Student: Ana Isabel Novo de Figueiredo

Supervisor: Paula Maria Lima Castro

Co-supervisors: Irina Sousa Moreira e Isabel Vasconcelos

Thesis Tittle: Search of new enzymes for the oxidation of sulfur compounds

Abstract (max. 3000 characters, including spaces)

Bioremediation is considered a cost-effective solution for the clean-up of contaminated environments, such as decontamination of sulfide contaminated gases and wastewater through biological oxidation.
Some heterotrophic *Proteobacteria* possess the enzymes sulfide: quinone oxidoreductase (SQR) and persulfide dioxygenase (PDO), which are responsible for the oxidation of sulfide via sulfite and thiosulfate [1,2]. This system is not energy efficient, being considered as a mechanism of detoxification.
On the other hand, members of *Roseobacter* clade possess the SQR-PDO system, as well as sulfite oxidase, which further oxidize sulfite and thiosulfate to sulfate, as energy generation systems [3].
Throughout the *P. koreensis* strain A9 genome analysis it was detected the presence of a genetic cluster for the oxidation of sulfide to sulfite and thiosulfate (SQR_PDO: peg's 4520_4521) regulated by a transcription factor of the Fis family (peg. 4922). In addition to the cluster associated with sulfide detoxification, the A9 genome carries a sulfite oxidase (EC 1.8.3.1) homolog gene (peg. 4921), that is probably responsible for the oxidation of sulfite to sulfate with associated energy gain.
Bacterial strain A9 was isolated from a sulfide enrichment and previously exhibits potential for the oxidation of sulfide to sulfate. The sulfide oxidase activity was been measured based on sulfate production, as the major end-product [4]. Maximum sulfide removal (99,8%) and degradation rate (1,6 $mmol.h^{-1}$) was achieved in GY medium with 16 mM of sulfide which are very promising results when compared to bibliography [1,4].
As the genetic analysis evidences, it was needed to confirm that the sulfide oxidation pathway go through the production of sulfite and thiosulfate as intermediates as the presence and organization of these enzymes coding genes appear in *P. koreensis* A9 genome.
In an assay performed in minimal mineral medium without any other source of sulfur except the sulfide added as a $Na_2S.9H_2O$ aqueous solution it was observed that the sulfide oxidation by A9 led to the formation of sulfite and thiosulfate while in the abiotic oxidation controls this does not happens.
Furthermore, analysis of A9 genome evidenced the presence of genes coding rhodanese-like, glutathione:sulfur transferase, thiol peroxidase, which point out the ability and versatility of this bacterium for bioremediation of sulfide contaminated streams.

References:

[1] Xu, X. *et al.* (2016) Characterization of a newly isolated strain *Pseudomonas* sp. C27 for sulfide oxidation: Reaction kinetics and stoichiometry. *Nat. Publ. Gr.* 1–10. DOI:10.1038/srep21032 DOI: 10.1038/srep21032.

[2] Liu, Y. *et al.* (2016) Genomic Analysis Unravels Reduced Inorganic Sulfur Compound Oxidation of Heterotrophic Acidophilic Acidicaldus sp. Strain DX-1. *Biomed Res. Int.* DOI: 10.1155/2016/8137012.

[3] Xin, Y., Liu, H., Cui, F., Liu, H. & Xun, L. (2016) Recombinant *Escherichia coli* with sulfide: Quinone oxidoreductase and persulfide dioxygenase rapidly oxidizes sulfide to sulfite and thiosulfate via a new pathway. *Environ. Microbiol.* **18,** 5123–5136. DOI: 10.1111/1462-2920.13511.

[4] Mohapatra, B. R., Gould, W. D., Dinardo, O., Papavinasam, S. & Revie, R. W. (2006) Optimization of culture conditions and properties of immobilized sulfide oxidase from Arthrobacter species. *J. Biotechnol.* **124,** 523–531. DOI: 10.1016/j.jbiotec.2006.01.031.

Student: Ana Luiza Rodrigues Fontes

Supervisor(s): Prof.ª Doutora Ana Gomes; Doutor Luis Rodríguez-Alcalá; Prof.ª Doutora Maria do Rosário Domingues

Thesis Tittle: Development of a new functional dairy product enriched in microbial bioactive conjugated fatty acids through an industrial-based approach

Abstract (max. 3000 characters, including spaces)

Unbalanced diets are among the major risk factors in the development of certain non-communicable diseases such as cardiovascular diseases and some types of cancer. Nevertheless, several food-derived compounds with potential bioactive properties have been identified, including those from the lipid fraction. These include conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) isomers, which have been reported as promising new functional ingredients with anti-inflammatory and energy metabolism modulation properties. Due to limitations in terms of concentration and availability of CLA and CLNA in its natural sources (e.g. ruminants' milk and meat or vegetable oils), the *in situ* microbial production may reveal itself to be a good strategy to increment CLA/CLNA daily intake: several lactobacilli, bifidobacteria and propionibacteria strains have demonstrated the ability to produce CLA/CLNA isomers using linoleic (LA) and alpha-linolenic (α-LNA) acids as substrates, respectively. Very few studies have been focused on this subject, let alone on its bioactive potential, therefore, the aims of this research study are i) the identification of CLA/CLNA-producing strains from among a wide range of collections including lactobacilli, bifidobacteria and propionibacteria from food and human origins, ii) characterization and understanding of the transformation pathways involved in the CLA/CLNA microbial production, iii) development and characterization of a dairy product enriched in microbial conjugated fatty acids, iv) assessment of its stability throughout storage and v) evaluation of its anti-carcinogenic activity.

To fulfill these objectives, a novel approach has been assayed for the identification of CLA/CLNA-producing strains through two screening techniques: i) molecular detection on genes encoding enzymes involved in LA/LNA isomerization and ii) determination of substrate high-tolerant strains, followed by production assays. From a range of 85 strains, 39 were selected according to the screening results. Each one was further cultured in the presence of LA: 4 strains out of 39 showed CLA production. Thereafter, those CLA-producing bacteria were further tested with LNA and all but one revealed CLNA production capacity. The 4 strains identified as CLA/CLNA-producers were then selected for the following tasks of this thesis research work.

Having concluded the first objective of this work (i), this Ph.D student and the supervising team are currently moving forward to the characterization and understanding of CLA/CLNA microbial production pathway and impact of type of substrate on CLA/CLNA production yield.

Student: Ana Rita Monforte

Supervisor(s): António César Silva Ferreira, Sara Martins, Rasmus Bro

Thesis Tittle: _ Chemiomics: A systems chemistry approach to unravel the interface pathways between Oxidation and Maillard mechanism responsible for flavour modulation during wine storage

Abstract (max. 3000 characters, including spaces)

In foods and in particular in wines, phenylacetaldehyde (PA) is reported to be particularly important, contributing to “honey” like aroma notes perceived as an off-flavor by the consumer. Its formation is related to the Strecker degradation (SD), which consists on the reaction between several compounds such as α-dicarbonyls formed during the Maillard reaction and o-quinones (vinylogs of α-dicarbonyls) formed during phenolic oxidation. Even though both reactions occur simultaneously in food systems, most research studies investigate them separately. The mechanistic understanding between the two reactions in the formation of PA is not well understood. To simultaneously determine the impact of Maillard reaction and the oxidation substrates on PA, a full factorial design has been performed. A duplicated completely randomized design, evaluating six factors (glucose, phenylalanine, gallic acid, metals, oxygen) at two levels (present or absence) at pH 3.2 and 7 was used. The formation of PA was monitored by solid phase micro extraction (SPME) in combination with GC-MS. The results seem to indicate that phenylacetaldehyde formation is promoted by the phenolic oxidation. Although all parameters showed an impact on PA formation, phenylalanine, gallic acid, pH and metals were the most significant factors. The present study allowed not only determining the critical parameters on PA formation in different model systems, but also the kinetics of PA formation is presented.

Student: Ana Sofia Teixeira Oliveira

Supervisor(s): Paula M.L. Castro, Catarina L. Amorim, Mark van Loosdrecht

Thesis Tittle: EPS_AuGmentS - Aerobic Granular Sludge technology combined with bioaugmentation with immobilized bacteria as a tool to degrade micropollutants from wastewater

Abstract (max. 3000 characters, including spaces)

The industrial sector generates high loads of effluents which composition is extremely heterogeneous. Moreover, industrial wastewater often contains simultaneously high salinity and high organic and toxic content, which represents a major challenge for its treatment.

Aerobic granular sludge sequencing batch reactors (AGS-SBR) are innovative wastewater treatment systems economically outcompeting the conventional activated sludge. AGS is considered a special case of suspended biofilms, composed of self-immobilized microorganisms in an extracellular polymeric substances (EPS) matrix, forming spherical sludge aggregates without the need of any carrier. AGS indigenous microbial communities are very efficient in the removal of certain nutrients and chemical species, but often are unable to remove xenobiotic contaminants. 2-Fluorophenol (2-FP) is an example of a toxic compound that can appear in several industrial effluents.

This work aims to develop 2-FP degrading granules by immobilization of a specialized degrading strain *Rhodococcus* sp. FP1 in a EPS matrix for further bioreactor bioaugmentation.

The characterization of EPS produced by AGS from different sources (lab- and full-scale reactor) enabled us to evaluate the feasibility to recover a valuable product from the surplus biomass. The extracted EPS was the basis for the subsequent steps of the work. Granules with *Rhodococcus* sp. FP1 were produced using the extracted EPS as a natural immobilizing agent. The specialized granules were tested for their strength in a shear stress test and for their potential to biodegrade the toxic compound, 2-FP. Strong 2-FP degrading granules were produced, representing an interesting alternative for bioaugmentation strategies of AGS technology.

Currently, a lab-scale AGS-SBR is under operation for the treatment of a simulated industrial wastewater containing 2-FP and variable salt content. The bioreactor was recently bioaugmented with the produced granules. Reactor performance regarding organic carbon, nitrogen and phosphorus removal and 2-FP biodegradation is being monitored. *Rhodococcus* sp. FP1 persistency in the reactor will be evaluated using quantitative polymerase chain reaction (qPCR). Before bioaugmentation, phosphorous removal was affected by the presence of 2-FP in the influent, and nitrogen removal stopped completely leading to ammonium accumulation. After bioaugmentation, nitrogen removal seems to be resumed in the bioreactor.

This study presents a potential application of a recovered resource from wastewater treatment and will potentially assist the existing industrial processes of wastewater treatment with AGS.

Student: Débora Andreia Campelo Campos

Supervisor(s): Professora Maria Manuela Pintado, Lorenzo Pastrana e José Teixeira

Thesis Tittle: Development and characterization of functional ingredients through valorisation of pineapple by-products: production, bioactivity and potential application

Abstract (max. 3000 characters, including spaces)

Pineapple (*Ananas comosus*) is the third most important tropical fruit in the world production and one of the most processed fruit, but is the most important among generated fruit residues. These residues (skins, crowns, cylinders, stems and pulp remnants) contain high content of bioactive compounds, but generally not directly available and for that reason is necessary to extract and characterize the feasible bioactive compounds [1].

Therefore, the focus of this research work was to perform an extended study on the prebiotic activity of pineapple by-products (peels and stems) to explore the potential of the development of a new functional ingredient. Frozen pineapples by-products were submitted to a milling and pressing processes, creating a pineapple juice and a solid semi-dried extract. The soluble fraction (juice) was submitted to extraction of the pineapple enzymes (Bromelain) and after this process the soluble fraction was assessed for the presence of soluble carbohydrates.

An initial screening was performed using six different probiotic strains from two different genera, *Lactobacillus sp.* and *Bifidobacterium sp.,* all the microorganisms showed a positive growth towards the pineapple by-products juices (PBJ), with an exception of *Lactobacillus acidophilus*. The activity was in the range of positive control (frutooligosaccharides - FOS).

To evaluate the maintenance of bioavailability of prebiotic compounds, the PBJ were submitted to gastrointestinal tract simulation, and analysis of polysaccharides molecular weight (MW) was made by HPLC, as well prebiotic capacity was tested after each step (mouth, stomach and small bowel simulation). A major picture of the potential should be made; thus, the insoluble fraction was evaluated in terms of percentage of lignin, cellulose and hemicellulose.

To evaluate the MW of polysaccharides an ultra-hydrogel column was used, and the results showed that two major peaks of polysaccharides comprising MW of 2000 and 600 Da. Also, when using an Aminex® column for simple sugars analysis, the fractions presented high concentration of two monosaccharides glucose and fructose, as expected. The polysaccharides of higher MW were identified as been galactomannan [2]. The insoluble fractions contained ca. 30% (w/w) of lignin being the most part soluble lignin, hemicellulose and cellulose represents ca. 35% (w/w).

Through this research work it was possible to characterize the carbohydrates presents in pineapple by-products, establish prebiotic related activity and study the maintenance of prebiotic activity through gastrointestinal tract simulation.

References

1. A. P. do Espírito Santo et al., Int J Food Microbiol, **2012**, *154(3)*, 135-144.
2. Salunkhe, O. L. et al., Handbook of fruit science and technology, **1995**, *1(1),* 5-9.

Student: Gianuario Fortunato

Supervisor: Prof. Célia Manaia, Dr. Ivone Vaz-Moreira, Prof. Olga Nunes

Thesis Tittle: Measurement of the impact of AR discharge in WW and in soil: ecological aspects

Abstract (max. 3000 characters, including spaces)

The irrigation of agriculture fields with treated wastewater has the potential to contribute to the increase of antibiotic resistance in the soil. This effect can be due to the discharge of antibiotic resistant bacteria and/or of selective pressures, such as metals. This type of can create conditions for the successful spread of antibiotic resistance in soil.

This study had two major objectives. One was the determination of the quantification limit of antibiotic resistance genes in soil. The other was the assessment of the stability of exogenous wastewater antibiotic resistant bacteria and resistance genes (ARB & ARG) in soil. To accomplish the first objective, were used soil microcosm assays spiked with different amounts of known ARB (from 10^2 to 10^7). The microcosms where analyzed by culture-dependent and qPCR to quantify the ARB and ARGs and set the limit of quantification for the ARGs in soil. These results supported the experimental design to accomplish the second objective. With this aim, microcosms inoculated with a known concentration (10^7) of ARB harboring known ARGs were incubated at constant temperature (25°C) for 1 month. The selective pressure was analyzed under the same conditions, with amendments with 1mM and 2mM for metals ($CuSO_4$ and $ZnSO_4$). The limits of quantification (LOQ) of the ARGs (vanA, qnrS, bla_{TEM}, bla_{OXA}, bla_{IMP}, bla_{VIM}) were observed to be approximately 4 log-units ARGs copies per gram of soil dry weight. Regarding the survival of the selected ARB (*Pseudomonas aeruginosa* and *Acinetobacter johnsonii*), the preliminary results showed the decrease of ARB and ARG after 1-2 weeks of incubation, an effect that was observed in the absence or in the presence of selective pressure ($CuSO_4$ and $ZnSO_4$). The effect of selective pressures will be further explored. The survival of wastewater bacteria in soil, even if only for one week, may be an issue of concern if antibiotic resistance transfer is possible in that kind of environment.

Student: Ingrid Pascale Collombel

Supervisor(s): Tim A. Hogg, Francisco M. Campos

Thesis Title: Vinification and post vinification micro-ecology of wines; the role of phenolic composition and the effects on quality

Abstract (max. 3000 characters, including spaces)

The overall objective of this project is to investigate the interactions between naturally present phenolics and the post alcoholic fermentation wine microbiota, with a focus on quality aspects. In a first study, the effect of an increase in phenolics on microbial growth and metabolism in (non-inoculated and inoculated) wines was followed during the malolactic fermentation (MLF) and subsequent storage. Along MLF, the bacterial population was inhibited by flavonols and hydroxycinnamic acids (HCA). In spontaneous MLF, these compounds also affected the metabolism. Flavan-3-ols, depending of the initial concentration of (+)-catechin added, enhanced the bacterial growth, or had an inhibitory effect on growth and metabolism. Citrate consumption was delayed by most of the phenolics in the inoculated wines. *Trans*-resveratrol seemed to cause a shift in the heterofermentative pathway towards the production of acetic acid. Three months after the initiation of MLF, HCA and *trans*-resveratrol appeared to cause a decrease in pH, anthocyanins, and yeasts concentrations. The addition of phenolics at the beginning of MLF impacted on the aroma profile of the stored wines. The results suggest that the pre-fermentation steps, which will affect the type of phenolic compounds of a wine, can directly impact its microbial population and its metabolism, and therefore its final quality. In a second study, the repercussion of the malolactic microbiota on the phenolics composition was analyzed. Some strains of *O. oeni* have been found to possess cinnamoyl esterase activity that can liberate phenolic acids, precursors of volatile phenols responsible for sensory faults. The aim here was to better understand the basis of this differential activity between strains. 5 strains were selected, 3 exhibiting cinnamoyl-esterase activity (CE+) and 2 not (CE-). Pasteurized wine was used as source of cinnamate esters in growth experiments whilst trans-caftaric acid was used as substrate for enzyme assay. On analysis of full genome sequences provided for all strains, no specific gene could be found that was common only to the CE+ strains. Initial results shown that, unlike the free HCA form, trans-caftaric acid is not toxic toward *O. oeni*. The CE activity is apparently intracellular and present in all wine-exposed and unexposed strains. Only in the case of the CE+ strains exposed to wine did the cell debris contain higher protein concentrations that the unexposed ones. Cell free extracts of the 3 CE+ strains, totally degraded trans-caftaric acid to trans-caffeic acid, whilst extracts of CE- strains exhibited a lower activity, albeit higher for one of these strains in experiments where there had been no prior exposure. In the case of CE+ strains, wine-exposed samples showed a more rapid degradation than the unexposed ones. These results highlight the possible implication of more than one enzyme and of membrane transport proteins in CE activity shown by CE+, *O. oeni* strains studied here.

Student: Jaqueline Rocha

Supervisor(s): Dr. Célia Manaia, Dr. Isabel Henriques and Dr. Margarita Gomila

Thesis Tittle: Novel approaches on the characterization of the wastewater resistome: possible implications on human health and water quality management

Abstract (max. 3000 characters, including spaces)

Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are considered contaminants of emerging concern, nowadays widely disseminated in the environment. Among other sources, urban wastewater treatment plants (UWTPs) are major reservoirs of these contaminants. In UWTPs, wastewater is subjected to different types of treatment that can reduce the levels of ARB and ARGs, although not to negligible levels. Quantitative PCR (qPCR) is nowadays the gold standard method used to quantify ARGs in environmental samples, however the lack of harmonization of qPCR methods between laboratories makes the comparison of results obtained worldwide difficult. This work aimed to compare the ARGs removal efficiency of several wastewater disinfection methods. The disinfection treatment UV/PMS was the most efficient removing total bacteria, a gene involved in site-specific genetic recombination (*intI1*), and ARGs encoding resistance to sulfonamides; while the treatments UV and UV/H_2O_2 were the most efficient removing ARGs encoding resistance to β-lactams and quinolones. It was also aimed to compare the influence of two different DNA extraction methods on ARGs recovery by qPCR, and to assess how variable qPCR results could be among different laboratories when the same DNA extracts, qPCR protocols and reagents are used. The comparison of two different DNA extraction kits (NZY Tissue gDNA Isolation kit and PowerWater® DNA Isolation Kit) suggested that ARGs recovery might be different with the different kits. This difference was more evident for putatively plasmid-associated genes in cleaner water samples (river) in comparison to wastewater samples (hospital effluent). The assessment of inter-laboratory variation showed that gene quantification variations could reach 28% and the qPCR equipment seemed to be the most important factor influencing the inter-lab variations. Thus, considering the variations observed it was intended to develop a novel approach for qPCR methods, based on the use of an internal standard that would allow the harmonization of results among laboratories. In average, the losses of the internal standard in ultrapure water were around 3.8%, and in environmental water samples, where the samples matrix is more complex, these losses could reach higher values such as 13.9%.

In conclusion, the comparison of the ARGs removal efficiency of different wastewater disinfection treatments will contribute to improve the control of the propagation of ARGs in the environment. The use of different DNA extraction kits helped to understand which ones could be used, for example, when

higher yields of plasmid DNA need to be recovered. The use of an internal standard is a promising approach to measure the losses that may occur during samples processing and genes quantification for environmental water samples and will improve the quality of comparative studies with data obtained in different laboratories.

Student: Joana Ribeiro Costa

Supervisor(s): Prof. Doutora Manuela Pintado, Lorenzo Pastrana & Lourdes Cabral

Thesis Tittle: Valorization of grape pomace through extraction of xylooligossacharides for potential application in functional ingredients

Abstract (max. 3000 characters, including spaces)

Grapes are one of the most cultivated fruit crops worldwide, from which more than 70% is generated in wine industry in the form of grape skin, seeds, stems and residual pulp, known as grape pomace (GP)[1,2]. Recently, extraction of xylooligosaccharides (XOS) from lignocellulosic feedstocks has become very common, as these molecules have a deep impact on gastrointestinal health, mainly due to their selectively stimulation of gut microflora – prebiotic and antimicrobial activities - but also for their antioxidant activity[3,4]. The objective of this work is to evaluate the biological properties of a xylooligosaccharide-rich grape pomace extract.

Grape pomace extract was obtained through enzymatic extraction using 100 IU/g of an enzymatic cocktail produced by *Aspergillus niger* 3T5B8, containing xylanase activity. Extraction was performed using citrate buffer with pH 5 as solvent, and extraction was performed at 40°C for 4 hours, under agitation. The extract was filtered under vacuum and lyophilized. Extract was characterized for its chemical composition and different bioactivities were evaluated: Antioxidant capacity via ABTS and ORAC methods; antimicrobial activity against MSSA, MRSA, *E. coli* and *P. aeruginosa*; prebiotic effect on *Bifidobacterium animalis* Bo, *Bifidobacterium longum* BG3, *Bifidobacterium animalis* spp. *lactis* Bb12, *Lactobacillus casei 01*and *Lactobacillus rhamnosus R11.* Cytotoxicity on Caco-2 cells (MTT assay) and an *in vitro* simulation of the GP extract gastrointestinal digestion were also analyzed.

Freeze-dried GP presents 24.81 ± 1.65 g/ 100 g of total dietary fibre, from which xylobiose at 5.57g/ 100 g of extract, xylotriose and xylotetraose are also present but could not be quantified (< 1.0 g/ 100 g). Other polysaccharides with molecular weights around 400 Da, between 5 and 10 kDa, and near 100 kDa are also present. Total phenolic compounds in the extract are 1.404 ± 0.03 mg Gallic Acid/ g sample. Antioxidant capacity of GP extract was 2.326 ±0.05 mg Ascorbic Acid/ g sample though ABTS method and 2049.8 ± 37.7 µmol Trolox Eq./ g sample. The Minimal Inhibitory Concentrations (MIC) of the GP extract were 14 mg/ mL for Gram negative bacteria (*E. coli* and *P. aeruginosa*) and 16 mg/ mL for Gram positive (MSSA and MRSA). The use of 2% (w/v) of GP inhibited the growth of different pathogenic bacteria and at concentration of 1% (w/v) GP showed bacteriostatic activity. The GP XOS-rich extract at concentration of 20 mg/mL stimulated the growth of both *Bifidobacterium* and *Lactobacillus* spp. and

promoted a higher growth rate of *Bifidobacterium* spp. when compared to FOS control. GP extract at concentrations below 10 mg/ mL showed no cytotoxicity upon Caco-2 cells.

References:

1. Spanghero, M. *et al.*, 2009. Anim Feed Sci Technol. 152 (3-4), 243 – 255.
2. Corbin, K. R. *et al*., 2015. Bioresour Technol. 193, 76 – 83.
3. Llobera & Cañellas, 2007. Food Chem. 101 (2): 659 – 666.
4. Saura-Calixto, F., 1998. J Agric Food Chem 46 (10): 4303 – 4306.

Student: Manuel João Rebelo Araújo Oliveira

Supervisor(s): Susana Carvalho and Marta Vasconcelos

Thesis Tittle: New challenges on the control of Flavescence dorée in grapevine: exploiting genetic resources and the use of elicitors

Abstract (max. 3000 characters, including spaces)

Flavescence dorée (FD) is an epidemic disease of the grapevine, associated with large production losses, lower grape quality and death of the most susceptible cultivars. FD infection is caused by a phytoplasma, which is spread epidemically by the insect Scaphoideus titanus Ball. Currently, there measures for this disease control have been shown to be ineffective as new cases continue to be reported in Europe. This study had two general objectives: (1) to compare a set of physiological, biochemical and molecular parameters in healthy and infected plants; (2) and to evaluate the effect of methyl jasmonate (MeJA) on the induction of defense mechanisms of grapevines against FD. Thus, with cultivar "Loureiro" was made physiological, biochemical and genetic analyses. In the first two analyses, the effect of MeJA (12.5 and 25 mM) was evaluated on healthy (FD-) and infected (FD+) grapevines. Genetic assay was focused on the evaluation the expression of genes encoding pathogenicity-related proteins (Thaumatin I, Thaumatin II, Osmotin, PBSP, CHIT4c, PIN, PGIP and GLU) involved in energy production (Rubisco) and protein degradation (subunit 5 α of proteasome) and genes of the phenylpropanoid pathway (PAL, STS). In this study, 12.5 mM of MeJA treatment was shown an induction of saponins production in infected plants. In contrast, MeJA application showed a positively induction of proline synthesis, only in healthy plants. Regarding gene expression, it was found that 12.5 mM of MeJA induced a greater number of expressed genes 24 hours after application, in pre-elicited infected plants, and 24 hours after treatment with 25 mM of MeJA on infected plants, elicited only in veraison.

Keywords: Flavescence Doreé, phytoplasma, methyl jasmonate, metabolites, gene expression.

Student: Mariana Roriz Lemos Costa

Supervisor(s): Marta Vasconcelos, Paula Castro, Susana Carvalho

Thesis Tittle: UTIL - Utilization of plant-growth promoting bacteria (PGPB) to ameliorate iron nutrition in the legumes

Abstract (max. 3000 characters, including spaces)

Legumes are important crops for humans and animals, having a positive impact on environmental health. Iron deficiency chlorosis (IDC) is a serious nutritional problem affecting legumes, particularly when grown in calcareous soils. Recent evidence suggests that the utilization of biofertilizers enriched with plant-growth promoting bacteria (PGPB) may be an efficient strategy to enhance iron nutrition in these crops. In this project, we aim at understanding the impact of PGPB as IDC control agents through morphological, physiological and molecular analysis in soybean.

Twenty-four PGPB isolates from a CBQF collection were first selected based on NH_3 and siderophore production, and phosphate solubilization, and tested for *in vitro* traits related to iron nutrition. Two isolates (*Sphingobium fuliginis* and *Pseudomonas jessenii*) were further selected based on the best capacity to reduce Fe(II). In parallel, 76 bacteria were isolated from shoots (18%) and roots (53%) of field grown soybean plants, and from rhizospheric soil (29%), 41 of which were identified based on the 16S rRNA gene sequencing. Results showed that *Brachybacterium, Kocuria, Luteimonas, Methylobacterium, Microbacterium, Pseudoclavibacter* and *Streptomyces* genus were isolated from shoots; *Agrococcus, Aliihoeflea, Bacillus, Brachybacterium, Cellulomonas, Dyadobacter, Flavobacterium, Flexithrix, Luteimonas, Microbacter, Microbacterium, Paenibacillus, Pseudomonas, Rheinheimera, Sphingobacterium, Staphylococcus, Stenotrophomonas* and *Vitreoscilla* genus were found in roots; and *Agrococcus, Arthrobacter, Bacillus, Fictibacillus, Kocuria, Microbacterium, Ochrobactrum, Paenibacillus, Sphingomonas, Sporosarcina* and *Stenotrophomonas* were isolated from soil.

The best candidates selected within the CBQF collection and soybean isolates are being used in soil experiments, in order to assess the effect of bacteria inoculation on IDC symptoms alleviation. Inoculations are being performed in a calcareous soil (pH 7.4) from the North of Portugal. Plants are being evaluated for several IDC and plant growth parameters, such as: SPAD, chlorophyll content, plant weight, root iron reductase activity, mineral content, expression of genes related to iron nutrition and PGPB colonization. The results of the first experiment with the two CBQF isolates have shown a statistically significant increase in the root iron reductase activity when plants were inoculated with both isolates alone, and in the iron concentration in the trifoliates when plants were inoculated with a mixture of both isolates and in the roots when inoculated with *S. fuliginis*. Also, a statistically significant increase was verified in the manganese concentration in the trifoliates of plants inoculated with a mixture of both isolates.

The bacterial PGP abilities of soybean isolates will also be tested applying techniques currently used in CBQF (amount of IAA, ACC-deaminase activity, phosphate solubilization, and ammonia and siderophore production) and those with the best PGP abilities will be tested for the same analysis related to Fe nutrition already performed to the CBQF isolates.

Student: Miguel António Marcos Ramos

Supervisor(s): Nadine R. Sousa, Paula M. L. Castro

Thesis Tittle: Selection of strains of edible mycorrhizal fungi for improved field persistence and mycelial expansion

Abstract (max. 3000 characters, including spaces)

Ectomycorrhizal Fungi (EcM) may play an important role in improving tree vigor and enhancing ecosystem services delivered by trees. It is important to develop resilient EcM-inocula to improve tree health, including urban trees. The use of native strains with strong adaptive skills to different abiotic and biotic challenges could be determinant for the success of tree establishment. The present work focus on screening high performance strains of edible ectomycorrhizal species *Lactarius deliciosus* (*L. deliciosus*) and assess its performance and ability to grow and adapt to stresses scenarios to ensure a more sustainable choice of isolates. The ability of *L. deliciosus* to grow and acclimate to abiotic stresses was studied by analyzing the effects of exposure on growth and biochemical traits. Fungal plugs were placed in fresh medium, on top of a cellophane sheet and grown for four weeks at 3 levels of temperature, (15°C, 22.5°C, 30°C), water stress (0%, 15% and 30% of PEG 6000) and pH (5.5, 6.5, 7.5). Box-PCR technique was used as a molecular tool for fingerprinting to differentiate L. del strains. BOX-PCR Fingerprinting showed that different strains of L. del were isolated at different locations (Maia, Macedo de Cavaleiros, Portugal and Saint Laurent du Cros, France). Optimal *L. deliciosus* growth conditions were 21ºC, pH of 7,5 and 0% of PEG6000 and the highest growth performance was observed for LDA strain. Mycelium cellular activity is higher when EcM are exposed to the higher temperature. Changes in morphological traits, such as circularity, pointed out to signs of abiotic stresses, and different mycelium colors were observed under stress conditions. The growth pattern and cellular activity indicate that the fungal mycelium is more stressed at higher temperatures. Morphological traits other than fungal growth, a classic and well-accepted form to assess fungal activity, may be important indicators of the fungal vitality and promptness to establish symbiosis. The present study will contribute to the understanding of what triggers mycelium development and ultimately mushroom formation.

Student: Nazareno Scaccia

Supervisor(s): Prof. Célia Manaia and Dr. Ivone Vaz-moreira

Thesis Tittle: Evaluation of possible risks of antibiotic resistance transmission to humans by treated WW-irrigated crops

Abstract (max. 3000 characters, including spaces)

The use of treated wastewater to irrigate crops may be a source of antibiotic resistant bacteria and antibiotic resistance genes (ARB&ARGs) capable of contaminating the human food chain, and therefore threat human health. The major objective of this work is to assess the risks of wastewater ARB transmission to humans via gastrointestinal tract. The research hypotheses of this work, regard on i) whether environmental ARB&ARGs might outcompete with the autochthonous human microbiota and ii) whether wastewater ARB may colonize plants and reach humans through the consumption of raw vegetables. In order to address these hypotheses, we have been following two main lines of research. The first, was a gut microbiota focused research based on faecal microcosm assays (FMAs) aiming to assess the fitness of environmental ARB in the presence of the complex human gut microbiota. For that, FMAs were inoculated with wastewater ARB (*Escherichia coli* A2FCC14 and *Enterococcus faecalis* H1EV10) known to harbor ARGs, performed under aerobic and anaerobic conditions, with or without sub-inhibitory concentrations of cefotaxime and vancomycin. FMAs were followed for at least 7 days, based on cultivation, quantitative PCR and bacterial community analyses. It was observed that the spiked ARB survived in the presence of the faecal microbiota for a week and their ARGs could be detected and quantified at least for one month. The trend of the bacterial growth in aerobic or anaerobic FMAs is quite similar, although anaerobically the bacteria population had a higher decrease. The presence of sub-inhibitory concentrations of antibiotics did not affect the survival of the ARB. However, to have a better understanding of the effects that selective pressure may have on the autochthonous community composition, microbiome analyses are ongoing.

The second research line focused on plant-associated bacteria and aims to investigate the possibility that ARB&ARGs is internalized from the environment by plants. For that, an extensive literature review of endophytic bacteria commonly found in edible plants, was carried out. More than 20 phyla in 22 different edible plants were found and commonly found endophytic bacteria were identified.

Overall, these results suggested the need of control strategies such as risk-assessment guidelines in order to ensure the public health and promote the safe reuse of treated wastewater for different purposes.

Student: Sofia Alexandra Araújo Pereira

Supervisor(s): Paula Teixeira, Vânia Ferreira (co-supervisor)

Thesis Tittle: Virulence traits of *Listeria monocytogenes* relevant to food safety

Abstract (max. 3000 characters, including spaces)

Listeria monocytogenes is an environmental saprotroph bacterium, commonly isolated from soil and decaying vegetation, and simultaneously a threatening food-borne pathogen able to cause listeriosis disease in both humans and other animals. In 2016, the European Food Safety Authority and the European Centre for Disease Prevention and Control reported a European Union (EU) notification rate of 0.47 cases of invasive listeriosis per 100,000 population, plus representing the most severe human disease in terms of hospitalization, with the highest case fatality rate (16.2%) of all the zoonotic diseases under EU surveillance [1]. From a public health perspective, this fact stresses the importance of the implementation of an effective surveillance system that assures a prompt statement of the clinical cases, as well as the monitoring of *L. monocytogenes* in food products. This study aims to obtain notification of the clinical listeriosis cases and corresponding epidemiological data, occurring in Portugal from 2015 through 2017, essential for the identification of contamination sources and transmission routes of this pathogen to humans.

Considering the workfront, clinical *L. monocytogenes* isolates were collected from 12 voluntary collaborating hospitals and were characterized by molecular subtyping techniques: genoserotyping by multiplex polymerase chain reaction; DNA macrorestriction pulsed-field gel electrophoresis; and whole genome sequencing. During that period, 45 cases of human invasive infection by *L. monocytogenes* were identified, with an average annual incidence rate of 0.14 cases per 100,000 inhabitants and an overall case fatality rate of 4.44%. Among the available information, 35 cases (77.8%) corresponded to nonmaternal/neonatal (non-MN) infection, mostly collected from blood (40.0%) and cerebrospinal fluid (25.7%). The mean age of the non-MN cases with documented age was 67 years, and 66.7% occurred in patients aged over 60 years. Characterization of *L. monocytogenes* by PFGE typing attested that majority cases of listeriosis were caused by genoserogroup IVb isolates, and further revealed a high molecular diversity, suggesting that most cases were sporadic. Several isolates with different geographic and time distributions still presented closely related PFGE types and were thus grouped into major clusters.

Prevention strategies are crucial since increasing listeriosis cases are expected given the demographic changes observed in the population, caused by aging and longer life expectancy. Further studies are of significance to human safety as they will evaluate the adaptive response of *L. monocytogenes* to single and sequential stresses, including those encountered in human body, and may aid reducing the

uncertainty of microbial risk assessments, associated with lack of knowledge on *L. monocytogenes* resistance to stress and proliferation.

1. EFSA. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA J. 2017;15(12).

Student: Tânia Isabel Bragança Ribeiro

Supervisor(s): Doutora Manuela Pintado, Professor Doutor António Augusto Vicente, Doutor João Miguel dos Santos Almeida Nunes

Thesis Tittle: Development and characterization of functional ingredients from olive pomace: bioactivity and potential application as bioactive edible films

Abstract (max. 3000 characters, including spaces)

Waste management is critical for the food industry for which there is increasing interest in food waste valorisation processes. Olive production is a major agricultural sector in the Mediterranean area, including Portugal. The production of olive oil generates 15-35% of semi solid waste, known as olive pomace (OP). The current options for its treatment (energy pathways, landfill or fertilizer) reveal operational and environmental weaknesses.

Olive Pomace from two olive mills (OM1 and OM2), an abundant agro-industrial byproduct was investigated as a low-cost material for the generation of different value-added products (food ingredients and energy) to achieve the “zero waste” goal. The application of simple separation processes (centrifugation and sieving) followed by a drying process allowed to obtain new food ingredients using the edible fraction from olive pomace: a phenolic-rich extract from the liquid fraction (L-OP) and an antioxidant fibre powder from the edible solid fraction (S-OP) and a solid biofuel (stones) from non-edible fraction. The stones comprising 21% of dried olive pomace, exhibited higher heating values of 18.94 and 18.65 MJ/kg. The phenolic-rich powder from L-OP obtained containing high amounts of hydroxytyrosol (625.76 ± 51.33 - 513.61 ± 27.85 mg/ 100 g dw), as well as the antioxidant fibre-rich powder obtained from S-OP (53.29 ± 0.46 - 59.28 ± 1.98 g fibre/ 100 g dw).

Considering the content of hydroxytyrosol and its derivatives in the phenolic-rich powder from L-OP a daily consumption of less than 1 g would provide the amount of hydroxytyrosol (5 mg) that would be needed to protect LDL particles from oxidative damage, according to the health claim approved by the EFSA.

The studies performed during the last year were focused in the improvement of the phenolic-extract obtained from L-OP and antioxidant fiber powder obtained from S-OP. The study of particle size from antioxidant fiber powder from S-OP revealed the presence of small particle of stones and peel in 30% of total weight (<1mm to > 400 µm). The remaining 70% of antioxidant fiber powder obtained from S-OP showed a particle size in the range of <400 µm to > 75 µm. The antioxidant fiber powder <400 µm and the phenolic-extract obtained from L-OP were studied relatively to its bioactivity, functionality and chemical composition. The bioavailability of sugars, organic acids and Hydroxytyrosol and other phenolic compounds were also achieved. The minor fraction of small stones and peel will be explored in the future

to extract polysaccharides (e.g. pectins). The next steps will include the study of the size of particle and the application of blanching processes to improve the functionality and bioactivity of antioxidant fiber powder. The incorporation of the food ingredients in a fat yogurt will be performed to study the influence of food matrix on the bioavailability and bioactivity of bioactive compounds.

Student: Tatiana Paula Vilela

Supervisor(s): Prof. João Paulo Ferreira and Prof. Ana Maria Gomes

Thesis Tittle: Development of New Added-Value Dairy Products from Cheese Surpluses - Biochemical, Structural and Sensorial Characterization

Abstract (max. 3000 characters, including spaces)

This project has as its main objective the development of novel dairy products incorporating surpluses of cheese production, or their off-standards items, or yet the pieces remaining after slicing large units. The basic ingredients for the targeted products are melted cheese bases (MCBs), that is, melted cheese pastes or slurries, obtained by heating cheese with starch or other hydrocolloids in milk. The MCBs prepared so far were optimized for different types of milk, cheese (soft, semi-hard and hard), concentration and nature of starch or other hydrocolloids.

The physical parameters in the process were also optimized using maize and waxy rice starches, by making temperature-time tests with different agitation paddles (a helix paddle and a U shaped paddle). In these tests, the heating temperature was varied, as well as the time allowed for starch gelatinization, while the agitation speed was kept constant. Optimum final temperature was found to be 85°C for maize starch and over 90°C, held for at least 2 minutes, for waxy rice starch.

Furthermore, studies were made regarding the viability of incorporating the MCBs in cheese-fortified yogurts (yogucheeses). The best results in terms of consistency, texture and cheese dispersion were obtained using maize starch, rice starch and waxy rice starch. Experiments with guar gum also showed promising results.
A microscopic analysis of the different MCBs was made using light microscopy and fluorescence microscopy, in order to visualize their structure. A continuous starch matrix was observed, with cheese protein aggregates of size 40 – 900 μm dispersed in that gelatinized matrix.

The study of interactions between carbohydrates and milk proteins are currently being studied, in order to understand the function of starch in the dispersion of cheese. We have started by studying the dissociation of cheese with several chemical agents (namely urea, SDS, EDTA, NaOH solutions). First, the conditions for cheese dispersion in these dissociating media were optimized (concentration, temperature and agitation), in processes that resemble the preparation of MCBs. The obtained suspensions are centrifuged, and the solubilized protein content measured using several alternative methods: Kjeldahl, colorimetric methods (Biuret, Bradford), and UV-280 nm. After this, we will be

pursuing a particular type of interaction in the formation of MCBs, namely carbohydrate-π interactions, which have not been looked into in studies of dairy proteins – hydrocolloid interactions. The results obtained from these studies will be reported on a scientific paper to be published within a year.