities has increased greatly especially after the revolution in the molecular microbial identification techniques. However, it is still challenging to answer questions such as: what is typical community of a healthy mouth? What species appear or disappear as oral or systemic diseases evolve? Can we associate a health or disease status to a community? What is the role of the microbial community in the normal or pathological pathways?

Large scale studies, such as the Human Microbiome Project, have produced data which facilitated the answer to these questions (1). Smaller scale studies also exist providing results from molecular identification of microorganisms from the different microhabitats in the mouth. However not only are these results scattered through the literature but also provide snapshots of what is present in that particular environment, under those particular conditions. The Computational Biology group at Institute of Health Sciences in Viseu is focused on creating resources which integrate the data produced by the different projects and studies to allow a true systems analysis of the oral cavity (2,3).

Our work has been centered on the analysis of proteomics results and the identification of the proteins (human and microbial) present in the oral cavity which resulted in a database with over 10 000 proteins (OralOme) along with an associated interface (ORALCARD) which allows the exploration of that information. The ongoing work aims at the inclusion of the genomic information from 16S studies in order to allow the analysis of the oral microbial communities' composition both in health and disease. An extension of this work, currently being improved by our group, is the study of the protein-protein interactions (PPIs) present in this ecosystem. The tool OralInt explores the data present in the OralOme database to predict PPIs between human and microbial proteins. These predictions have obvious impact on the understanding of the Oral Ecosystem and are crucial for diagnosis and therapeutics of oral and possibly systemic diseases.

The work so far has led to the following conclusions: the amount of information present in the literature for human proteins is much greater than for microbial ones; the species identified as present in the oral cavity by proteomics data are much less than those identified by 16S data, which reveals the limitations of microbial protein identification in oral samples; the human mouth has very diverse communities both in health and in disease depending on the specific habitat which is colonized.

The main conclusion is that the complexity and variability of this ecosystem requires new bioinformatics and visualization tools and strategies in order to convert the large amount of data from studies providing snapshots of the system, into a true whole system level platform which allows the understanding of pathways and processes (the Oral Physiome).

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## Systems Biology

P357

YEASTRACT: AN UPGRADED INFORMATION SYSTEM FOR THE ANALYSIS OF GENE AND GENOMIC TRANSCRIPTION REGULATION IN *SACCHAROMYCES CEREVISIAE*

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The YEASTRACT ([www.yeasttract.com](http://www.yeasttract.com)) information system is a tool for the analysis and prediction of transcription regulatory associations in *Saccharomyces cerevisiae*. Since its first version [1], this information system has been regularly upgraded [2,3]. In its new release [4], YEASTRACT contains 206.299 regulatory associations between Transcription Factors (TFs) and target genes, including 326 DNA binding sites for 113 TFs. This data was retrieved from exhaustive expert curation of 1.337 articles published in international peer-reviewed journals. All regulatory associations stored in YEASTRACT are linked to information on the experimental setup and environmental conditions in which those associations take place and on whether the transcription factor is acting upon its target genes as activator or repressor. Based on this information new queries were developed allowing the selection of specific environmental conditions, experimental evidence or positive/negative regulatory effect. This release further offers tools to rank the TFs controlling a gene or genome-wide response by their relative importance, based on: 1) the percentage of target genes in the dataset; 2) the enrichment of the TF regulon in the dataset when compared to the genome; or 3) the score computed using the TFRank system, which selects and prioritizes the relevant TFs by walking through the yeast regulatory network.

Since its initial release, the YEASTRACT website has been accessed by around 8,500 researchers, performing nearly 170,000 queries per year. We expect that with the new data and services made available, the system will continue to be instrumental for Yeast Biologists and Systems Biology researchers.

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## Session 8

# Emergent technologies

# Plenary Lecture

## CULTURED MEAT

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The global population is estimated to increase with 50% during the next 35 years. This population increase will be accompanied with an almost doubling of the greenhouse gas emissions if no actions are taken. It is anticipated that also the global meat consumption will double during the next 35 years if societies worldwide become more affluent. Meat production requires a relatively high proportion of land, energy, and fresh water use. Moreover, livestock contributes significantly to the emission of greenhouse gases and, in many countries, to the pollution of water and soil. An obvious solution to the problem would be to consume less products of animal origin. This may, certainly in Western societies where the consumption of (animal) proteins is very high, be part of the solution. Replacement of dietary animal (vertebrate) proteins by plant, fungal, or even insect proteins can be another part of the solution. Yet another possibility is to culture large amounts of muscle cells derived from stem cells of farm animal species and to produce cultured meat (in vitro meat). The advantage of this technology is that for the making of most products of animal origin no animals are needed. Products prepared with the latter technology may combine a favorable ecological footprint with similar nutritional values as conventional products. Ultimately, cultured meat products may be made with similar sensory qualities as some of the conventional products.



# Keynote Lecture

## KL09

# HIGH PRESSURE TECHNOLOGY: 20 YEARS OF INCREASINGLY FOOD COMMERCIAL SUCCESSES AND FUTURE FOOD AND BIOTECHNOLOGICAL POTENTIAL APPLICATIONS

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High pressure (HP) technology industrial applications and scientific maturity had considerable developments in the last decade and is now clearly the fast growing novel food technology worldwide. HP food pasteurization/shelf life extension, at room temperature or even under cold conditions, is now a well established and increasingly growing industrial practice (in 2011 over 3000 million euros of sales). Envisaged novel short coming industrial applications include a new method to achieve food commercial sterility level, applicable to canned foods, called Pressure Assisted Thermal Sterilization (PATP), already approved by FDA that allows the production of better quality sterilized foods. Other food-related HP potential applications comprise the creation of new products, with distinctive characteristics, such as possible new textural properties, improvements of protein digestibility [3], and reduced protein allergenicity.

Apart from food preservation and processing applications, HP is now also viewed as having diverse potential biotechnological applications, *lato sensu*, ranging from new fermentation processes with modulation of metabolic patterns of cells, therapeutic proteins, biocatalysis, and improved vaccines, to name a few.

Working for more than 10 year in HP applications, University of Aveiro, is now creating a High Pressure Based Multidisciplinary Research and Technological Platform, based on a portfolio of three HP equipments (lab, semi-pilot and pilot scales – see below), aiming to create a center of international competitiveness in Portugal, to promote research and industrial applications of HP.

This presentation summarizes the main outcomes and evolution of HP for food preservation and processing applications, since the first commercial product in Japan, 20 years ago, such as number of commercial equipments in operation and products commercially available, as well as presents very recent new potentials, as is the case of a new possible food preservation method (at room temperature as a potential substitute of refrigeration) that appeared last year and food biotechnological applications, as the production of fermented food products under pressure, like yogurt.



The three equipments at University of Aveiro, from left to right: lab scale, semi-pilot, and pilot/industrial scale.

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# Oral Presentations

## OP35

## DELIVERY OF DNA BIOPHARMACEUTICALS BY NON-VIRAL NANOPARTICULATED CARRIERS

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Non-viral gene therapy currently arises as an exceptionally promising approach for the treatment of a wide spectrum of incurable pathologies that have striking worldwide occurrence, such as cancer or HIV [1]. However, regardless of its unique therapeutic potential, the translation of nucleic acid-based biopharmaceuticals onto realistic clinical applications remains largely hindered due to issues associated with the purity of DNA formulations and also their limited biological efficiency [1]. In order to surpass these limitations, in this work we describe an approach that addresses these key limitations through the establishment of high throughput biopharmaceutical purification platforms in conjugation with an application-oriented design of novel nanoparticulated carriers for DNA delivery into cancer cells [2]. Particularly, herein we synthesised biocompatible and biodegradable chitosan gene delivery carriers functionalized with amino acid moieties [3]. The synthesized polymers had the ability to condense DNA biopharmaceuticals and spontaneously assemble into stable nanoparticulated polyplexes with positive surface charge density and spherical morphology. Flow cytometry data revealed that the DNA-nanocarriers were efficiently internalized by cancer cells. Additionally, by following the cellular entry routes of these gene delivery systems, the carriers demonstrated their ability to escape from degradative lysosomal trafficking pathways and promoted a remarkable increase in transgene expression. Collectively, these important strategies emphasise the relevance of DNA delivery by biocompatible materials, and thus open the possibility to design a new generation of application-oriented nanocarriers that can transport highly pure biopharmaceuticals for cancer gene therapy.

**Keywords:** DNA Biopharmaceuticals, Nanoparticles, Biocompatibility, Gene Expression, Cancer

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## OP41

## CARBOHYDRATE-DRIVEN PORPHYRINOIDS TO INDUCE CELL DEMISE BY PHOTODYNAMIC THERAPY

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Conventional Photodynamic Therapy (PDT) combines a non-toxic photosensitizer (PS) drug, light irradiation at a specific wavelength and tissue molecular oxygen to generate *in situ* reactive oxygen species (ROS) which are able to destroy the targeted cancer cells. In order to enhance the specific delivery of PSs in cancer cells, our research group has developed carbohydrate-based porphyrinoids (porphyrins, phthalocyanines and chlorins) for bladder cancer treatment by PDT. Carbohydrates have a strong potential as PS-delivery systems, since they provide water solubility, being compatible molecules with a rapid cellular uptake and specific recognition by lectin proteins, which play a key role in cancer-related biochemical pathways.

Our group has chemically modified the core of the porphyrinoids by derivatization of them with galactodendritic and cyclodextrin units. These conjugates have demonstrated outstanding photo-physical and -chemical properties being water soluble, fluorescents, efficient generators of ROS, photo-stable and able to interact with human serum albumin (HSA). The promising photo-physical data of the derivatives have prompted us to validate them as PSs against bladder cancer cells (UM-UC-3 and HT-1376).

The PS drugs have demonstrated co-localization with carbohydrate receptors (galectin-1 and GLUT1) and induction of phototoxicity in both bladder cancer cell lines by a ROS-mediated mechanism. A galactodendritic phthalocyanine exhibited a particular “dark” mode of action by decreasing carbohydrate receptors (galectin-1 and GLUT1) after its uptake in cancer cells. The phototoxicity induced after PDT was dependent on the conjugate and on the cell line, being higher in UM-UC-3 than in HT-1376 cells. The resistance observed in HT-1376 cells after PDT was due to the a) low expression of carbohydrate-binding proteins (namely galectin-1), b) presence of a population with stem-like properties overexpressing the multi-drug resistant pump ABCG2 and c) activation of antioxidant enzymes after PDT.

The *in vitro* results show that galactodendritic-PS and cyclodextrin-PS conjugates are promising therapeutic agents for the treatment of bladder cancer by PDT. *In vivo* studies are being performed to validate the effectiveness of our conjugates, contributing for a possible potential on PDT clinical practice.

# Poster Presentations

## Cell culture, Stem Cell and Tissue Engineering

P358

## URINARY RHABDOSPHINCTER BIOENGINEERING – A DECELLULARIZED URETHRA MATRIX FOR MODELING SUI IN VITRO AND TISSUE ENGINEERING APPLICATIONS

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**Introduction:** Stress urinary incontinence (SUI) is related, among others, with the malfunction of the closure mechanism of the rhabdosphincter, which is the external urethral muscle layer and is constituted by skeletal muscle cells.[1, 2] There are several *in vivo* models for SUI, but no *in vitro* model has been developed so far.[2] The aim of this study is to establish a reliable *in vitro* model of rhabdosphincter for the development and screening of new drugs and therapies for SUI.

**Materials and Methods:** The urethras from piglets were harvested and decellularized using different concentrations of Triton X–100 or SDS and a system based on agitation (60 rpm) and perfusion (40 mL/min). The tissue was exposed to 5 cycles of 24 hours each. The efficiency of cell removal on decellularized urethras was assessed by Hematoxylin & Eosin (H&E) staining and confirmed by DNA extraction. The presence and localization of collagens I–IV, elastin, fibronectin and laminin was evaluated by immunofluorescence staining. Total collagen, elastin and glycosaminoglycans (GAGs) were also quantified by specific colorimetric assays.

**Results and Discussion:** From all the solutions that were tested, only 0.5% and 1% SDS solutions successfully decellularized the urethra, as shown by H&E, which revealed no purple basophilic staining (nuclear material). DNA quantification revealed that 0.5% SDS solution removed  $93.4 \pm 2.6\%$  of DNA material, while 1% SDS removed  $96.1 \pm 0.0\%$ . Both solutions removed more than 90% of total DNA (similar to the values reported in the decellularization of liver, heart, lungs and kidney) [3–5][4], nevertheless the studies were pursued with the 0.5% SDS solution since it has a lower concentration of detergent and it is less aggressive over the proteins of the extracellular matrix (ECM). Immunofluorescence staining indicated no major loss or modification of ECM proteins localization; however, the presence of elastin and fibronectin in the acellular ECM was lower compared to native tissue. Quantification of the total amount of ECM proteins showed no major differences between native tissue and acellular scaffold.

**Conclusions:** Our results indicate that this dynamic decellularization of the urethra was able to remove up to 93% of the total DNA while preserving ECM chemistry and localization. Future studies include recellularization of the scaffold, where cell adhesion, proliferation and most importantly, recovery of rhabdosphincter function, will be assessed.

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## Cell culture, Stem Cell and Tissue Engineering

P359/F36

### GENE DELIVERY STRATEGIES TO EFFICIENTLY OVER-EXPRESS FUNCTIONAL HLA-G IN BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS

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**Introduction** Mesenchymal stromal cells (MSC) have unique properties, such the relative ease of isolation and the ability to be extensively expanded in culture, which make them ideally suited both for regenerative medicine and gene delivery. Furthermore, MSC have been reported to reduce local inflammation, blunt immune response, and counteract the chemotactic signals released to recruit immune cells to the site of injury/inflammation. HLA-G, expressed at low levels on MSC, is one of the molecules responsible for MSC immunomodulatory properties; it suppresses allogeneic T-cell response, inhibits natural killer (NK) cell cytotoxicity, and modifies maturation of antigen-presenting cells. Here we hypothesized that, by over-expressing HLA-G on bone marrow derived MSC (MSC-G1), we could improve their immune inhibitory function, thus increasing their therapeutic potential. In addition, and in order to investigate which gene transfer system was best suited for delivering this immunomodulatory protein, we performed a side-by-side comparison of several non-viral and viral delivery strategies, assessing function, efficiency, safety, and duration of gene expression.

**Methods** A lentiviral vector (Lentiv-HLA-G1), and three non-viral plasmids, including a conventional plasmid (pmax-HLA-G1), a minicircle (MC-HLA-G1; plasmid vectors that lack bacterial elements), and an episomal plasmid (EP-HLAG1; persists in the nucleus in an extra-chromosomal state) were tested. The immunological characterization of MSC-G1 using the different gene transfer strategies was assessed by: 1) evaluating expression of immunoreceptors; 2) NK cytotoxic assays; 3) Cytokine production; and 4) Mixed-lymphocyte reaction (MLR)

**Results** Over-expression of HLA-G1 by Nucleofecting MC-HLA-G1 or pmax-HLA-G1 plasmid constructs resulted in maximal levels of HLA-G1 expression by 48h post-transfection (MC-HLA-G1:57±2.8%; pmax-HLA-G1:54±3.9%). At 12 days post-transfection, the percentage of MSC expressing HLA-G1 was 32±4.0% and 36±3.2%, respectively. Nucleofection using EP-HLAG1 resulted in 13±1.1% of MSC expressing HLA-G1 at 48h, decreasing to 1±1% at 12 days. In order to perform a side-by-side comparison between non-viral and viral systems, we stably transduced MSC with Lentiv-HLA-G1 at an MOI that yielded similar levels of HLA-G1-expressing MSC (49±7.8%) between the delivery systems. NK cell-mediated lysis assays showed a significant difference in the susceptibility of Lentiv-HLA-G1-MSC and non-modified MSC to NK cells; the former were protected from NK-lysis when compared to non-modified cells. However, for non-viral approaches, regardless of the presence of enhanced HLA-G1 levels, an opposite effect was observed. For MC-HLA-G1-MSC and pmax-HLA-G1-MSC, a slight increase in susceptibility to NK cell cytotoxicity was detected, whereas for EP-HLA-G1-MSC, a significant increase in NK-mediated lysis was observed. Since expression of NKG2D and DNAM-1 was not altered in transfected MSC, other pathways leading to NK activation such Toll-Like Receptors were investigated, and we found a significant increase in TLR-9 in all non-virally transfected cells. Nevertheless, no upregulation of production of pro-inflammatory cytokines was observed in these cells. One-way and two-way MLR demonstrated that only Lentiv-HLA-G1-MSC significantly reduced T cell proliferation when compared with non-modified cells.

**Conclusion:** MSC can be efficiently modified to over-express HLA-G1 using viral and non-viral strategies, but only HLA-G1-lentiviral transduction resulted in an improvement of BM-MSC immunomodulatory properties.



## Cell culture, Stem Cell and Tissue Engineering

### P360/F37

#### PROLIFERATIVE AND MINERALOGENIC ACTIVITIES OF SEAWEEDS AND MARINE PLANTS EXTRACTS IN A FISH IN VITRO CELL SYSTEM

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Seaweeds and halophytes have been identified as an under-exploited resource, although they have long been recognized as valuable sources of diverse bioactive compounds. Moreover, several phytochemicals have been recently found to present antioxidant capacities and to stimulate bone formation. Bone diseases, in particular osteoporosis, are considered one of the major human health issues worldwide. In this regard, an increasing number of studies were performed to get insights into the mechanisms of bone formation and uncover molecules with therapeutic potential. The aim of this work was to test the mineralogenic, proliferative and antioxidant effects of marine plant extracts.

Extracts from three halophytes (*Salicornia fragilis*, *Salicornia ramossissima*, *Spartina alterniflora*) and two green algae (*Cladophora rupestris* and *Codium fragile*) were prepared by liquid/liquid extraction. Three different extracts from each species were tested: i) a crude hydroalcoholic extract, and two liquid/liquid purified fractions, ii) ethyl acetate fraction (EAF) and iii) aqueous fraction (AF). *In vitro* assays to determine mineralogenic and proliferative activities were performed using a bone-derived cell line from gilthead seabream (*Sparus aurata*) supplemented with the marine plant extracts. Antioxidant activities were assessed by radical scavenging activity (using DPPH procedure) and reducing power assays.

Cell proliferation (assessed through XTT assay) was stimulated upon exposure to *S. fragilis* EAF. Pro-mineralogenic effect, detected by extracellular matrix mineralization (assessed with Alizarin red staining and quantification), was induced by 2.5 folds, upon exposure to *S. alterniflora* EAF. Moreover, EAF of halophytes presented the highest antioxidant activities compared to seaweeds, as *Salicornia* species EAF are 1.5–1.9 more active than  $\alpha$ -tocopherol for the reducing power assay.

This study shows promising results for both mineralogenic and antioxidant activities of marine species extracts. Further investigations are following in order to identify the molecules responsible for the pro-mineralogenic and antioxidant effects.

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## Bionanotechnology

### P361

## AN *IN-VIVO* STUDY: THE ANTITUMOR EFFECT OF BIOSYNTHETIC SILVER NANOPARTICLES BY STIMULATING THE IMMUNE SYSTEM

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### Introduction

Many tumors have resistances to the usual antitumor medicines. Nanoparticles could help to reduce these resistances. This study was focused on the immune stimulatory effects of biosynthetic silver nanoparticle (AgNPs) against 4T1 breast cancer cells.

### Material and methods

The AgNPs were synthesized by adding extracellular supernatant of fresh *Klebsiella pneumoniae* into silver nitrate solution under the sunlight and filtered to eliminate the remain of particles of microorganisms. 4T1 breast cancer was cultured in 96 well treated tissue culture plates in suitable condition (37°C with 5% CO<sub>2</sub>) and to determine the cytotoxicity effect of AgNPs, the MTT-based assay was performed. The tumor growth and DTH responses was investigated into 4 groups of female inbred BALB/c mice, each containing 20 mice. Group 1 was as control, non-treated mice without any induction of tumor, group2 was the mice that only transplanted cancer cells without any treated, group3 was tumor-induced mice treated once daily with AgNPs through IP route up to 20 days after the tumor cells induction and group4 was tumor control mice that was treated without AgNPs. Tumor growth volume was measured twice a week. To evaluate the DTH response, the tumor antigen was prepared by homogenizing and dialyzing in PBS. Then the mice were confronted with tumor antigen on the right footpad and with PBS on the left one. After that the swelling size was measured.

### Results

In this investigation, the average size of AgNps was 28nm. The associated IC50 values were 40 µg/ml for AgNPs/4T1 breast cancer cells. The relative tumor volume size in AgNPs treated group and control group was  $0.750 \pm 0.132$  cm<sup>3</sup> and  $2.351 \pm 0.391$  cm<sup>3</sup> respectively and the swelling in DTH assay was  $18.51 \pm 1.78$  mm and  $8.01 \pm 1.98$  mm respectively that showed the immune stimulatory in test group was higher than control.

### Conclusion

This study showed that the life span of tumor induced mice treated with AgNPs was increased about 75% in compare with tumor control mice. This study demonstrates that the anticancer activity of biogenic AgNPs is due to immune stimulatory effect. However more studies should be conducted to find other mechanisms of action on the other cancer cells.

## Bionanotechnology

P362

### EFFECT OF SILVER NANOPARTICLES IN THE MICROBIOME OF THE ISOPOD *PORCELLIONIDES PRUINOSUS*

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**Background** Due to the widespread use of silver nanoparticles (AgNPs) in daily products, the knowledge of its toxicity on organisms and associated microbial communities is mandatory. Disturbances induced by antimicrobial AgNPs and its counterparts may have severe consequences on the composition of the gut microbiome of isopods, possibly causing impairment of soil function.

**Methods** Experiments were performed to determine the possible effects of AgNPs in the gut microbiome of the terrestrial isopod *Porcellionides pruinosus*. Isopods were exposed to 50mg/Kg (NOEC– no observed effect concentration) and 5µg/Kg (PEC– predicted environmental concentration) of AgNPs and the ionic counterpart (AgNO<sub>3</sub>). Gut microbial communities were analysed by denaturing gradient gel electrophoresis of PCR– amplified fragments of the 16S rRNA gene.

**Results** Results revealed that exposure to both Ag forms alter the *Porcellio* gut microbial community. No evident differences on the antimicrobial effects of AgNPs and AgNO<sub>3</sub> were detected. Also, by using this methodology, no distinct effects were observed on the microbial community composition when exposing isopods to the two tested concentrations. Although PCR–DGGE analysis detects only ribotypes that represent at least 1% of the total community, it allowed to identify effects of AgNPs on the microbial community of isopods' gut. Other molecular techniques allowing to explore the detailed structure of the community will be applied to further characterize this disturbance and to identify the most affected bacterial groups.

**Conclusions** This study indicates that AgNPs impact the isopod's gut microbial community. Thus, NP contamination of soils, particularly by silver NPs, is a concern and future investigation for establishing a link between microbiota changes and possible impairment of soil function are needed.

## Bionanotechnology

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### ELECTROSPUN SILK-ELASTIN-LIKE FIBER MATS FOR TISSUE ENGINEERING APPLICATIONS

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Protein-based polymers are ubiquitous macromolecules present in all living organisms, fulfilling structural and mechanical roles. Advances in protein engineering and recombinant DNA technology allow the design and production of recombinant protein-based polymers (rPBPs) with an absolute control of its composition. Molecular genetics tools allow to combine, in the same polypeptide chain, the properties of two or more different protein domains. Although the application of recombinant proteins as scaffolds is still an emerging technology, the possibilities are limitless and far superior to natural or synthetic materials, as the complexity of the structural design can be fully customized. In this work we report the electrospinning of two new genetically engineered silk-elastin-like proteins (SELPs) consisting of alternate silk- and elastin-like blocks. Electrospinning was performed with formic acid and aqueous solutions at different concentrations without addition of further agents. The size and morphology of the electrospun structures were characterized by scanning electron microscopy showing to be dependent of concentration and solvent used. Treatment with air saturated with methanol was employed to stabilize the structure and promote water insolubility through a time-dependent conversion of random coils into  $\beta$ -sheets (FTIR). The resultant methanol-treated electrospun mats were able to absorb large amounts of water displaying a degree of swelling in the range of 570–720%. Further, the methanol-treated mats were characterized by a water vapour transmission rate of 1083 g/m<sup>2</sup>/day and a modulus of elasticity of ~126 MPa. The methanol-treated SELP fiber mats showed no cytotoxicity and were able to support adhesion and proliferation of normal human skin fibroblasts. Adhesion was characterized by a filopodia-mediated mechanism. These results demonstrate that SELP fiber mats can provide promising solutions for the development of novel biomaterials suitable for skin regeneration applications, especially considering moderately exuding wounds.

#### ACKNOWLEDGEMENTS

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#### INTEGRATED EXTRACTION, CONCENTRATION AND QUANTIFICATION OF OCHRATOXIN A IN WINES USING A MICROFLUIDIC ATPE COUPLED TO A FLISA

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Ochratoxin A (OTA) is a secondary metabolite of a variety of fungi, particularly those belonging to *Aspergillus* and *Penicillium* genera. These fungi have been found to contaminate a wide variety of food commodities at a worldwide scale, such as wine, coffee and cereal grains [1]. Hepatotoxic, nephrotoxic, neurotoxic, teratogenic immunotoxic and carcinogenic effects have been observed in several animal models after exposure to OTA [1].

Given its potential harmful effects, the regulatory limit for OTA in wine has been set at 2ng/mL [1]. In order to enforce this regulation, it would be desirable to quickly screen the levels of OTA “on site” and, if necessary, send the sample to a certified laboratory for quantification. Screening for food safety and quality control is an emerging demand given the increase in world population and merchandise mobility.

To approach this problem, an integrated microfluidic device comprising two modules was developed. The first aims to perform an aqueous two phase extraction (ATPE) and the second an immunoassay, allowing matrix extraction, OTA pre-concentration and quantification in a single assay. The ATPE module is based on the insertion of raw wine spiked with phosphate salt and a polyethylene glycol (PEG)-rich solution in two separate inlets that converge into a single channel. Both solutions flow in parallel while OTA is concentrated in the PEG rich phase and the polyphenols are bound to PEG. The PEG-rich phase is then converged with a third flow containing an anti-OTA antibody in order to perform an indirect competitive fluorescence linked immunosorbent assay (icFLISA). Using this strategy a limit of detection (LoD) of 0.26 ng/mL for OTA in red wine was obtained, improved from 1.9ng/mL using thin layer chromatography extraction and having a linear response spanning 3 orders of magnitude (0.1–100 ng/mL) in a logarithmic concentration scale. Furthermore, this extraction/concentration method provides similar detection curves and LoDs for pronouncedly different matrixes such as red wine, white wine and beer.

This novel approach based in aqueous two phase systems may potentiate the development of a simple, low-cost matrix extraction and concentration platform at both macroscale and microscale. These results also seem to indicate that such strategy can be broadened to other mycotoxins and biomolecules.

[1] *Mycotoxins: Risks in Plant, Animal, and Human Systems*. Ames, IA: Council for Agricultural Science and Technology, 2003.

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### MIP NANOPARTICLES: A STRATEGY TO BLOCK QUORUM SENSING?

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Quorum sensing (QS) is a phenomenal system of cell-to-cell communication in bacteria, and is used to coordinate gene expression in response to environment fluctuations in cell-population density. Pathogenesis, antibiotic production, bioluminescence, conjugation, sporulation, swarming, biofilm formation and the expression of several virulence factors are phenotypes correlated with QS.

The mechanism of QS is mediated by signalling molecules like N-acyl homoserine lactone (AHLs); this mechanism is highly precise and specific due to the interactions between these molecules and their receptors. Inhibition or disruption of QS might result in an excellent strategy to control bacterial pathogenesis and also for the development of antimicrobial agents.

Molecular imprinted polymers (MIP) are attractive for several applications mainly due their high affinity, selectivity, stability and simplicity of preparation. Molecularly imprinted nanoparticles (MIP NPs) are an innovative material that fits better with surface imprinting strategies, making the design for highly specific targets easier.

As main aim of this project we propose the development of MIP NPs with affinity for AHLs involved in QS. The capture of AHLs molecules decreases bacterial communication thus preventing the coordinate gene expression that results from QS.

The synthesis of MIP NPs was performed in a solid phase containing the immobilised template with methacrylic acid as the functional monomer under UV polymerisation. Particle size and affinity were evaluated respectively by Dynamic Light Scattering and Surface Plasmon Resonance.

MIP NPs with sizes around 110nm (DLS) showed nanomolar affinity towards AHLs (SPR). No cytopathic effects were detected when MIP NPs coated coverslips were tested in Vero cells cultures. QS-associated phenotypes such as biofilm formation and bioluminescence were investigated in the presence of MIP NPs.

These results provide basis to propose MIP NPs as a new approach to block several QS- controlled phenotypes, including those involved in bacterial virulence.

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### NANO, MICRO AND MACRO MAGNETIC MATERIALS IN BIOTECHNOLOGY

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In the last decades, much attention has been dedicated to the study of iron oxide magnetic nanoparticles (MNP). Their unique physical proprieties, namely large surface to volume ratio and superparamagnetism, allied with their biocompatibility, make MNP attractive for biomedical applications such as magnetic resonance imaging (MRI), hyperthermia, targeted drug and gene delivery, tissue engineering, as well as in bioseparation and biosensing. Surface chemistry is important to confer colloidal stability and to provide chemical groups amenable of further surface modification. Hydrophilic coatings, usually composed by polysaccharides (e.g. dextran) or synthetic polymers (e.g. polyethylene glycol (PEG)) are widely used for this purpose. According with the synthesis procedure, magnetic particles will present different properties and this will influence its application. In this work, it will be shown how magnetic particles can be prepared at the nanoscale level for biomedical applications (1), at the microscale level for bioseparation processes (2), for the creation of antimicrobial devices or magnetic biocatalysts (3), and finally at a macromolecular level when incorporated onto macroporous structures with magnetic-responsive properties. For these applications, different polymers and biopolymers were used, with distinct surface modifications and targeting agents specific for each application.

(1) Dias, AMGC, Hussain A, Marcos AS, Roque ACA. 2011. A biotechnological perspective on the application of iron oxide magnetic colloids modified with polysaccharides. *Biotechnology Advances*. 29:142–155; Bicho, A, Roque ACA, Cardoso AS, Domingos P, Batalha ÍL. 2010. In vitro studies with mammalian cell lines and gum arabic-coated magnetic nanoparticles. *Journal of Molecular Recognition*. 23:536–542.

(2) Santana, SDF, Dhadge VL, Roque ACA. 2012. Dextran-Coated Magnetic Supports Modified with a Biomimetic Ligand for IgG Purification. *ACS Applied Materials and Interfaces*. 4(11):5907–5914.

(3) Santana, SDF, Pina AS, Roque ACA. 2012. Immobilization of enterokinase on magnetic supports for the cleavage of fusion proteins. *Journal of Biotechnology*. 161:378–382.

## Bionanotechnology

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### NEW BIOCOMPATIBLE AND BIOACTIVE NANOCOMPOSITES OF BACTERIAL CELLULOSE AND POLYMETHACRYLATES

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Bacterial cellulose (BC) is produced by several bacteria of the *Gluconacetobacter* genus. BC shows unique properties such as high water holding ability, mechanical strength which are due to its tridimensional micro- and nanofibrillar structure, and it is also intrinsically biodegradable and biocompatible (D. Klemm, F. Kramer, S. Moritz, T. Lindström, M. Ankerfors, D. Gray, and A. Dorris, *Angewandte Chemie (International ed. in English)*, 2011, **50**, 5438–66; E. Trovatti, L. S. Serafim, C. S. R. Freire, A. J. D. Silvestre, and C. P. Neto, *Carbohydrate Polymers*, 2011, **86**, 1417–1420). One of its main applications is as wound dressing material as it favors healing and protects the wound, reducing the risk of infections (W. Czaja, A. Krystynowicz, S. Bielecki, and R. M. Brown, *Biomaterials*, 2006, **27**, 145–51).

Besides the application in its native form, research is being developed in order to produce BC nanocomposite materials with improved and new functional properties for biomedical applications (R. J. B. Pinto, P. a a P. Marques, C. P. Neto, T. Trindade, S. Daina, and P. Sadocco, *Acta Biomaterialia*, 2009, **5**, 2279–2289).

This communication describes the preparation of new BC nanocomposites with two synthetic polymers poly(2-hydroxyethyl methacrylate) PHEMA (A. G. P. R. Figueiredo, A. Alonso-Varonab, S. C. M. Fernandes, T. Palomares, E. Rubio, A. Barros-Timmons, A. J. D. Silvestre, C. P. Neto, and C. S. R. Freire, *BioMed Research International*, 2013), and poly(2-aminoethyl methacrylate) PAEMA, by an *in situ* polymerization approach; their chemical and physical characterization and the assessment of their biocompatibility and antimicrobial activity.

BC/PHEMA and BC/PAEMA nanocomposites were thermally stable up to 250 and 200 °C respectively, which allow their use in biomedical application in situations where thermal sterilization might be required.

The potential antibacterial activity of BC/PAEM nanocomposites was evaluated towards luminescent *E. coli*, showing good antibacterial activity; while biocompatibility studies revealed that BC/PHEMA materials are no cytotoxic providing a favorable cell environment for optimal adhesion and proliferation of human adipocyte-derived stem cells (ADSCs).

Finally, the attained nanocomposite materials show properties that confer them high potential for several biomedical applications namely as antibacterial wound dressing or as scaffold for tissue engineering.



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### RELEASE OF POLYPHENOLS FROM CARNAUBA WAX NANOPARTICLES

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The inclusion in foods of compounds with antioxidant activity from rich sources (fruits, aromatic herbs, etc.) has become a common procedure of the food industry. However, when incorporated in food matrix these compounds may interact with matrix components and reduce or loss bioactivity. Hence, the formulation of loaded polyphenols nanoparticles may offer a way to protect such compounds against degradation. In this work, the releasing rates from Solid Lipid Nanoparticles (SLNs) of rosmarinic acid (RA) were tested. Moreover, the effect of selected SLNs upon DNA *i.e.* antioxidant and pro-oxidant effects were also evaluated. Different formulations of SLNs were tested, using 0.5% (w/v) of carnauba wax and 1 and 2% (v/v) of polysorbate 80, prepared by a hot melt ultrasonication method.

Two types of *in vitro* release tests were performed. The first approach used a cellulose acetate dialysis bag with molecular weight cut-off of 12 kDa, to evaluate the direct release of RA from the SLNs. The experiment was conducted during 12 h, at  $37 \pm 0.5$  °C with continuous homogenization. The SLNs were dispersed in PBS (0.1 M, pH 7.4), and samples were withdrawn at different time points during the experiment (0, 1, 2, 4, 6, 8, 10 and 12 h). The polyphenol content of samples was analysed by HPLC. The second test using intestinal-based cell co-culture (Caco-2 and HT29-MTX cells), was performed to study the cells permeability to the RA and the possible presence of secondary metabolites from RA. The passage through the cell monolayer and cytotoxicity were evaluated by HPLC and TEER monitoring, respectively. The SLNs were diluted in sterilized ultrapure water, the solution was placed in the apical zone of the cells and samples were taken from the sub-apical zone, at different times.

In both *in vitro* release tests the results have shown high % of RA release. The test using dialysis bag showed 50% RA release after 4h of experiment, and by 12h the % of release reached ca. 90%. On the other side, tests using intestinal cell lines showed that the Caco-2 cells have higher % of passage of RA (ca. 60%) for the sub-apical zone, when compared with the set of Caco-2 and HT29-MTX cells.

Finally, SLNs do not attack DNA chains, but also do not protect the DNA, demonstrating that SLNs have a neutral effect, whereas free RA demonstrated a protective effect on the DNA chain.

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### TOWARDS THE MINIATURIZATION OF CELL ASSAYS FOR GPCR MONITORING

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G–protein coupled receptor (GPCRs) drug discovery is a thriving strategy in the pharmaceutical industry. The standard approach uses living cells to test millions of compounds in a high–throughput format. Typically, changes in the intracellular levels of key elements in the signaling cascade are monitored using fluorescence or luminescence read–out systems, which require external equipment for signal acquisition.

In this context, miniaturization of such a system can be an important step towards faster testing systems that use considerable smaller amounts of reagents and allow more parallel testing. Integration of miniaturized systems with electronic components with the capacity to measure fluorescence or bioluminescence would allow testing without large and complex external devices. However the development of this kind of devices has its own challenges where a suitable method for macro *vs.* micro scale comparison is of great importance.

In this work, a standard fluorescence microscopy approach is used as a benchmark tool for the development of a miniaturized platform for GPCR monitoring. HEK 293T cells are stained with the Fluorescent dye Fluo4 AM Direct and the intracellular calcium responses to drugs targeting the Muscarinic M1 Receptor such as Carbachol and Pirenzepine are recorded. The characterization of the average behavior of the cell population was described in terms of dose–response curves with EC50 calculations. A method of collecting data of individual cell behavior within an activation assay was developed combining several tools in the ImageJ software to assess biological limitations to the miniaturization of the system related with cell behavior heterogeneity. Individual cell behavior was measured in several conditions of surface coating, cell densities and drug concentrations. Estimates made on the basis of these experiments indicate that within a system containing around 50 cells there is a 95% chance of getting one cell with the average behavior of the initial 1000 cell population.

Following the establishment of the miniaturization degree, GPCR response was characterized using thin film amorphous silicon photodiodes which are more compatible with the reduced cell number in such miniaturized fluorescence intracellular calcium assays.

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#### USING BRAIN-DERIVED CELLS TO INVESTIGATE BEHAVIOUR WITH CHEMICAL AND ELECTRICAL STIMULATIONS

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This work reports on the behaviour of neuronal cells when seeded into planar electronic devices based on thin film transistor architectures. These sensing devices are able to record extracellular activity when the cells are subjected to electrical and/or chemical stimulations. The goal is to develop non-invasive electrical techniques suitable to detect physiological cell responses for future implantable regenerative devices. Neuro-2a cells lines obtained from ATCC were seeded on poly-lysine coated sensing devices specifically developed in our laboratory and allowed to settle and adhere for 2 hours prior to use. Neuro-2a cells were electrical monitored upon exposure to chemicals namely KCl and tetrodotoxin (TTX). Dramatic changes in cell activity were recorded using electrical noise and small signal impedance techniques. Our findings show a good response of Neuro-2a cells after addition of 30 mM of KCl and 2 $\mu$ M TTX.

Array of sensing devices are now being developed to be used as diagnostic tool to assess effects of neurotransmitters and other chemical substances on the electrical activity of a variety of neuronal cells.

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Cost action session

EP4Bio2Med

# Keynote Lecture

## KL06

### PULSED ELECTRIC FIELD TECHNOLOGY: CURRENT APPLICATIONS IN THE AGRO-FOOD SECTOR AND POTENTIAL USE IN BIOTECHNOLOGY

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Biological cells exposed to a sufficiently strong external electric field show an increase of the permeability of the membrane. This phenomenon, referred to as electroporation, is well known and the technology based on electroporation became attractive in the last decades and is nowadays widely used in different fields such as medicine, e.g. in cancer treatment and gene transfection, food processing, e.g. for microbial inactivation and vegetable tissues permeabilization, biomass processing, e.g. in the recovery of valuable compounds from algae and/or by-products, environmental applications, e.g. in waste water treatment.

As far as the agro-food industry is concerned, in order to respond to the consumers needs of safe minimally processed foods with good sensorial properties and high content of health beneficial compounds, in recent years new non thermal processing technologies have been proposed as an alternative to traditional thermal processing methods. The latter, although having been recognized and approved by regulatory authorities as reliable and robust food processing methods, cause detrimental effects on food quality due to the loss or the strong reduction of thermo sensitive and thermo labile nutritional compounds. Among the non thermal technologies high hydrostatic pressure (HHP), pulsed light (HILP), high pressure carbon dioxide (HPCD) and pulsed electric fields (PEF) have been proposed.

Numerous research activities on the utilization of pulsed electric fields technology in food processing have been promoted, in academia as well as in R&D industrial departments, to investigate its use in two main fields, namely microbial inactivation and cell membrane permeabilization, and the results are reported in publications available in the literature.

Depending on the kind of application considered, PEF treatments can be categorized as high intensity field treatments (20 – 50 kV/cm), as in pasteurization, and moderate intensity field treatments (1 –10 kV/cm) as in tissue permeabilization.

However, whatever is the field intensity applied, the treatment consists on the discharge of trains of ultra-short pulses (of the order of microseconds) of high-strength electric field through a product (liquid foods or liquid containing particulate solids) flowing between two electrodes in a treatment chamber.

The effectiveness of the PEF treatments to achieve the microbial inactivation or vegetable tissue permeabilization is affected by the electrical parameters, mainly the electric field strength and total energy input, but also by the type of microorganism, the nature of the food matrix, the chemical and physical properties of the product considered. In this lecture, the mechanism of PEF microbial inactivation and the mechanism of action of moderate electric field on cell membrane permeabilization is presented and some example on the application of PEF technology in the food as well as in the biotechnology sectors is given and discussed.

# Oral Presentations

## OP22

### FUNDAMENTALS OF MICROBIAL INACTIVATION BY PULSED ELECTRIC FIELDS

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Pulsed electric field technology (PEF) is viewed as one of the most promising nonthermal methods for microbial inactivation. The capability of PEF to inactivate vegetative form of microorganisms at temperatures that do not affect the sensorial and nutritional food properties is very attractive for food pasteurization.

The mechanism underlying microbial inactivation by PEF has not been fully elucidated. It is believed that the local defects or pores created by the application of an external electric field lead to the loss of the membrane integrity and uncontrolled molecular transport across the membrane may occur impairing maintenance of the microbial homeostasis. However, in microbial cells, cytoplasmic membrane is not the only barrier that separates the cytoplasm from the environment. The measurement of the uptake of the fluorescent dye propidium iodine after PEF treatments has demonstrated that the relationship between electroporation and loss of microbial viability is influenced by the envelopes surrounding the cytoplasmic membrane and the pH of the treatment medium. The capability of the microorganism of recovering the damage caused by PEF in the cytoplasmic membrane has been correlated with the occurrence of sublethal injury after the PEF treatment. Currently it is well established that PEF causes sublethal injury depending on the microorganism investigated and pH of the treatment medium. Generally, greater number of sublethally injured cells are detected in a population of Gram-negative bacteria when treated by PEF at pH 4 than at pH 7 but in Gram-positive bacteria occurrence of sublethal injury is greater at pH 7 than at pH 4.

A proper understanding of the mechanism involved in microbial inactivation by PEF may contribute to obtaining safe foods of high quality at reduced economic cost. This paper reviews the current state of the art in the proposed microbial inactivation mechanisms by PEF.



## OP23

### LIBERATION OF WATER-SOLUBLE PROTEINS FROM YEAST BY PULSED ELECTRIC FIELD TREATMENT

Valentina Ganeva

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Yeasts have been used for many years to produce large amounts of enzymes for industrial and biopharmaceutical use. There are becoming also some of the most utilized hosts for recombinant protein production. Many of the proteins of interest are with intracellular or periplasmic localization and for their recovery usually mechanical disintegration or chemical extraction is applied. Although applicable on large scale, these procedures can lead to protein inactivation and complicate the downstream purification process, thus leading to higher production costs.

Pulsed electric field (PEF) treatment that induced irreversible plasma membrane permeabilization gained great popularity during recent years as an efficient method for non-thermal liquid food preservation and for extraction of biologically active compounds from microorganisms and plant cells. Recently we demonstrated that PEF applied in continuous mode can be utilized as an efficient method for recovery of intracellular enzymes from different yeast species. The electrical conditions applied for plasma membrane permeabilization are relatively mild and do not provoke cell disintegration.

A very important factor determining the efficiency of protein liberation from electropermeabilized cells is the yeast cell wall – its charge and porosity. Despite induction of irreversible plasma membrane permeabilization the proteins could be largely retained inside the cell, because of their size or due to electrostatic interaction with cell wall components. It was found that the efficiency of total protein liberation depends strongly on the pH and the composition of the postpulse incubation media. A similar tendency is observed for different yeast species. This opens possibilities to increase the selectivity of protein liberation on the base of its isoelectric point and size.

PEF treatment suitable for intracellular and periplasmic enzyme liberation provokes not only plasma membrane permeabilization, but enhances the cell wall porosity of late and stationary phase cells. This renders the electropermeabilized cells more sensitive to cell wall degrading enzymes like lyticase. In dependence on the strain and the electrical conditions, cell lysis can be obtained at 2 – 8 times lower enzyme concentration in comparison with control untreated cells. Furthermore, the incubation of electropermeabilized cells with a very low concentration of lyticase enhanced both the rate and the efficiency of protein release, without significant cell lysis. Such combined treatments can be helpful for selective liberation of intracellular and periplasmic compounds from cells with very strong walls or when the liberation of very large molecules or supramolecular complexes is desired.

Suitable combination of several factors: electrical parameters, cell growth phase, pH and composition of the postpulse incubation buffer, low concentration of lytic enzymes etc. enables the development of a simple procedure for efficient recovery and partial purification of water-soluble native and recombinant proteins from yeast.

## OP24

## PULSED ELECTRIC FIELD ASSISTED EXTRACTION OF BIOLOGICALLY ACTIVE CONSTITUENTS FROM FRESH BLUEBERRIES

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Pulsed electric field (PEF) technology has the potential to be used for the improvement of mass transfer processes from plant tissues. Pretreatment of plant materials with short, high power electrical pulses induces the permeabilization of cell membranes that may enhance extraction of bioactive metabolites. In this study we aimed to examine whether PEF can be used to enhance mass transfer processes of secondary metabolites from fresh blueberries (*Vaccinium myrtillus* L.) and their press cake.

The degree of blueberry cell's permeabilization was determined on the basis of impedance measurement and consequently the optimal PEF parameters were estimated. The effect of PEF pretreatment on juice yield and other characteristics (°Brix, pH, total phenolics, total anthocyanins, antiradical activity) were analyzed. In addition, berry press cake obtained after juice pressing was subjected to further extraction (acidified aqueous ethanol for 24 h at ambient temperature) and the quality of obtained extracts, in terms of bioactive compounds content, was evaluated.

The best results in fresh blueberry permeabilization were achieved when electric fields of 1, 3 and 5 kV/cm were used, and energy input was 10 kJ/kg. The pretreatment of blueberries with PEF increased juice yield from 23 to 30%. Furthermore, higher total phenolics content (from 36 up to 48%) in fresh pressed blueberry juice was obtained from PEF pretreated berries. Fresh juice obtained after PEF pretreatment had also resulted in significantly higher content of total anthocyanins (from 38 up to 74%). The highest total anthocyanins content (514 mg/L) was obtained with electric field strength of 3 kV/cm and energy input of 10 kJ/kg. Finally, in comparison to juice of control berries, juice of PEF pretreated berries had higher antiradical activity.

Blueberry press cake represents a low-cost source of biologically active compounds. Significantly higher amounts of total phenolics and anthocyanins were extracted from press cake of PEF pretreated berries. Extraction enhancement positively correlated with the electric field strength applied.

In this study we demonstrated that PEF technology can increase juice yield from blueberries as well as juice and press cake extracts quality.

## OP25

## ELECTRICALLY ASSISTED EXTRACTION OF POLYPHENOLS: APPLICATION OF PULSED ELECTRIC FIELDS AND HIGH VOLTAGE ELECTRICAL DISCHARGES

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Recently, the use of electrical pulsed energy (EPE) for treatment of food and agricultural products became very popular. Disruption of nanometer-sized biological membranes by EPE application results in critical acceleration of mass transfer. Acceleration of mass transfer in foods by traditional methods (ultrasonic, pressure, thermal, chemical, etc.) are commonly accompanied by many undesirable factors, degradation, necessity in using of unsafe organic solvents, enzymes, detergents, temperature elevation or unreasonably high energy consumption. The application of high voltage electrical discharges (HVED) and pulses electric fields (PEF) has been shown to be appropriate for the extraction of polyphenols from various products such as grape by-products, oilseeds and lignocellulosic biomass.

Depending on the type of product but also on the localization of polyphenols at the cellular level, different extraction protocols have been proposed. The extraction of soluble pigments (anthocyanins) could be performed by using gentle conditions and rather low energy input. The extraction of molecules that could be linked to the cell wall or cell membrane (stilbenes or tannins) required the combination of the electrically assisted extraction with a basic or acidic hydrolysis. Various synergetic effects of electrical treatment and solvent (ethanol) have been determined. The selective release of some compounds by using the electrically assisted extraction could be obtained by varying the operating parameters. For example, the HPLC results could reflect the presence or absence of some polyphenols depending on the applied treatment. The impact of the electrically assisted extraction on the extract quality has also been studied. It has been shown for example that the application of high HVED treatment energy input (> 200 kJ/kg) on a grape pomace suspension could result on the oxidation of the main polyphenols (catechin, epicatechin, quercetin-3-o-glucoside, kaempferol-3-o-glucoside). The optimization of the treatment operating parameters is thus required for each studied product.

## OP26

### PRODUCTION OF WHEY PROTEIN HYDROGELS THROUGH APPLICATION OF ELECTRIC FIELDS

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Whey protein based gelling systems may present several functional roles in food formulations by enhancing textural properties (e.g. mouthfeel), acting as stabilizing agents or by being used as carrier of bioactive substances (e.g. nutraceuticals). The rates and pathways for the production of a protein gel system are controlled by heating conditions, protein concentration, pH, ionic strength, and solvent medium. The combination of heat and electric treatment has the potential to interfere with unfolding and aggregation of whey proteins and thus with protein-protein interactions. The general objective of this study was to evaluate and understand the effects of electric fields (EF) during thermal aggregation of liquid dispersions of whey protein isolate (WPI) and hydrogels made thereof. The main goal of this study was to induce thermal aggregation of a liquid dispersion of WPI into a three-dimensional network, a so called hydrogel, through combined application of instantaneous heating and EF. Nanostructures and nano-scale phenomena of the initial steps of whey protein aggregation were assessed by nano-tracking analysis and dynamic light scattering techniques. To assess the effects of EF on macroscopic properties of the produced hydrogels, rheological measurements were performed under steady shear flow. This study shows that EF interferes with inter- and intra-molecular protein interactions producing a marked reduction in whey protein aggregation. This particle size reduction can be controlled by the intensity of electrical treatment applied (e.g. EF treatments of 0, 3 and 10 V/cm have determined particle sizes of 156.9, 141.1 and 117.7 nm, respectively). The rheological measurements show that apparent viscosity of the produced hydrogels is also affected by the presence and magnitude of the EF applied. In conclusion, EF induces changes from a nanometer to micrometer range offering a great potential to the development of GRAS (generally recognized as safe) engineered nanotechnology-based delivery systems for food applications.



# Last minute Posters

## Food Microbiology & Biotechnology

P371

### MICROBIOLOGICAL PROFILE OF ARTISANAL CHEESE PREPARED FROM RAW MILK WITH OREGANO HERBS

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For commercial purposes, herbs, spices, vegetables, fruits, and other condiments are being added to cheese to diversify flavor or presentation. In line with this, the effect of oregano (*Origanum vulgare*) herbs addition on the microflora of cheese prepared from raw milk (mixture of goat and sheep milk) was assessed. The viable numbers of lactic acid bacteria, mesophilic aerobic microorganisms, total coliforms, *E. coli*, enterobacteria, staphylococci, and yeasts and moulds were evaluated after a 42-day period of ripening, on traditional cheese and cheese with oregano herbs, with TEMPO automated system (bioMerieux™). The microbial loads of Portuguese traditional cheeses are diverse from each other, but high, since they are manufactured from raw milk and are not inoculated with standardized starter cultures. The results showed that lactic acid bacteria and mesophilic microorganisms were the predominant groups in both artisanal cheeses (approximately 8 log CFU). Similar loads of enterobacteria, yeasts and moulds (approximately 6 log CFU) were also found in traditional cheese and cheese with oregano. However the addition of *Origanum vulgare* increased total coliforms, *E. coli*, and staphylococci counts, probably due to the microbiological quality of the herbs.

P372

MONITORING ANTIMICROBIAL RESISTANCE IN *SALMONELLA SPP* STRAINS ISOLATED AT THE NATIONAL INSTITUTE OF PUBLIC HEALTH IN ANGOLA

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Bacterial resistance to antimicrobials, as well as continuing to be a topical issue, it is also a subject widely studied, especially when the disease caused by the bacterial agent has high potential for endemicity such as typhoid. The incidence of outbreaks constitute a stimulating factor for the increase in abusive wrong administration of antimicrobial agents, thereby providing a favorable environment for the emergence of resistant bacterial strains. This study evaluated the resistance to antimicrobials commonly used to treat Salmonella infection; 10 Salmonella strains isolated in the period from January to November 2013. Strains identified and confirmed as *Salmonella spp*, were tested for antimicrobial susceptibility by antibiogram, using the disc diffusion method, according to the norms of the document M2 – A8, of the National Committee for Clinical Laboratory Standards – NCCLS. As a control reference were used *Escherichia coli* ATCC. The 10 strains analyzed showed resistance to one or more antimicrobials, 80 % showed resistance to ceftriaxone, 40 % to tetracycline and 30 % to chloramphenicol. The nalidixic acid and ciprofloxacin showed intermediate resistance of 50 % each. Eith different patterns of resistance were evidenced. The results indicate a progressive increase resistance strains of *Salmonella spp*, thus suggesting the need for proper and proper and responsible use of antimicrobials.



## Microbial Pathogenesis

### P373/F38

#### EVALUATION OF THE STRESS-INDUCED GROWTH OF THE PORTUGUESE CLINICAL ISOLATE *HELICOBACTER PYLORI* CI-5 STRAIN – A STRUCTURAL CHARACTERIZATION OF CELL-SURFACE LIPOPOLYSACCHARIDES

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*Helicobacter pylori* is a microaerophilic, and spiral-shaped Gram-negative bacterium, commonly associated with the development of gastro-intestinal pathologies, such as chronic gastritis, gastric and duodenal peptic ulcers, and ultimately gastric cancer. Nowadays, more than half of world's population is infected, and to date, there is no vaccine against *H. pylori*. The treatment is limited to antibiotic administration, whose efficiency is decreasing. Lipopolysaccharides (LPS) are the major cell-surface antigens of Gram-negative bacterial species, and their role in the pathogenesis remains to be fully clarified. Chemically, LPS are composed of a variable polysaccharide moiety (O-chain), normally expressing Lewis blood group determinants, and a structurally conserved core oligosaccharide linked to a lipid A moiety. It has been reported that the structural variation and the degree of polymerization of the O-chain are influenced by the bacterial growth medium and by the pH of the environment. In the present work, we evaluated the *H. pylori* CI-5 strain LPS structural composition during its normal (37°C, 48 h) and stress-induced growth triggered by lack of nutrients (37°C, 168 h) in a solid agar medium. Structural analysis of glycosidic linkages carried out on both normal and stressed CI-5 cultures revealed the presence of terminal fucose, 3-substituted galactose, 2-substituted ribose, 4-, and 3,4-substituted *N*-acetylglucosamine as the main sugar residues, in the approximate molar ratio of 16:25:16:8:10, and 16:29:18:5:4, respectively. This lower variability in the LPS structural composition suggests that the CI-5 clinical isolate presents a higher tolerance to stress induced by lack of nutrients.

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## Drug Resistance Targets and Biotechnology

P374

### INDICATOR GENES TO ASSESS ANTIBIOTIC RESISTANCE ECOLOGY IN A HOSPITAL-URBAN WASTEWATER TREATMENT PLANT SYSTEM

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Antibiotics are important micropollutants and antibiotic resistance genes are considered emerging pollutants. Municipal and hospital effluents (HE) are major sources of both antimicrobial residues and resistance genes in urban areas. Resistance increase relies on a complex set of factors in which horizontal gene transfer and selection are especially important, although not the unique drivers. The type of resistance, the bacterial hosts and the abiotic conditions are examples of important determinants of the fate of resistance genes and bacteria.

Four genes, *int11*, *bla*TEM, *vanA*, *marA*, related with antimicrobial resistance, but associated to different hosts and types of resistance were monitored in HE and in raw (RWW) and treated wastewater (TWW) from the treatment plant that receives those effluents. The abundance of those and of the 16S rRNA genes was estimated by real-time PCR and analyzed in function of the concentration of antimicrobial residues, antibiotic resistance prevalence and 16S rRNA gene-DGGE patterns. Gene copy numbers were normalized with the abundance of the 16S rRNA gene.

The relative abundance of *int11*, which codes for an integrase responsible for recombination events in class1 integrons, did not differ in HE, RWW and TWW. For *bla*TEM, one of the most common beta-lactamases genes occurring in enterobacteria, the relative abundance decreased significantly from the HE to TWW. The determinant *vanA*, associated with vancomycin resistance in enterococci, was significantly more prevalent in HE than in RWW or TWW. The relative abundance of *marA*, which is part of a multidrug resistance locus in *Escherichia coli*, was significantly higher in HE than in RWW, becoming significantly more prevalent in TWW. The normalized abundance of *bla*TEM was correlated with the concentration of tetracycline and sulfamethoxazole residues, the prevalence of the amoxicillin and ciprofloxacin resistant bacteria and bacterial populations represented by three DGGE bands. On the other hand, the normalized abundance of *vanA* was correlated with bacterial populations represented by two DGGE bands.

Major conclusions were that i) HE was an important source of *bla*TEM and *vanA*, while wastewater treatment selected for *marA*; ii) an identical potential for gene acquisition in HE, RWW or TWW was hinted by the stable prevalence of *int11*; iii) *bla*TEM and *vanA* variations were associated to antibiotic resistance sources, antimicrobial residues and bacterial populations.

## P375

C2-PHYTOCERAMIDE PERTURBS LIPID RAFTS AND CELL INTEGRITY IN *SACCHAROMYCES CEREVISIAE* IN A STEROL-DEPENDENT MANNER

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Specific ceramides are key regulators of cell fate, and extensive studies aimed to develop therapies based on ceramide-induced cell death. However, the mechanisms regulating ceramide cytotoxicity are not yet fully elucidated. Since ceramides also regulate growth and stress responses in yeast, we studied how different exogenous ceramides affect yeast cells.

We tested the effect of exogenously added soluble and cell-permeable phytoceramides and ceramides on clonogenic survival of *Saccharomyces cerevisiae* W303-1A. In particular, the effects of C2-phytoceramide on cell cycle, cell death and autophagy induction were evaluated. Cell wall defects and changes in organization of lipid rafts were evaluated by sensitivity to zymolyase digestion, filipin staining and Pma1p-GFP plasma membrane distribution. The impact of perturbing lipid rafts on the response to C2-phytoceramide was assessed using a *rvs161Δ* mutant. Involvement of MAP kinase pathways was tested using mutant strains lacking components of these pathways. Clotrimazole and ketoconazole, ergosterol biosynthesis inhibitors, methyl- $\beta$ -cyclodextrin, which extracts ergosterol from membranes, or amphotericin B, an ergosterol-binding antibiotic, were used to evaluate the sterol dependence of C2-phytoceramide effects.

C2-phytoceramide, the yeast counterpart of mammalian ceramide, greatly reduced clonogenic survival, particularly in the G2/M phase, but did not induce autophagy nor increase apoptotic markers. Rather, the loss of clonogenic survival was associated with PI positive staining, disorganization of lipid rafts and cell wall weakening. Sensitivity to C2-phytoceramide was exacerbated in mutants lacking Hog1p, the MAP kinase homolog of human p38 kinase. However, deficiency in components of the CWI pathway did not lead to differences in response to this compound. Decreasing sterol membrane content reduced sensitivity to C2-phytoceramide, suggesting sterols are the targets of this compound.

This study identified a new function of C2-phytoceramide through disorganization of lipid rafts and induction of a necrotic cell death under hypo-osmotic conditions. Since lipid rafts are important in mammalian cell signaling and adhesion, our findings further support pursuing the exploitation of yeast to understand the basis of synthetic ceramides' cytotoxicity to provide novel strategies for therapeutic intervention in cancer and other diseases.

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