Universidade de Aveiro Departamento de Biologia
2017

Beatriz Luciana Tavares de Almeida

Investigating the antibacterial activity of *Pedobacter lusitanus* NL19

Investigando a atividade antibacteriana de *Pedobacter lusitanus* NL19

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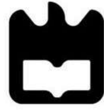

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Investigating the antibacterial activity of *Pedobacter lusitanus* NL19

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica de Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e de Tânia Isabel Sousa Caetano, bolseira de Pós-Doutoramento no Departamento de Biologia da Universidade de Aveiro.

Dedicado aos meus pais, irmãos e avós.

o júri

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agradecimentos

Queria agradecer primeiro à Professora Sónia Mendo por me ter aceite no LBM para realizar este trabalho que tanto prazer me deu ao longo deste ano e meio.
À Tânia por me ter orientado/ aturado durante o trabalho e por estar sempre disposta a ajudar durante todo o trabalho.
Queria agradecer à Cláudia por ter sido a minha “iniciadora” não só no LBM como também no trabalho no laboratório, e ter tirado tanto do seu tempo para me ensinar como tudo funcionava. Só graças a ela consegui desenvolver este trabalho.
À Ana Teresa por ser a minha eterna “parceira de laboratório” e uma verdadeira amiga para os bons e maus momentos… dois cursos feitos juntas!
A toda a equipa do LBM por terem sido verdadeiramente uma segunda família, por todos os momentos divertidos que passámos juntos.
Um grande agradecimento a todos os meus amigos, especialmente a Maria e a Mariana, por terem sempre sido uma distração (por vezes necessária) desta tese, e por me terem sempre apoiado.
Esta tese é dedicada aos meus pais, por nunca terem questionado as minhas escolhas e terem sempre confiado em mim.
Aos meus irmãos, por quem faço esta tese.

palavras-chave

atividade antibacteriana, meio de crescimento, cromatografia líquida, espectrometria de massa, péptidos antibacterianos, péptidos não-ribossomais, péptido sintetases não-ribossomais, análise da expressão génica.

resumo

NL19, a estirpe tipo da espécie recentemente descrita *Pedobacter lusitanus*, possui uma atividade antibacteriana significante contra diferentes bactérias patogénicas clinicamente revelantes, incluindo *Staphylococcus aureus* resistente à meticilina (MRSA). Esta bioatividade foi inicialmente identificada em placas de agar tríptico de soja (TSA). Quando crescida no meio líquido equivalente (TSB100%), não foi observada atividade antibacteriana. Contudo, quando a estirpe foi crescida em meio TSB diluído (TSB25%) a atividade antibacteriana foi restabelecida.
O objetivo deste estudo foi investigar esta atividade antibacteriana de P. lusitanus NL19 e perceber se as suas espécies mais próximas e a estirpe tipo do género são também produtoras de compostos antibacterianos. Assim, a atividade antibacteriana de *P. lusitanus* NL19, *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM14825^T, *P. hartonius* DSM 19033^T, *P. westerhofensis* DSM 19036^T e *P. heparinus* DSM2366^T foi estudada em diferentes meios sólidos e líquidos. *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM14825 *e P. hartonius* DSM 19033^T mostraram atividade contra algumas estirpes indicadoras, quando crescidas em meio sólido. No entanto, quando crescidas no meio líquido equivalente, a atividade antibacteriana já não foi observada, ou, para *P. himalayensis* MTCC 6384^T, foi muito baixa. Os compostos responsáveis pela atividade de *P. lusitanus* nos diferentes meios foram separados por Cromatografia Líquida de Alta-Pressão (HPLC) e as frações recolhidas foram analisadas por Ionização e dessorção a laser assistida por matriz (MALDI-ToF), tendo sido identificados como sendo pedopeptinas. Pedopeptinas são uma mistura de três péptidos não ribossomais e cujas péptido sintetases envolvidas na sua biossíntese foram identificadas no genoma de *P. lusitanus* NL19 (*pedo_nrps*). As diferenças de produção de pedopeptinas em TSB100% e PC25% foram investigadas, tendo-se concluído que uma elevada concentração de peptona de caseína (PC) reprime a produção de pedopeptinas em TSB100%. Foi investigada a expressão dos dois genes *pedo_nrps*, por análise por Reação em Cadeia da Polimerase Quantitativa em Tempo Real (RT-qPCR), em células que foram crescidas em meios de cultura com elevada e baixa concentração de PC. Verificou-se um aumento de 10 vezes nos níveis de expressão de ambos os genes *pedo_nrps* no meio com menor concentração de PC.
Este estudo confirma que as pedopeptinas são péptidos não-ribossomais antibacterianos e que estes não são produzidos por todas as estirpes de *Pedobacter* spp. estudadas. Para além disso, mostramos que a produção de antibacterianos pode depender do tipo de cultivo (sólido vs. líquido), mesmo quando são usadas as mesmas proporções de fontes de carbono e azoto. A produção de pedopeptinas parece ser inibida pela repressão de transcrição dos genes *pedo_nrps*, devido à alta concentração de PC no meio de crescimento.

keywords antibacterial activity, growth media, liquid chromatography, mass spectrometry, bioactive peptides, non-ribosomal peptides, non-ribosomal peptide synthetases, genetic expression analysis

abstract

NL19, the type strain of the recently described species *Pedobacter lusitanus*, exhibits significant antibacterial activity against several relevant pathogenic bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA). This bioactivity was first identified in tryptic soy agar (TSA). When grown in its equivalent broth (TSB100%), no activity was detected. However, antibacterial activity is observed when cells are grown in 4fold diluted TSB (TSB25%).

The aim of the present study was to further investigate the antibacterial activity of *P. lusitanus* NL19 and understand if its closely-related strains and the genus type strain were antibacterial producers. Thus, the antibacterial activity of *P. lusitanus* NL19, *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM14825^T, *P. hartonius* DSM 19033^T, *P. westerhofensis* DSM 19036^T and *P. heparinus* DSM2366^T, was assessed in different solid and liquid media. *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM14825 and *P. hartonius* DSM 19033^T were active against some indicator strains when grown in some agarized media. However, when the equivalent broth media was used, no antibacterial activity was observed, or, for *P. himalayensis* MTCC 6384^T, it was very low. The compounds responsible for the activity of *P. lusitanus* in different media were identified by High-Pressure Liquid Chromatography (HPLC) separation followed by matrix-assisted laser desorption/ionization (MALDI-ToF) of the HPLC collected fractions as pedopeptins. Pedopeptins are a mixture of three peptides and we suggest that they are nonribosomal peptides. The genes of the nonribosomal peptide synthetases involved in their biosynthesis were identified in the genome of *P. lusitanus* NL19 (*pedo_nrps*). The differences between the production of pedopeptins in TSB100% and TSB25% were investigated and it was concluded that high concentration of peptone from casein (PC) represses the production of pedopeptins in TSB100%. Also, the expression of the two *pedo_nrps* genes at the transcriptional level was investigated by Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) analysis, in cells that were grown in media containing high and low concentrations of PC. It was found that the expression levels of both *pedo_nrps* increased up to 10-fold in cells grown in lower concentrations of PC.

Our study confirms that pedopeptins are nonribosomal antibacterial peptides that are not produced by all the *Pedobacter* spp. strains studied. In addition, we show that the production of antibacterial compounds can depend on the type of cultivation (liquid vs solid), even when the same proportions of carbon and nitrogen sources are used. Pedopeptin production can be inhibited due to transcription repression of *pedo_nrps* genes, caused by high concentrations of PC.

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Abbreviation List

4’ PP cofactor_ 4'-phospho-pantethein cofactor
A domain_ Adenylation domain
AC_ Affinity Chromatography
ACN_ Acetonitrile
antiSMASH_ Antibiotics and Secondary Metabolite Analysis Shell
AraC family_ Transcriptional activator family
Asp_ Aspartic acid
BTI_ Bis (1,1-trifluoroacetoxy) iodobenzene
C domain_ Condensation domain
CD14_ Cluster of differentiation 14
CHCA_ α-cyano-4-hydroxycinnamic acid
CNS_ Central Nervous System
Cq level_ Quantification cycle level
Dab_ Diaminobutyric acid
Dap_ Dehydroamino acid
Dap_ Diaminopropionic acid
Dhb_ Dehydrobutyrine
EARS-Net_ European Antimicrobial Resistance Surveillance Network
ECDC_ European Centre for Disease Prevention and Control
Glu_ Glutamine
HlyD_ Hemolysin secretion protein D
HPLC_ High Pressure Liquid Chromatography
IEX_ Ion-Exchange Chromatography
LA_ Lysogeny agar
LB_ Lysogeny broth
Leu_ Leucine
LPS_ Lipopolysaccharide
MALDI-TOF_ Matrix assisted laser desorption ionization-time of flight mass spectrometry
MarR family_ Multiple antibiotic resistance regulator family
MFS_ Major facilitator superfamily
mins._Minutes
MQ-H_2O_ Milli-Q Ultrapure Water
MRSA_ Methicillin resistant *Staphylococcus aureus*
MS_ Mass Spectrometry
NA_ Nutrient Agar
NaCl_ Sodium Chloride
NB_ Nutrient Broth
NRP_ Non-ribosomal peptide
NRPS_ Non-ribosomal peptide synthetase
NTF2_ Nuclear transport factor 2
OD_{600}_ Optical density measured at 600nm absorbance

PAP2_ Type 2 phosphatidic acid phosphatase
PBP_ Penicillin-binding protein
PC_ Peptone from casein
PCP domain_ Peptide Carrier Protein domain
PCR_ Polymerase Chain Reaction
PES_ Polyethersulfone
Phe_ Phenylalanine
PK_ Polyketide
PLP_ pyridoxal phosphate
PLP_ Pyridoxal phosphate
PS_ Soy peptone
R2A_ R2V agar medium
R2V_ R2V liquid medium
RPE_ Reverse-Phase Chromatography
RT-qPCR_ Real Time Quantitative Polymerase Chain Reaction
Ser_ Serine
TE domain_ Termination domain
TFA_ Trifluoracetic acid
Thr_ Threonine
TolC_ Outer membrane protein
t_R_ Retention time
TSA_ Tryptic Soy Agar
TSB_ Tryptic Soy Broth
TSS_ Toxic Shock Syndrome
UTI_ Urinary Tract Infection
UV absorption_ Ultraviolet absorption
Val_ Valine
Val_ Valine
WHO_ World Health Organization
X-Gal_ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
β-OH Val_ β -hydroxyvaline

Introduction

Chapter I: Antibiotic resistance – a global crisis

1. Overview

According to the World Health Organization (WHO) "antibiotics are medicines used to prevent and treat bacterial infections, and antibiotic resistance occurs when bacteria change in response to the antibiotics used to treat bacterial infections (such as urinary tract infections, pneumonia, bloodstream infections, etc.) making them ineffective" (WHO 2016). This "change" is one of the most worrying problems in public health nowadays (Bartlett et al. 2013). Even though resistance to antibiotics is not a recent process for bacteria, instead, it has been rising at a much faster speed in recent decades (Ventola 2015).

Antibiotics have different modes of action, targeting bacteria at different structures (Walsh 2000; Brown & Wright 2016). Therefore, the mechanisms of bacterial resistance to antibiotics are also different, given the different classes of compounds (Neu 1992; Bbosa et al. 2014; Lupo et al. 2012). An overview of some of the major antibiotics classes, their mode of action in bacterial cells and their resistance mechanisms can be seen in Table 1.

Table 1 - An overview of some of the major antibiotic classes, categorized by function, and associated resistance mechanisms (Walsh 2000).

Classes of antibiotics	**Mode of action**	**Associated resistance mechanisms**	**References**
Beta-lactams (including penicillins, cephalosporins, monobactams and carbapenems)	Cell-wall synthesis inhibitors	Enzymatic inactivation (β-lactamases), target mutations (mutation of the penicillin binding proteins-PBPs), reduced permeability	(Neu 1992; Walsh 2000)
Aminoglycosides	Protein synthesis inhibitors	Enzymatic modification	(Bbosa et al. 2014)
Glycopeptides (includes vancomycin)	Cell-wall synthesis inhibitors	Target modification by enzymes, reduction of the uptake of the compound	(Bbosa et al. 2014; Walsh 2000; Blair et al. 2015)
Macrolides	Protein synthesis inhibitors	rRNA methylation (alters the binding-site), inactivation of the drug by hydrolysis, increased efflux	(Walsh 2000; Blair et al. 2015)

Quinolones	Inhibitors of DNA synthesis (block DNA replication by acting on DNA gyrase)	Mutations, reduced permeability	(Walsh 2000; Brown & Wright 2016; Bbosa et al. 2014)
Tetracyclines	Protein synthesis inhibitors	Increased efflux	(Walsh 2000)

In February 2017 the WHO published a document highlighting the major concerns in terms of resistant bacteria, and dividing them by priority (Tacconelli et al. 2017):

- **Priority 1 – Critical** includes *Acinetobacter baumannii* carbapenem-resistant, *Pseudomonas aeruginosa* carbapenem-resistant and *Enterobacteriaceae* (including *Klebsiella pneumonia*e, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp. and *Morganella* spp.) carbapenem-resistant and 3rd generation cephalosporin-resistant
- **Priority 2 – High** includes *Enterococcus faecium* vancomycin-resistant, *Staphylococcus aureus* methicillin-resistant, vancomycin intermediate and resistant, *Helicobacter pylori* clarithromycin-resistant, *Campylobacter* fluoroquinolone-resistant, *Salmonella* spp. fluoroquinolone-resistant and *Neisseria gonorrhoeae* 3rd generation cephalosporin-resistant and fluoroquinolone-resistant
- **Priority 3 – Medium** includes *Streptococcus pneumoniae* penicillin-non-susceptible, *Haemophilus influenzae* ampicillin-resistant and *Shigella* spp. fluoroquinolone-resistant

The latest data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) comes from their 2015 annual report, and shows the real impact of antibiotic resistance in this continent (Figure 1) (ECDC 2015). In this figure we can clearly see that lower resistance percentages occur in northern Europe countries, and higher percentages in southern and eastern countries (ECDC 2015). These differences might have something to do with the antimicrobial use, infection control and healthcare practices in the different regions (ECDC 2015).

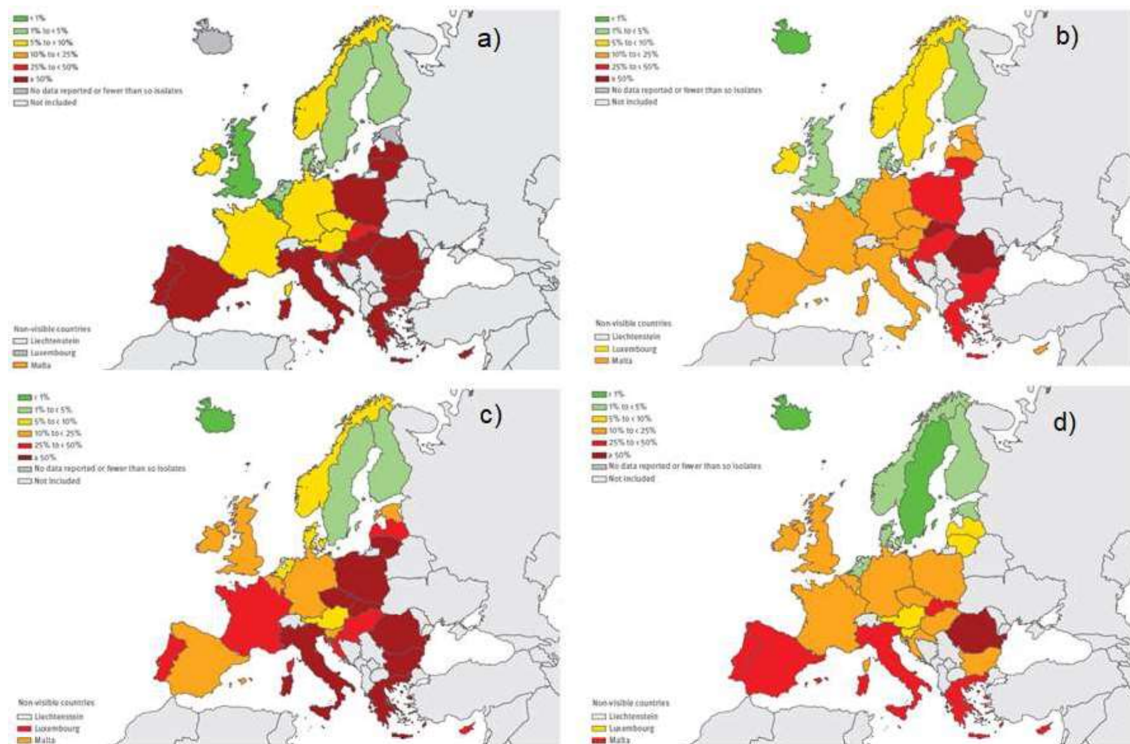

Figure 1 - Percentage (%) of invasive isolates of a) carbapenem resistant *Acinetobacter* spp., b) carbapenem resistant *Pseudomonas aeruginosa*, c) *Klebsiella pneumoniae* resistant to third-generation cephalosporins and d) methicillin-resistant *Staphylococcus aureus* (MRSA), in EU/EEA countries. Source: European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2015. Annual Report of the EARS-Net, 2015

With a prediction of 390 000 deaths attributed to antimicrobial resistance by 2050 in Europe alone, this problem is nowadays one of the priorities of health organizations, academia and health professionals (O'Neill 2014).

One of the first clinically available antibiotics was penicillin. This compound was first discovered by Alexander Fleming, in 1928, and after works of Howard Florey, Ernst Chain, and Norman Heatley in its purification, it became widely available in 1946 (Torok et al. 2009). But even as soon as 1940, an enzyme extracted from a pathogenic bacterium was described as capable of destroying this antibiotic, as seen in the work of Edward Penley Abraham and Ernst Chain (Abraham & Chain 1940). In fact, antibiotic resistance has been proven to predate the use of antibiotics by mankind, and to be a natural phenomenon widespread in the environment, since the similarity between resistance genes found in permafrost samples and current resistance genes was confirmed (D'Costa et al. 2011). So, what has led to the massive increase in antibiotic resistance?

2. Main causes for the antibiotic resistance crisis

Since their discovery, antibiotics have been seen as “wonder drugs“, capable of treating every infection affecting humans (Laxminarayan et al. 2013). But overuse of these drugs, inappropriate prescribing (wrong type of antibiotic or wrong dosage), wrong use (uncompleted treatments, use of antibiotics to treat non bacterial infections) and extensive use in agriculture and aquaculture, led to a major increase in resistance, and facilitated its spread (Davies & Davies 2010; Laxminarayan et al. 2013; Ventola 2015).

Since resistance became a serious public health concern, the agencies responsible for the approval of new drugs have created stricter regulation for the production of new antibiotics, making antibiotic research and development a lengthy and expensive ordeal (Hay et al. 2014; Nathan 2004). This led to a growing disinterest by big pharmaceutical companies in investing in the search for new antibiotics, because this activity is not profitable enough compared with the production of other drugs, especially long-term treatments and medicine for chronic diseases, such as heart disease or diabetes (Nathan 2004). So, even though the need for new antibiotic compounds has never been greater, the phenomenon we’re observing now is the complete opposite: a rapid decline in research and production of new antibiotics (Amin & Deruelle 2015).

3. Search for new antibiotics as a solution for the resistance crisis

Secondary metabolites have long been a source of new bioactive compounds, used by society in health, nutrition and other diverse economically important activities (Demain & Fang 2000). Unlike primary metabolites, that are the products resultant of all biochemical processes essential for cell growth, secondary metabolites serve different survival functions in the producer organisms, and their production is dependent on the characteristics of the growth environment of the organism (Ruiz et al. 2010; Gokulan et al. 2014; Demain & Fang 2000). Microbial secondary metabolites are low molecular mass products, divided into different classes, such as polyketides (PKs), non-ribosomal

peptides (NRPs), terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, β-lactams, siderophores and others, and are used by society as antibiotics, pigments, toxins, pesticides, antitumor agents and growth promoters of animals and plants (Medema et al. 2011; Gokulan et al. 2014).

The first antibiotics were obtained by the screening the secondary metabolites produced by soil bacteria (Lewis 2013). For example, streptomycin, the first compound discovered that was effective against tuberculosis, was found by the simple Waksman platform, that screened streptomycetes for antimicrobial activity (Lewis 2013). This screening platform led to the discovery of the major antibiotics classes known today, initiating the so called "golden era of antibiotics" (Lewis 2013; Brown & Wright 2016). But problems started to arise with the discovery of antibiotics using this method, mostly because they are not designed to be used in humans, leading to pharmacological and toxicological problems (Brown & Wright 2016). So, scientists started to develop synthetic versions of these compounds, engineering them to be less toxic and more effective (Brown & Wright 2016).

The search for new antibiotics is one of the answers to the antibiotic resistance crisis (Nathan 2004). Knowing that bacteria are an excellent source of new compounds, one possible source for new antibacterial compounds is the bacterial community living in extreme environments (Li & Vederas 2009). In these environments, good competitive tools are extremely important for bacterial survival; extreme environments have a physical or chemical extreme characteristics, such as temperature, pressure, radiation, salinity, pH or heavy metal concentration (Nies 2000; Van den Burg 2003), and some microorganisms are able to survive and thrive in these extreme environments (Rothschild & Mancinelli 2001). Knowing that the production of secondary metabolites by bacteria is very dependent on the external conditions of the producing organism, it seems logical to infer that, besides having adapted themselves to live in different, extreme situations, the microorganisms inhabiting extreme environments can produce new compounds with antibiotic properties (Seufferheld et al. 2008). This, added to the fact that the microorganisms in extreme environments are still a big mystery for researchers, makes these environments great sources for the discovery of new antibiotics (Seufferheld et al. 2008). For example, glionitrin A is an antibiotic-antitumor secondary metabolite

produced when *Sphingomonas* bacterial strain KMK-001, and *Aspergillus fumigatus* fungal strain KMC-901, extracted from the drainages of an abandoned coal mine, are co-cultivated (Figure 2) (Park et al. 2009). This compound showed good activity against methicillin-resistant *Staphylococcus aureus* (MIC=0.78 µg/mL), and cytotoxic activity against cancerous cell lines (Park et al. 2009).

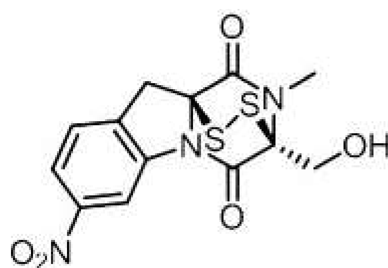

Figure 2 - Structural representation of glionitrin A. Adapted from Park et al. 2009.

3.1. Nonribosomal peptides

Nonribosomal Peptides (NRPs) are a class of secondary metabolites whose biosynthesis is independent of the ribosome (Schwarzer et al. 2003; Medema et al. 2011). Instead, they are synthetized by large enzymes called Nonribosomal Peptide Synthetases (NRPSs) (Weissman 2015). The structure of these peptides is varied, and so are their activities (Schwarzer et al. 2003). They are produced by organisms from all domains of life (Wang et al. 2015). Clinically, NRPs have several different applications, including antibiotics (daptomycin, tyrocidine, gramicidin and vancomycin), immunosuppressants or suppressors (such as ciclosporin), and antitumor drugs (bleomycin or epothilone) (Strieker et al. 2010; Schwarzer et al. 2003).

NRPs are synthetized by NRPSs, biosynthetic systems that include the peptide template and the necessary machinery for its production (Schwarzer et al. 2003). These enzymatic complexes are made up of protein domains organized in modules that operate in a process similar to an assembly line to construct the final peptide (Weissman 2015). The module is the section that incorporates each amino acid into the final product, and it is divided into domains, each of these responsible for a different step in the synthesis of NRPs (Schwarzer et al. 2003). Three domains are essential for the activity of NRPSs and

consequent NRP production: the adenylation domain (A), the peptidyl carrier protein (PCP), also known as thiolation domain (T), and the condensation domain (C) (Schwarzer et al. 2003). Also, the last module of the NRPS contains, in most cases, a thioesterase domain (TE), responsible for the release and cyclization of the product (Finking & Marahiel 2004; Schwarzer et al. 2003). Usually, the first module of an NRPS does not have a C domain (initiation modules), because they don't need to connect to any previous amino acid, serving only for the recognition and activation of the first amino acid of the peptide chain (Schwarzer et al. 2003). A representation of a basic NRPS, with initiation and extension modules, and A, PCP, C and TE domains can be seen in Figure 2.

The NRP synthesis is a multi-step process, with the following main steps (Schwarzer et al. 2003):

1) The PCP domain is initially present as inactive apo-PCP, so it must be activated to its holo-PCP form; this happens by modification with the 4'-phosphopantetheine (4'PP) cofactor.
2) The A domain recognizes the substrate and activates it using ATP, and the holo-PCP is loaded with the substrate (the activated amino acid) (the PCP works as a transporter, allowing the product to move between catalytic centres).
3) Synthesis of the peptide is continued by formation of peptide bonds (by the C domain) between the amino acid bound to the first module and the adjacent module.
4) Elongation continues until the product reaches the TE of the last module, where it undergoes macrocyclization and release.
5) In this final step, the NRPS is regenerated and the final product is usually further modified.

Figure 3 - Representation of NRPS initiation and extension modules, with the basic domains. Chemical modifications can be introduced during or after chain extension by tailoring domains such as epimerases, which are not represented. Adapted from Finking & Marahiel 2004).

Nonribosomal peptides are molecules that have an extremely diverse structure (Grünewald & Marahiel 2013). This structural diversity can arise from several sources, including monomer diversity, modifications to the peptide before and after synthesis, diversity in chain termination, and post synthesis tailoring (Grünewald & Marahiel 2013). A large number of monomers that make up the structure of the NRPs are non-proteinogenic amino acids, meaning that they are not naturally encoded by the organism (Grünewald & Marahiel 2013). These amino acids are created by enzymatic reactions, from pre-existent proteinogenic amino acids, or her are newly constructed (Walsh et al. 2013). These non-proteinogenic amino acids include 2,3-diaminopropionate (Dap), that is formed by the pyridoxal phosphate (PLP) mediated enzymatic dehydration of serine, diaminobutyric acid (Dab), resultant from the BTI-mediated conversion of glutamine or D-amino acids (Walsh et al. 2013; Kuhn et al. 1996).

During chain assembly NRPs can suffer several modifications, including epimerization (Figure 4) (by the optional NRPS domain E), heterocyclization (by the Cy domain), oxidation (by the Ox domain), or reduction (by the R domain) (Schwarzer et al. 2003).

This variety of modifications, during and/or after synthesis, that these peptides can suffer, creates an immense variety of compounds, most of them unknown, which makes them extremely interesting in the research of new compounds with different applications (Strieker et al. 2010; Schwarzer et al. 2003).

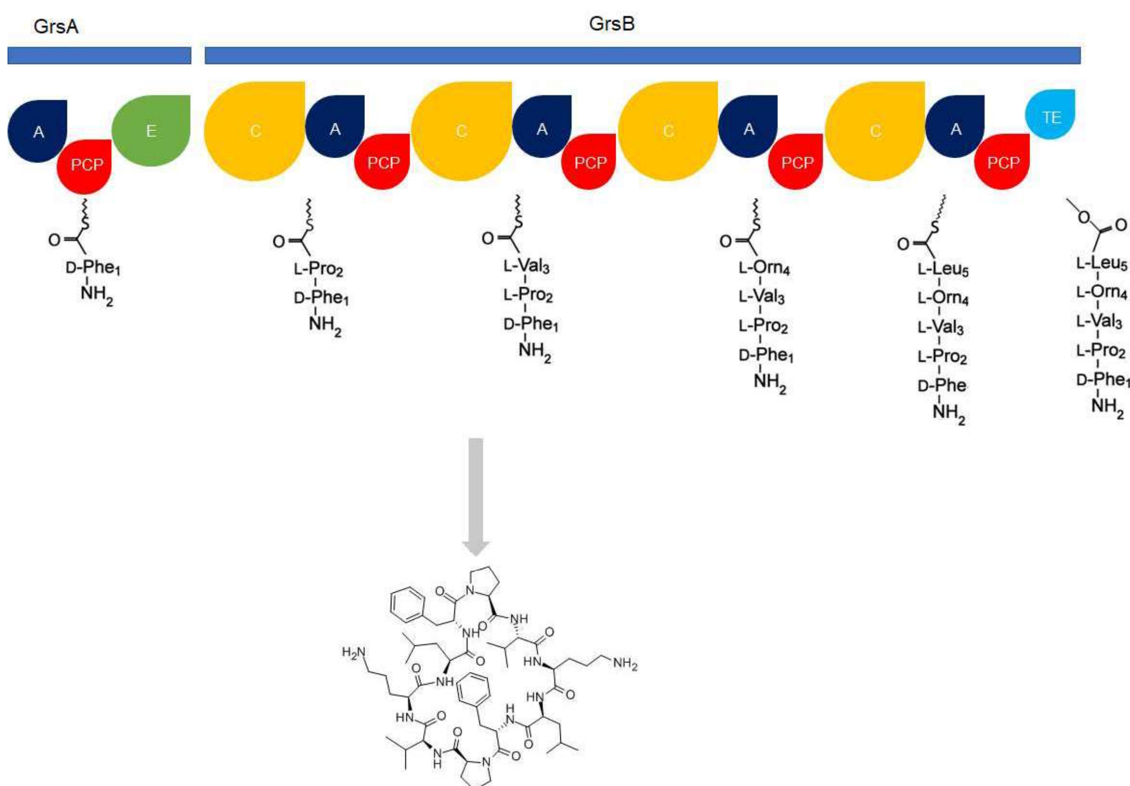

Figure 4 - Representation of the enzymatic pathway leading to the synthesis of the peptide Gramicidin S. This peptide is assembled by two NRPSs (GrsA and GrsB), with modules that produce the structure cyclo (-D-Phe1- L-Pro2- L-Val3- L-Orn4- L-Leu5 -D Phe6- L- Pro7- L-Val8- L-Orn9- L-Leu10-). The first amino acid is an epimerized phenylalanine, epimerized by the E domain present in the first module. Legend: A, adenylation domain; PCP, peptidyl carrier protein; E, epimerization domain; C, condensation domain; TE, thioesterase domain. Adapted from Hoyer et al., 2007 (Hoyer et al. 2007)

3.2. Genetic expression analysis

Along with polyketides synthetases (PKS), NRPS are the major targets when screening genomic DNA for the production of novel secondary metabolites (Bode & Müller 2005). As these enzymes have common domains, they are easy to screen using, for example, PCR, or bioinformatic identification of genes encoding conserved domains, such as the C, A or PCP domains of NRPSs (Ayuso-Sacido & Genilloud 2005; Weber & Kim 2016).

After the identification of the genes encoding a given compound or NRPS or PKS, their genetic expression can be analysed. Gene expression analysis allows to understand the expression of a gene at a given time, in a specific cell or in a specific condition, connecting the genotypes to the phenotypes (Lovén et al. 2012; Marguerat & Bähler 2010). Several tools can be used for this analysis, such as digital PCR, microarrays and real-time quantitative Polymerase Chain Reaction (RT-qPCR). The latter is considered the best tool for this type of work, due to its accuracy, sensitivity, and speed (Derveaux et al. 2010).
RT-qPCR uses probes, such as SYBR Green, hydrolysis probes, molecular beacons or scorpion probes, to quantify the expression levels of a specific gene by PCR (Jozefczuk & Adjaye 2011; Arya et al. 2005). The different probes used give a fluorescent signal when hybridized with the PCR product, and when the fluorescent signal surpasses the background threshold levels we obtain the quantification cycle (Cq) level (D'haene et al. 2010; Jozefczuk & Adjaye 2011). This value is dependent on the initial amount of DNA (Jozefczuk & Adjaye 2011). Therefore, if a gene has a large number of copies in a sample the Cq level will be low, and vice-versa (D'haene et al. 2010). In RT-qPCR, two different methods are used to quantify gene expression: absolute and relative quantification (Schmittgen & Livak 2008). Absolute quantification allows the user to know the exact initial copy number of a gene by comparing the PCR signal to a known standards, whose copy number is known, presenting the results as copy number (Arya et al. 2005; Schmittgen & Livak 2008). In relative quantification the expression levels of a gene are quantified relatively to internal controls, such as housekeeping genes (Arya et al. 2005; Schmittgen & Livak 2008).

3.3. Peptide purification

After a strain is recognized as the producer of a specific peptide, to be able to study this compound, first it must be extracted and purified. Peptides can be extracted by precipitation, using organic solvents such as ethanol, methanol or acetone or acids solutions with trifluoroacetic acid (TFA), for example (Nollet & Toldra 2012). After extraction, the peptides must be separated and purified, for further analysis by mass spectrometry (MS) (Nollet & Toldra 2012). This process is usually based on a

chromatographic techniques, such as Ion Exchange Chromatography (IEX), Affinity Chromatography (AC) or Reversed-Phase Chromatography (RPE) (Nollet & Toldra 2012).

3.3.1. High-Pressure Liquid Chromatography (HPLC)

Liquid chromatography is a technique used to separate the individual components of a sample (Betancourt & Gottlieb 2014). This technique was first described in the early 1900s, by Mikhail Tswett, a Russian botanist that used it to separate plant pigments (Jandera & Henze 2012). The term "chromatography" was introduced 3 years later by Tswett himself (Sakodynskii & Chmutov 1972). The separation is based on the movement of the mobile phase (liquid) through a stationary phase (solid) (Jandera & Henze 2012; USP 2007). The affinity between the sample components and stationary phase is reflected in the distribution of the sample compounds between the stationary and mobile phases (Jandera & Henze 2012).

In liquid chromatography the mobile phase is, a mixture of solvents that have low viscosity (due to pressure requirements) and a good solubility for the compounds yo be analysed (Guiochon & Trapp 2012). The stationary phase has to be compatible with the mobile phase, but it can't be soluble in the mobile phase (Guiochon & Trapp 2012). The chromatographic column contains the stationary phase and the mobile phase is poured into it (Jandera & Henze 2012). This process used to rely only on gravity or sometimes suction to drag the mobile phase and sample through the stationary phase (Jandera & Henze 2012), making the process very time consuming, leading to a need for more practical and time saving updates to this very helpful technique (Snyder et al. 2009). That is why nowadays the most commonly used liquid chromatography technique is HPLC, or High-Pressure Liquid Chromatography (Jandera & Henze 2012). The stationary phase is formed by fine solid particles packed together into a column (metal, glass or plastic) and the mobile phase, usually a mixture of two or more components, is forced through the column (Jandera & Henze 2012). The sample is separated along the column by flowing with the mobile phase, and, as each component leaves the column, it is detected by

ultraviolet absorption (UV) (Snyder et al. 2009). The time at which each compound exits the column is designated the retention time (t_R), and different t_R usually indicate different compounds (Snyder et al. 2009).

The mixture of solvents for HPLC usually includes water (solvent A) and an organic solvent (solvent B), such as acetonitrile or methanol, whose concentration (%) usually increases during the run, as solvent A concentration decreases (Snyder et al. 2009). Both solvents are usually acidified with a buffer in the case of biochemical separation (Snyder et al. 2009). This buffer is usually an acid, such as trifluoroacetic acid (Snyder et al. 2009).

Chapter II- *Pedobacter lusitanus* NL19

1. Pedobacter lusitanus

Pedobacter lusitanus NL19^T was isolated from sludge from a deactivated uranium mine located in Quinta do Bispo, Viseu, Portugal (Covas et al. 2017). The mining of uranium ore was an economically important activity in Portugal since 1908 and until around 2001 (Pereira et al. 2009; Caetano et al. 2016). The mines have since been abandoned, suffered a process of chemical treatment and are monitored to reduce environmental impact (Covas et al. 2017; Pereira et al. 2014). Due to the years of exploration, the chemical treatment, and the improper removal of the radioactive material, in the areas surrounding these abandoned uranium mines, the water and sediment have a low pH and are polluted with several radionuclides and toxic metals, such as uranium, cadmium, arsenic, amongst others (Pollmann et al. 2006; Selenska-Pobell 2002; Pereira et al. 2014). But even in the most polluted areas that are still records of bacterial communities that adapted themselves to these environments, using several different survival mechanisms, including: oxidation and/or biomineralisation (Selenska-Pobell 2002).

When first studied, the antibacterial activity of this strain was observed in a simplified version of the Tryptic Soy Agar medium (TSA). But, when grown in the liquid version of this media (Tryptic Soy Broth), this activity was no longer visible. When the liquid media was diluted, this activity was restored.

2. The *Pedobacter* genus

The *Pedobacter* genus was proposed along with its family (*Sphingobacteriaceae*) by Steyn *et al.* in 1998 (Steyn et al. 1998; Covas et al. 2017). It comprises Gram-negative rod-shaped bacteria (Covas et al. 2017). The species in this genus are aerobic, rod-shaped, Gram-negative cells, do not form endospores and are either non-motile or motile by gliding (Margesin & Shivaji 2015). The isolates grow between 1°C and 37°C (Margesin 2003; Steyn et al. 1998). Most strains also produce heparinise, hence the name of the type strain *Pedobacter heparinus* (Farfan et al. 2014). This genus (and consequentially the *Sphingobacteriaceae* family) belongs to the *Bacteroidetes* phylum (Covas et al. 2017). The genus was initially described and included two bacterial species formerly assigned to the *Sphingobacterium* family (*Pedobacter heparinus comb. nov.* and *Pedobacter piscium comb. nov.*), plus two new species, *Pedobacter africanus* and *Pedobacter saltans* (Farfan et al. 2014). Nowadays there are 67 species in the *Pedobacter* genus (http://www.bacterio.net/index.html, accessed 17/10/17, (Euzéby 1997)), isolated everywhere from activated sludge and soil, to glacier cryoconite or glacier water, and even from fish (Margesin & Shivaji 2015).

Based on 16S rRNA gene sequence analysis, NL19's closest *Pedobacter* strains are *P. himalayensis* MTCC 6384^{T} (Shivaji et al. 2005), *P. cryoconitis* DSM 14825^{T} (Margesin 2003), *P. hartonius* DSM 19033^{T} (Muurholm et al. 2007) and *P. westerhofensis* DSM 19036^{T} (Muurholm et al. 2007) (Covas et al. 2017). A further characterization of these strains, NL19 and the genus type strain *P. heparinus* DSM 2366^{T} (Steyn et al. 1998) are shown in Table 2.

Table 2 - Characterization of NL19's closest strains *P. himalayensis* MTCC 6384^{T}, *P. cryoconitis* DSM 14825T, *P. hartonius* DSM 19033^{T}, and *P. westerhofensis* DSM 19036^{T}, NL19 and the genus type strain *P. heparinus* DSM 2366^{T}

***P. cryoconitis* DSM 14825^{T}**	
Place of isolation	Alpine glacier cryoconite (Margesin 2003)
Characteristics	Degrades diesel oil and produces high yields of protease at low temperature. DNase activity and assimilation of 2-ketogluconate (Margesin 2003)

Resistance to antibiotics (µg)	Amikacin (16), ampicillin (8), chloramphenicol (8), cephalothin (8), penicillin (0,3), tetracycline (2), gentamicin (4), cephamandole (16) and erythromycin (4) (Margesin 2003)
pH tolerance	Grows between 5 and 8, optimal growth between 6 and 7 (Margesin 2003)
Temperature range	Growth between 1 and 25°C, optimal growth at 20°C (Margesin 2003)
***P. hartonius* DSM 19033^T**	
Place of isolation	Hard water creek (Muurholm et al. 2007)
Characteristics	Hydrolyses gelatine (Muurholm et al. 2007)
Resistance to antibiotics (µg)	Ampicillin (10), ticarcillin (75), cefazolin (30), chloramphenicol (30), lincomycin (15), norfloxacin (10), nitrofurantoin (100), ofloxacin (5), penicillin G (6) (Muurholm et al. 2007)
pH tolerance	Grows at pH 5,7 to 7,8 (Muurholm et al. 2007)
Temperature range	Grows between 6 and 26°C, optimum growth at 12 to 16 °C (Muurholm et al. 2007)
***P. heparinus* DSM 2366^T**	
Place of isolation	Soil (Steyn et al. 1998)
Characteristics	Capable of growing on sorbitol and L-fucose, absence of valine arylamidase (Margesin & Shivaji 2015)
Resistance to antibiotics (µg)	Ampicillin (8) and gentamicin (4) (Steyn et al. 1998)
pH tolerance	Grows at pH between 6 and 8 (Margesin & Shivaji 2015)
Temperature range	Temperature range is normally between 5 and 30°C (Steyn et al. 1998)
***P. himalayensis* MTCC 6384^T**	
Place of isolation	Glacial water from the snout of the Hamta glacier located in the Himalayan mountain ranges of India (Shivaji et al. 2005)
Characteristics	Arginine dihydrolase activity, gluconate assimilation, production of acid from D-mannitol but not from D-arabinose and D-glucose (Shivaji et al. 2005)
Resistance to antibiotics (µg)	Penicillin (10), kanamycin (30), chloramphenicol (30), ampicillin (10), tetracycline (30) (Shivaji et al. 2005)
pH tolerance	Grows between pH 6 and 10. Optimum pH is 7 (Shivaji et al. 2005)
Temperature range	Grows between 4 and 25°C. Optimum temperature for growth is 22°C (Shivaji et al. 2005)
***P. westerhofensis* DSM 19036^T**	
Place of isolation	Hard water creek (Muurholm et al. 2007)
Characteristics	Hydrolyses gelatine, hydrolyses DNA weakly (Muurholm et al. 2007)
Resistance to antibiotics (µg)	Ampicillin (10), ticarcillin (75), cefazolin (30), norfloxacin (10), nitrofurantoin (100), ofloxacin (5), penicillin G (6) (Muurholm et al. 2007)
pH tolerance	Grows at pH 5,3–7,8 (Muurholm et al. 2007)
Temperature range	Grows between 9 and 28°C; optimum growth at 22–24°C (Muurholm et al. 2007)
***P. lusitanus* NL19^T**	
Place of isolation	Sludge of a deactivated uranium mine (Covas et al. 2017)
Characteristics	Antibacterial activity against a wide range of Gram negative and Gram positive bacteria (Covas et al. 2017)

Resistance to antibiotics (μg)	Amikacin (30), chloramphenicol (30), tetracycline (30), penicillin G (10), vancomycin (30), ticarcillin (75), and tobramycin (10) (Covas et al. 2017)
pH tolerance	Grows at pH between 6 and 8 (Covas et al. 2017)
Temperature range	Grows between 4 and 30ºC, with optimal growth between 18 and 30ºC (Covas et al. 2017)

3. Pedopeptins

The first compounds with antibacterial activity isolated from any *Pedobacter* strains were extracted from the *Pedobacter* sp. SANK 72003, and were named pedopeptins (Hirota-Takahata et al. 2014). These are described as lipopolysaccharide (LPS) inhibitors (Kozuma et al. 2014). LPS are components of the cell wall of Gram-negative bacteria, which consist of three different components: a lipid A domain, a core oligosaccharide and the O antigen (or O polysaccharide) (Hirota-Takahata et al. 2014; Bryant et al. 2009). They activate the immune response by binding to receptors and leading to the overexpression of inflammatory cytokines (Bryant et al. 2009; Hirota-Takahata et al. 2014). LPS are bacterial endotoxins, that are a possible target for the research of new antibacterials (Hirota-Takahata et al. 2014; Kozuma et al. 2014).

Pedopeptins A, B and C (Figure 5) are cyclic depsipeptides that were isolated from cultures of *Pedobacter* sp. SANK 72003 (Hirota-Takahata et al. 2014). Based on previous studies (Hirota-Takahata et al. 2014), the following amino acids are part of the structure of the pedopeptins: diaminopropionic acid (Dap, formed by the pyridoxal phosphate - PLP- mediated amination of serine), phenylalanine (Phe), diaminobutyric acid (Dab, product of the bis (1,1-trifluoroacetoxy) iodobenzene (BTI)-mediated conversion of glutamine), dehydrobutyrine (Dhb, dehydroamino acid derived from dehydration of threonine), leucine (Leu), Dap, Leu, β-hydroxyvaline (β-OH Val), and asparagine (Asp).

Pedopeptins inhibit the interaction between LPS and the receptor cluster of differentiation 14 (CD 14) in the cell (Kozuma et al. 2014). At the time of discovery these peptides showed good antimicrobial activities against *Escherichia coli* (Gram-negative), and pedopeptin B also showed strong antibacterial activity against *Staphylococcus aureus* (Gram-positive), while showing low cell toxicity (Kozuma et al. 2014). These results make pedopeptins extremely interesting products in the research of new antibacterials in this new era of bacterial resistance (Kozuma et al. 2014; Hirota-Takahata et al. 2014).

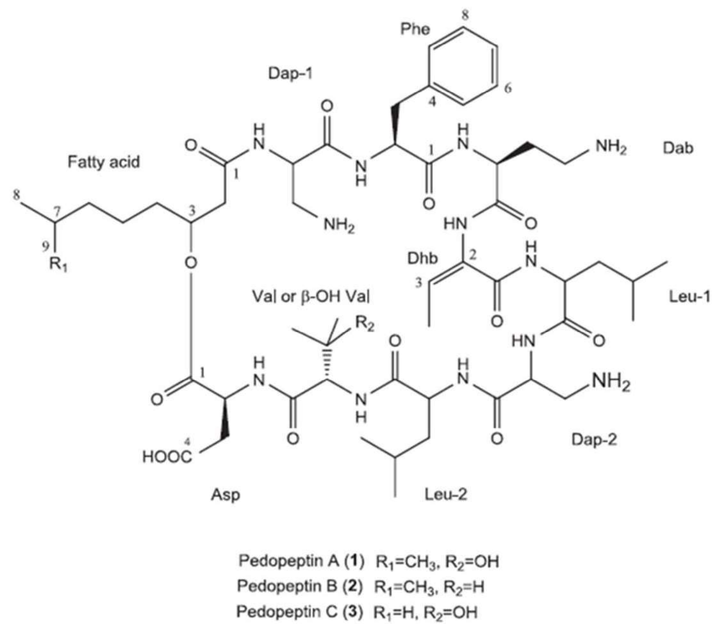

Figure 5 - Structure of pedopeptins (Hirota-Takahata et al. 2014)

Objectives

The main goals of this work are:

1. Determine the antibacterial activity of *Pedobacter* spp. closely related to *P. lusitanus*, in different culture media;
2. Investigate the production of antibacterial compounds by *P. lusitanus* in different solid and liquid media;
3. Identify the antibacterials produced by *P. lusitanus*, by MS analysis of the HPLC fractions;
4. Screen (*in silico*) the genome of *P. lusitanus* for the presence of natural products gene clusters;
5. Perform an integrated analysis of gene clusters identified and the antibacterial products.

Materials and Methods

1. Analysing the antibacterial activity of *Pedobacter lusitanus*

1.1. Solid media assay

The study of the antibacterial activity of *Pedobacter lusitanus* in solid media was carried-out using the double-layer method. The solid media chosen were Tryptic Soy Agar (TSA) at 25% (3.75 g/L peptone from casein, 1.25 g/L papaic digest of soybean, 1.25 g/L sodium chloride and 15 g/L agar), Nutrient Agar (NA) (3 g/L beef extract, 5 g/L peptone from casein and 15 g/L agar), and R2A (0.5 g/L yeast extract, 0.5 g/L peptone from casein, 0.5 g/L casamino acids, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L sodium pyruvate, 0.3 g/L potassium dihydrogen phosphate, 50 mg/L magnesium sulphate heptahidrated and 15 g/L agar). Each plate (55 mm diameter) was filled with 7 mL of the chosen solid medium and inoculated with 5µL of overnight cultures of *Pedobacter lusitanus* NL19 and its closely-related strains *P. himalayensis* MTCC 6384^T (Shivaji et al. 2005), *P. cryoconitis* DSM 14825^T (Margesin 2003), *P. hartonius* DSM 19033^T (Muurholm et al. 2007) and *P. westerhofensis* DSM 19036^T (Muurholm et al. 2007), and the genus type strain *P. heparinus* DSM 2366^T(Steyn et al. 1998). The overnight cultures were performed with the liquid versions of the respective solid media (no agar).

The growth temperature of the producer strains (18ºC) was obtained by studying the growth temperatures of all the *Pedobacter* strains used.

After growing at 18ºC for one week, each plate was overlaid with 7 mL of soft agar medium (TSB medium with 7.5 g/L of agar) inoculated with the indicator strains at a final OD_{600nm} of 0,02. Plates were grown at the appropriate temperature overnight and the results seen as clear zone with no growth around the *Pedobacter* culture indicating antibacterial activity. Because some *Pedobacter* grow at 26ºC, plates of *A. hydrophyla* and *B. cereus* were subjected to UV light for 15 mins. prior to overlay.

The indicator strains used throughout this study and their characteristics can be seen in Table 3 (Covas et al. 2017).

Table 3 - Indicator strains used in this study and an overview of their characteristics

Name	Family	Description	Habitat	Infection	Growth Temperature (in this study)	References
***Aeromonas hydrophyla* ATCC 7966**	*Aeromonadaceae*	Gram negative rods	Humans, animal, fish and fresh water	Opportunistic human pathogens, cause gastrointestinal diseases	26°C	(Huys et al. 2002)
***Bacillus cereus* AV2**	*Bacillaceae*	Gram positive rods	Organic matter, fresh and seawater, intestinal tract of invertebrates…	Food poisoning, eye infections, sepsis, central nervous system (CNS) infection	26°C	(Bottone 2010)
***Escherichia coli* ATCC 25922**	*Enterobacteriaceae*	Gram negative rods	Intestines and faeces of warm blooded animals and reptiles	Urinary tract infections (UTI), bacteraemia, various organ infections	37°C	(Rogers et al. 2011; Tenaillon et al. 2010; Yim et al. 2011)
***Enterococcus faecalis* ATCC 29212**	*Enterococcaceae*	Gram positive cocci	Gastrointestinal tract of humans and animals	UTI, soft tissue infections	37°C	(Kenneth & Ray 2004)
***Enterococcus faecium* 547261**	*Enterococcaceae*	Gram positive cocci	Gastrointestinal tract of humans and animals	UTI, soft tissue infections	37°C	(Kenneth & Ray 2004)
***Haemophilus influenzae* 121642**	*Pasteurellaceae*	Gram negative coccobacillus	Part of the nasopharyngeal microbiome	Systemic illnesses such as pneumonia, meningitis, arthritis, and others	37°C	(Kenneth & Ray 2004)
***Klebsiella pneumoniae* 100603**	*Enterobacteriaceae*	Gram negative rods	Microbiome of the mouth, skin and intestines	Pneumonia	37°C	(Kenneth & Ray 2004)
***Listeria monocytogenes* 71**	*Listeriaceae*	Gram positive rods	Gastrointestinal tract of animals and humans	Listeriosis (may lead to meningitis and encephalitis)	37°C	(Kenneth & Ray 2004)

Micrococcus luteus	*Micrococcaceae*	Gram positive cocci	Skin of mammals	UTI, pneumonia, meningitis	37°C	(Kocur et al. 2006)
Methicillin-resistant *Staphylococcus aureus* (MRSA)	*Staphylococcaceae*	Gram positive cocci	Anterior nostrils, perineum	Skin and respiratory infections, food poisoning, toxic shock syndrome (TSS)	37°C	(Kenneth & Ray 2004)
***Pseudomonas aeruginosa* PAO**	*Pseudomonadaceae*	Gram negative rods	Water, soil and vegetation	Pulmonary, urinary and soft tissue infections	37°C	(Kenneth & Ray 2004)
***Salmonella enterica* ATCC 13076**	*Enterobacteriaceae*	Gram negative rods	Eggs, poultry and red meat, microbiome of reptiles and amphibians, contaminated water	Gastroenteritis, diarrhoea, fever, abdominal cramps	37°C	(Kenneth & Ray 2004)
***Staphylococcus aureus* ATCC 292**	*Staphylococcaceae*	Gram positive cocci	Anterior nostrils, perineum	Skin infections, bone and joint infections	37°C	(Kenneth & Ray 2004)

1.2. Liquid media assay

To test the activity of the *Pedobacter* strains in liquid medium, a liquid media assay was designed.

The four producer strains that showed activity in solid media (*P. cryoconitis*, *P. hartonius*, *P. himalayensis* and *P. lusitanus*) were grown in the liquid versions of the media studied in the double-layer assay: Tryptic Soy Broth at 25% (TSB25%; 3.75 g/L peptone from casein, 1.25 g/L papaic digest of soybean and 1.25 g/L sodium chloride), Nutrient Broth (NB; 3 g/L beef extract and 5 g/L peptone from casein), and R2V (0.5 g/L yeast extract, 0.5 g/L peptone from casein, 0.5 g/L casamino acids, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L sodium pyruvate, 0.3 g/L potassium dihydrogen phosphate and 50 mg/L magnesium sulphate heptahidrated).

The studied strains were grown in 5 mL of the studied media at 18ºC, overnight, to prepare a pre-inoculum. This pre-inoculum was used to inoculate 30 mL of each of the studied media, at a concentration of 1/100 (300 µL of pre-inoculum to 30 mL of liquid medium). The culture was grown at 18ºC and 180 rpm for one week.

At each time point studied (24 hours, 48 hours, 72 hours and one week), the OD_{600} of the culture was measured. Additionally, 1 mL of culture was centrifuged at 12300 g for 10 mins., the supernatant was collected and stored at -20ºC until bioassay was performed (no more than 6 days).

The activity of the supernatants was investigated using the well diffusion assay. In this assay, 45mL of soft agar media was seeded with the indicator strains as described in section 1.1 of Materials and Methods. The seeded soft agar was poured onto a sterile Petri dish and wells were made with a sterile glass pipette. Each well was filled with 50µL of the supernatant and the plates were incubated at the appropriate temperatures overnight (See section 1.1 of Materials and Methods). The antibacterial activity was assessed through the measurement of the inhibition halos formed around the wells.

2. Identification of antibacterial compounds produced by *Pedobacter* strains

To identify the compounds responsible for the antibacterial activity of *Pedobacter* strains, a High-Pressure Liquid Chromatography (HPLC) experiment was planned.

The producer strains were grown in 30 mL of the TSB25% at 18ºC for 48 hours. After measuring the OD_{600}, the culture was centrifuged at 1520g for 25 min and the supernatant was collected. 1 mL of the supernatant was taken to assess the antibacterial activity using the well diffusion assay as described in section 1.2 of Materials and Methods, against *B. cereus*, *M. luteus* and *S. enterica*.

25 mL of the supernatant was mixed with 4.5 mL of 1-butanol at room temperature for one hour. The mixture was centrifuged at 1520g for 25 mins. and the organic top layer was collected. It was separated equally in 6 microcentrifuge tubes of 1.5 mL and evaporated at 50ºC for 12 hours using an evaporator EZ2-Standard (GeneVac).

After evaporation, each pellet was ressuspended in 400 µL of acetonitrile (ACN) at 40% (v/v). When completely dissolved, about 1.2 mL was obtained and sterilized by filtration with a 0.22 µm polyethersulfone (PES) filter. Part of the final sample was used for bioassay using well diffusion assay (as described in section 1.2 of Materials and Methods) against *B. cereus*, *M. luteus* and *S. enterica*. The remaining volume of the sample was used for HPLC.

The constituents of each sample were separated by HPLC with a C_{18} column, with detection at 215 nm and injection volume of 100µL. The flow rate was of 1mL/min and the profile of the solvent B was as following: 0% to 50% between 0 and 10 min, 50% to 70% between 10 and 30 min, 70% to 100% between 30 and 40 mins., and 0% between 30 mins. and the end of the run (50 mins.). The solvents used included a mixture of ultrapure water (MQ-H_2O) and trifluoroacetic acid (TFA) at 0.1% (solvent A) and a mixture of acetonitrile (ACN) and TFA also at 0.1% (solvent B). During the run, 1 mL per minute was collected during the first 30 mins. of the run. The collected samples were tested for antibacterial activity against *B. cereus*, *M. luteus* and *S. enterica* using the agar diffusion assay. The plates were prepared as described in section 1.2, but instead of creating wells in the agar, 5 µL of each HPLC fraction was placed on the top of the agar. The dots were dried and the plates incubated at the

appropriate temperature (see section 1.1 of Materials and Methods). Antibacterial activity was verified when the area where the dot was placed was clear (no growth). The controls used include ACN at 40% and 100%, and a control of the full gradient collected from a blank run.

3. Study of the compounds produced by *P. lusitanus* in different liquid media

Following the previous assay, the compound production by *P. lusitanus* in different media was investigated. Also, to improve the production of antibacterial compounds, the growth conditions of *P. lusitanus* were improved, with the temperature of growth being raised to 26ºC, which was found to be the ideal production temperature in other studies. *P. lusitanus* was grown in 30 mL of each media (TSB25%, NB and R2V), at 26ºC and 180 rpm for 48 hours. The remaining sample preparation and separation of compounds was as described in section 2 of Materials and Methods, except activity was assessed against all indicator strains.

4. Identification of the compounds responsible for the antibacterial activity of *P. lusitanus*

After the identification of the fractions with antibacterial activity, *P. lusitanus* was grown in TSB25% for 48h and 2 consecutive HPLC runs were performed to repeatedly collect only the peaks corresponding to those fractions.

After collection of the peaks in the first HPLC separation (cultures were prepared as described in section 2 of Materials and Methods), the solvents were evaporated by using a SpeedVac (Savant). Each fraction was dissolved in 400µL of ACN at 40% and submitted to another round of separation by HPLC, to concentrate the compound.

In both HPLC separations, only the central area of the peaks was collected in order to promote the recovery of a unique compound. After HPLC, the solvents were evaporated as previously described.

The pellets were dissolved in 50 µL of 0,1% TFA (in MQ-H_2O) and 1 µL was mixed with 9 µL of the α-cyano-4-hydroxycinnamic acid (CHCA) matrix. 0,7 µL of the mixture was applied to each spot of a MALDI plate. The CHCA matrix consisted of a solution of 10 mg/mL using 50% ACN/mqH2O with 0,1% TFA as a solvent. The spectra of each sample were acquired with a MALDI-TOF equipment.

5. Study of the effect of media compound concentration in the production of pedopeptins by *P. lusitanus*

5.1. Alterations in the concentration of media components

The antibacterial activity of *P. lusitanus* was first identified in a simplified version of TSA made up of 15 g/L of casein from peptone (PC), 5 g/L of soy peptone (PS), 5 g/L of sodium chloride (NaCl) and 15 g/L of agar. But, when grown in the liquid version of the same medium (no agar) this activity was no longer seen. Interestingly enough, when this media was diluted to 75%, 50% or 25% of the initial concentration antibacterial activity was restored. In order to find which compound was responsible for the inhibition of the bioactivity of *P. lusitanus* the concentration of each of the compounds of the simplified medium was reduced to 25%.

The antibacterial activity of *P. lusitanus* was tested in 6 different liquid media (see Table 4).

Table 4 - Media components and their respective concentration, used to test the component responsible for the elimination of antibacterial activity of *P. lusitanus* in TSB.

Media/components	PC	PS	NaCl
TSB100%	15 g/L	5 g/L	5 g/L
TSB25%	3,75 g/L	1,25 g/L	1,25 g/L
NaCl25%	15 g/L	5 g/L	1.25 g/L
PS25%	15 g/L	1.25 g/L	5 g/L
PC25%	3.75 g/L	5 g/L	5 g/L

P. lusitanus was grown in a falcon with 5 mL of each of the media, at 26ºC, overnight, to prepare a pre-inoculum. This pre-inoculum was used to inoculate 30 mL of each of the studied media, at a concentration of 1/100 (300 µL of pre-inoculum to 30 mL of liquid medium). The culture was grown at 26ºC and 180 rpm for 48 hours. After growth, the OD_{600} of the cultures was measured, and 1 mL of culture was obtained and centrifuged at 12300 g for 10 mins.. The supernatant was separated and the activity tested against all indicator strains using the well diffusion method described in section 2 of Materials and Methods. The supernatants of *P. lusitanus* grown in each of these media were separated via HPLC, as described in the previous sections, and the bioactivity before and after compound extraction and of the collected fractions (every minute for the first 30 mins. of the run) was studied against *B. cereus*, *M. luteus* and *S. enterica*.

5.2. *In silico* study of pedopeptin production

To study the production of pedopeptins by *P. lusitanus*, we had to first discover how these compounds are produced by the bacterium. The draft genome of *P. lusitanus* (Santos et al. 2015) was entered in the antiSMASH (Medema et al. 2011) tool and the NRPS found were investigated using the PKS/NRPS Analysis Web-site (Bachmann & Ravel 2009).

5.3. Study of the expression of pedopeptins by P. lusitanus in different liquid media

In order to assess if the effect of PC concentration in the antibacterial activity of *P. lusitanus* was due to an effect on pedopeptin production, a real time quantitative PCR (RT-qPCR) study was designed.

Cultures of *P. lusitanus* in TSB100% and PC25% (Table 2, section 5.1 of Materials and Methods) were grown until OD_{600} of 0,500 and RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA, U.S.A.). RNA concentration was obtained using the Qubit® Fluorometer (Invitrogen, USA) and integrity was checked by agarose gel electrophoresis.

cDNA was synthetized with 2 µg of RNA using the SuperScript™ IV VILO™ Master Mix (Invitrogen, USA), according to manufacturer's instructions.

Primers for the *pedo_nrps* genes were designed using OligoPerfect Designer (Invitrogen), for regions without secondary structures that were predicted with the mfold web server (Zuker 2003) (Table 5). All primers were designed purposefully for this study.

Table 5 - Primers designed for this RT-qPCR study.

Name	Primer (5'->3')	Target	Contig	Amplicon size (bp)
pedo_nrps1_fw	TACATCATGCTGTTCCTGCAA	TH53_08205	31	100
pedo_nrps1_rv	CAATCATTTCAATCAGAGCGTTT			
pedo_nrps2_fw	CCGTTTGGTTAGTTATTTGGGTA	TH53_08200	31	106
pedo_nrps2_rv	GGAGCTCCTGGTGATCATGT'			

The quantification was performed based on the method described by Whelan *et al.* (Whelan et al. 2003). The *pedo_nrps1* and *pedo_nrps2* genes copy number was determined using standard curves generated from fragments of these same genes. To achieve this, plasmids containing these two genes were constructed. The *pedo_nrps1* and *pedo_nrps2* genes were amplified from genomic DNA of *P. lusitanus* by PCR, using Platinum™ Green Hot Start PCR Master Mix (2X) (Invitrogen, USA). The PCR product was ligated into pTZ57R-T vector (Fermentas) and cloned in *Escherichia coli* strain DH5α. To do so, 2.5µL of the ligation were mixed with the chemically competent cells and incubated in ice for 15 mins.. Afterwards cells were transferred to a dry bath at 42ºC for 45 seconds and then returned to the ice for 2 mins.. After transformation, the cells were grown in 1 mL of commercial Lysogeny Broth (LB) medium for 1 hour at 37ºC, 180 rpm. The culture was plated onto Lysogeny Agar (LA) medium plates supplemented with ampicillin (100µg/mL) and X-Gal (40 µg/mL). Plates grew overnight at 37ºC and the positive colonies were identified as the colonies that grew white. These colonies were then grown in 10 mL of LB medium overnight at 37ºC and 180 rpm.

The plasmids were extracted using Fermentas GeneJet plasmid miniprep kit (Fermentas, Vilnius, Lithuania) and the plasmid concentration was measured using Qubit® Fluorometer (Invitrogen, USA). For each gene, RT-qPCR was performed using a dilution series of the corresponding plasmid to create a 10-fold standard curve that ranged from 10^{-3} to 10^{-9} in *pedo_nrps1* and 10^{-1} to 10^{-8} in *pedo_nrps2*.

RT-qPCR was performed using 1 µL of template (either cDNA or plasmid dilutions), 300 nM of each primer, 10 µL of PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and nuclease free water, making up a total volume of 20µL. Quantification was performed using the CFX96 real-time PCR system (Bio-Rad, Hercules, CA), under the following conditions: 2 min at 50ºC, 2 min at 95ºC, and 50 cycles of 15 sec at 95ºC and 1 min at 60ºC, with a plate read at the end of each cycle, plus, at the end of the 50 cycles, a melt curve. For each reaction, non-template controls (NTC) were included (without cDNA) as well as positive controls (genomic DNA as template). PCR efficiency was measured using the CFX Manager software (version 2.0; Bio-Rad). The RT-qPCR amplification was considered valid only when the standard curves had a correlation coefficient greater than 0.990 and a PCR efficiency within the range 90–110%.

Results and Discussion

1. Analysis of the antibacterial activity of *Pedobacter lusitanus* NL19

1.1. Solid Media Assay

The study of the antibacterial activity of *P. lusitanus* in solid media was carried out using the double-layer method. *P. lusitanus*, *P. cryoconitis*, *P. hartonius*, *P. hartonius*, *P. himalayensis* and *P. heparinus* were grown in TSA25%, NA and R2A for one week at 18ºC, and their antibacterial activity tested against all the indicator strains (Table 6 and Table 7).

P. westerhofensis and *P. heparinus* showed no antibacterial activity against the indicator strains, and *P. hartonius* was only active against *B. cereus*, which is the most sensitive of all the indicator strains used in this study (Table 7).

P. lusitanus was active against all the indicator strains except *P. aeruginosa*. This lack of activity against *P. aeruginosa* is constant for *P. lusitanus* in all the studies prior to this one and also during this specific study (Table 6). *P. cryoconitis* showed activity against most of the Gram-negative bacteria (except for *B. cereus* and *M. luteus*, both of them highly sensitive) (Table 6). *P. himalayensis* was only active against Gram positive bacteria (*B. cereus*, *E. faecalis*, *E. faecium*, *L. monocytogenes* -exclusively in NA and R2A-, *M. luteus*, MRSA and *S. aureus*) (Table 6).

The production of antibacterial compounds doesn't show major differences when the same strains were grown in different solid media. The spectrum of activity was wider when *P. cryoconitis* was grown in R2A medium, and when *P. himalayensis* was grown in NA medium. There were no major differences in production for *P. hartonius* and *P. lusitanus* between the tested media.

Table 6 - Antibacterial activity of *P. lusitanus*, *P. cryoconitis* and *P. himalayensis* grown in TSA25%, NA and R2A, against 13 indicator strains. Activity is evaluated as active (✓) or not active (✗).

		P. lusitanus			***P. cryoconitis***			***P. himalayensis***		
		TSA25%	NA	R2A	TSA25%	NA	R2A	TSA25%	NA	R2A
Gram negative	*A. hydrophila*	✓	✓	✓	✓	✓	✓	✗	✗	✗
	E. coli	✓	✓	✓	✓	✓	✓	✗	✗	✗
	H. influenzae	✓	✓	✓	✓	✓	✓	✗	✗	✗
	K. pneumoniae	✓	✓	✓	✗	✗	✓	✗	✗	✗
	P. aeruginosa	✗	✗	✗	✗	✗	✗	✗	✗	✗
	S. enterica	✓	✓	✗	✓	✗	✓	✗	✗	✗
Gram positive	*B. cereus*	✓	✓	✓	✓	✓	✓	✓	✓	✓
	E. faecalis	✓	✓	✓	✗	✗	✗	✓	✓	✓
	E. faecium	✓	✓	✓	✗	✗	✗	✓	✓	✓
	L. monocytogenes	✓	✓	✓	✗	✗	✗	✗	✓	✓
	M. luteus	✓	✓	✓	✗	✓	✗	✓	✓	✓
	MRSA	✓	✓	✓	✗	✗	✗	✓	✓	✗
	S. aureus	✓	✓	✓	✗	✗	✗	✓	✓	✗

Table 7 - Antibacterial activity of *P. hartonius*, *P. westerhofensis* and *P. heparinus* grown in TSA25%, NA and R2A, against 13 indicator strains. Activity is evaluated as active (✓) or not active (✗).

		P. hartonius			***P. westerhofensis***			***P. heparinus***		
		TSB25%	NA	R2A	TSB25%	NA	R2A	TSB25%	NA	R2A
Gram negative	*A. hydrophila*	✗	✗	✗	✗	✗	✗	✗	✗	✗
	E. coli	✗	✗	✗	✗	✗	✗	✗	✗	✗
	H. influenzae	✗	✗	✗	✗	✗	✗	✗	✗	✗
	K. pneumoniae	✗	✗	✗	✗	✗	✗	✗	✗	✗
	P. aeruginosa	✗	✗	✗	✗	✗	✗	✗	✗	✗
	S. enterica	✗	✗	✗	✗	✗	✗	✗	✗	✗
Gram positive	*B. cereus*	✓	✓	✓	✗	✗	✗	✗	✗	✗
	E. faecalis	✗	✗	✗	✗	✗	✗	✗	✗	✗
	E. faecium	✗	✗	✗	✗	✗	✗	✗	✗	✗
	L. monocytogenes	✗	✗	✗	✗	✗	✗	✗	✗	✗
	M. luteus	✗	✗	✗	✗	✗	✗	✗	✗	✗
	MRSA	✗	✗	✗	✗	✗	✗	✗	✗	✗
	S. aureus	✗	✗	✗	✗	✗	✗	✗	✗	✗

1.2. Liquid Medium Assay

According to the results obtained from the double-layer method, a liquid media assay was designed. The four producer strains that showed activity in solid media (*P. cryoconitis*, *P. hartonius*, *P. himalayensis* and *P. lusitanus*) were grown in the equivalent liquid media

(TSB25%, NB and R2V) and their activity was tested against all the indicator strains at each time point (24 hr, 48 hr, 72 hr and 1 week). Also, the bacterial growth of each strain was monitored by OD_{600} measurement at each time point (Figure 6). All strains seemed to grow less in the R2V medium whereas their growth rate was similar in NB and TSB25% media (Figure 6).

The antibacterial activity of *P. lusitanus* was different when studied in different liquid media, especially when the activity of the producer strain grown in R2V was compared to its activity when grown in TSB25% or NB (Figure 7). Even though *P. lusitanus* grown in R2V reached the "standard" of activity in this study (activity against all the indicator strains except *P. aeruginosa*), it only reached this value after a week, whereas the activity in TSB25% and NB activity was at its highest values after 48 hours of growth.

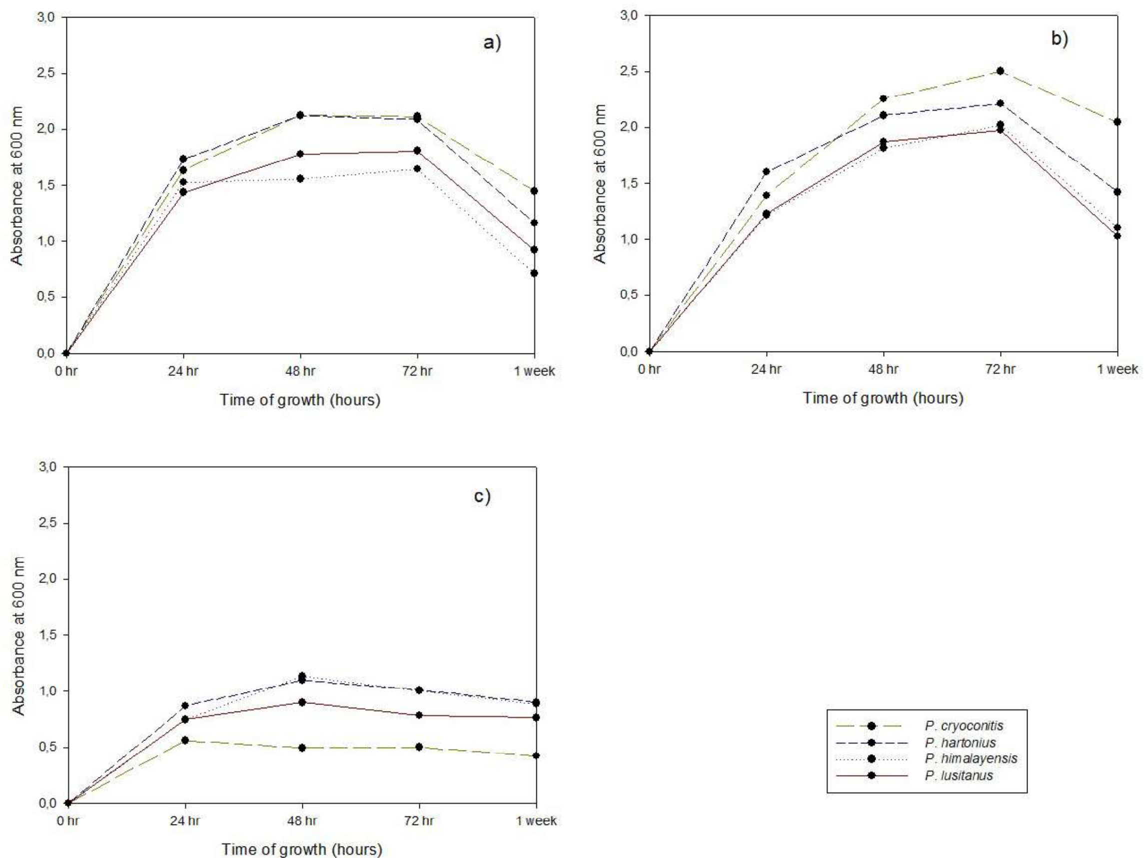

Figure 6 - Monitoring of the bacterial growth of *P. lusitanus*, *P. cryoconitis*, *P. hartonius* e *P. himalayensis* in a) TSB25%, b) NB and c) R2V, over the course of one week. Growth was measured with absorbance at 600nm.

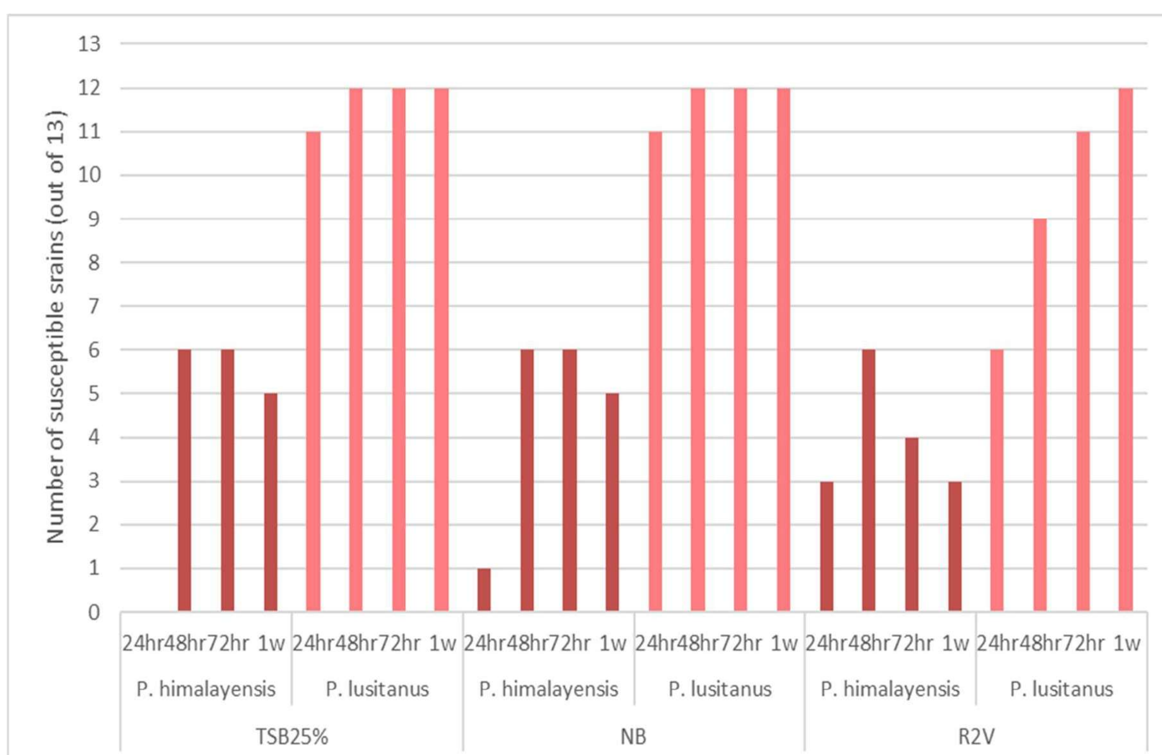

Figure 7 - Development of antibacterial activity of *P. himalayensis* and *P. lusitanus* over the course of the studied period. At each point of study (24, 48, 72 hours and one week) the number of susceptible strains (out of 13) is presented.

The antibacterial activity of *P. himalayensis* grown in different liquid media shows an interesting characteristic when compared with that of *P. lusitanus*: the activity decreased related with the number of cells. Also, in both solid and liquid media, *P. himalayensis* is only active against Gram-positive bacteria (*B. cereus*, *E. faecalis*, *E. faecium*, *M. luteus*, MRSA and *S. aureus*). From solid to liquid media *P. himalayensis* loses its activity against *L. monocytogenes*.

At 48 hours of growth *P. himalayensis* and *P. lusitanus* had both demonstrated antibacterial activity against the broadest amount of indicator strains (12 susceptible strains out of 13 for *P. lusitanus* and 6 out of 13 susceptible strains for *P. himalayensis*) in both TSB25% and NB media. *P. himalayensis* showed antibacterial activity against the broadest amount of indicator strains in R2V at 48 hr and *P. lusitanus* only exhibited antibacterial activity against 12 strains at 1 week of growth (Figure 7). In this study, neither *P. cryoconitis* nor *P. hartonius* showed antibacterial activity when grown in liquid media (Table 8).

Table 8 - Number of indicator species (out of 13) susceptible to *Pedobacter* strains grown in different liquid media.

	TSB25%	NB	R2V
P. cryoconitis	0	0	0
P. hartonius	0	0	0
P. himalayensis	6	6	6
P. lusitanus	12	12	12

1.3. Discussion

So far, in the *Pedobacter* genus, only *Pedobacte*r sp. SANK 72003 and *P. lusitanus* have been described as having antibacterial activity (Kozuma et al. 2014; Covas et al. 2017). The antibacterial activity of *P. cryoconitis, P. hartonius* and *P. himalayensis* is described in this study for the first time. A comparison of the results in solid and in liquid media assays, clearly shows that bioactivity is better when the producer strains are grown in agarized media. Both *P. cryoconitis* and *P. hartonius* lose their antibacterial activity when grown in the liquid media tested.

The differences between production in solid and liquid media can be explained by the physiology of bacteria in solid media. Although these differences are more studied for fungi and filamentous bacteria such as *Actinobacteria*, we know that bacteria show different characteristics in solid media (Cheung & Fischetti 1988). Also, the concentration of the compounds produced in solid media is usually higher than that in liquid media, and the characteristics of the products can also be different in solid media (Barrios-González 2012; Acuña-Argüelles et al. 1995).

So, the producer strains can either produce the same compounds in solid and liquid media but the concentration is higher in solid media, or produce different compounds in solid and liquid media. This can be further investigated with compound extraction from solid media and subsequent HPLC studies.

The antibacterial activity of *P. lusitanus* was different in the different liquid media, especially when comparing the activity of the producer strain grown in R2V with that of TSB25% or NB. This may be due to the growth response of the bacterium in this medium. Even though

there isn't a perceivable correlation between the density (number of cells) of the culture (measured by the OD_{600}) and the antibacterial activity, growth of *P. lusitanus* in R2V is very slow, thus a lower number of cells/less biomass, when compared to the density of the culture when the bacterium is grown in TSB 25% or in NB. Knowing that the production of secondary metabolites occurs at a late growth phase of bacteria, it is expectable that, when *P. lusitanus* grows in R2V the growth rate is lower and, therefore, it takes much longer to reach the same cell density and, consequently, the same level of activity observed in other production media, such as NB and TSB25%.

The antibacterial activity of *P. himalayensis* decreases towards the end of the growth period studied. When the OD_{600} of the culture is at its highest, the bioactivity is also at its highest. In liquid media and in the studied conditions, *P. himalayensis* inhibits growth of 6 out of the 13 indicator species. Once the density of the culture starts to decrease, the activity decreases as well. Also, in both solid and liquid media, *P. himalayensis* is active against Gram positive bacteria (*B. cereus*, *E. faecalis*, *E. faecium*, *L. monocytogenes* -only in NA and R2A-, *M. luteus*, MRSA and *S. aureus*). These observations might indicate that the compounds responsible for the antibacterial activity in *P. lusitanus* are different from those produced by *P. himalayensis*.

2. Identification of antibacterial compounds produced by *Pedobacter* strains

To identify the compounds responsible for the antibacterial activity detected in *Pedobacter* strains a High-Pressure Liquid Chromatography (HPLC) experiment was designed. Even though the only strains that showed activity in liquid media were *P. lusitanus* and *P. himalayensis* we decided to test all the *Pedobacter* spp. strains that were used in the previous liquid media assays (section 1.2 of Materials and Methods). The first medium tested was TSB25%.

The strains were grown in TSB25% at 18ºC for 48 hours, and the activity was tested against *B. cereus*, *M. luteus* and *S. enterica* at every step of the procedure (before and after compound extraction and after HPLC separation). The results of the antibacterial activity

assay before compound extraction showed low activity of *P. himalayensis* and only against *B. cereus* and *M. luteus* and good activity from *P. lusitanus* against all the indicators. As expected, no activity from *P. cryoconitis* and *P. hartonius* was identified (Table 9). After compound extraction, *P. himalayensis* supernatant lost its activity against *M. luteus* whereas *P. lusitanus* supernatant retained its activity against all the indicators tested (Table 9).

Table 9 - Bioassay results of the supernatant of the different *Pedobacter* strains grown in TSB 25%, before and after compound extraction with butanol. Activity was evaluated quantitatively: - stands for no activity, +/- stands for moderated activity, + and ++ stand for activity and high activity, respectively.

		Antibacterial activity before compound extraction	**Antibacterial activity after compound extraction**
P. cryoconitis	*B. cereus*	-	-
	M. luteus	-	-
	S. enterica	-	-
P. hartonius	*B. cereus*	-	-
	M. luteus	-	-
	S. enterica	-	-
P. himalayensis	*B. cereus*	+/-	+/-
	M. luteus	+/-	-
	S. enterica	-	-
P. lusitanus	*B. cereus*	++	++
	M. luteus	++	++
	S. enterica	+	+

When comparing chromatograms of the HPLC separation of *P. cryoconitis*, *P. hartonius* and *P. himalayensis* with that of *P. lusitanus,* it is observed that the latter has very intense peaks starting at a t_R of 14 min (Figure 8). These peaks are absent in the extracts of the other strains.

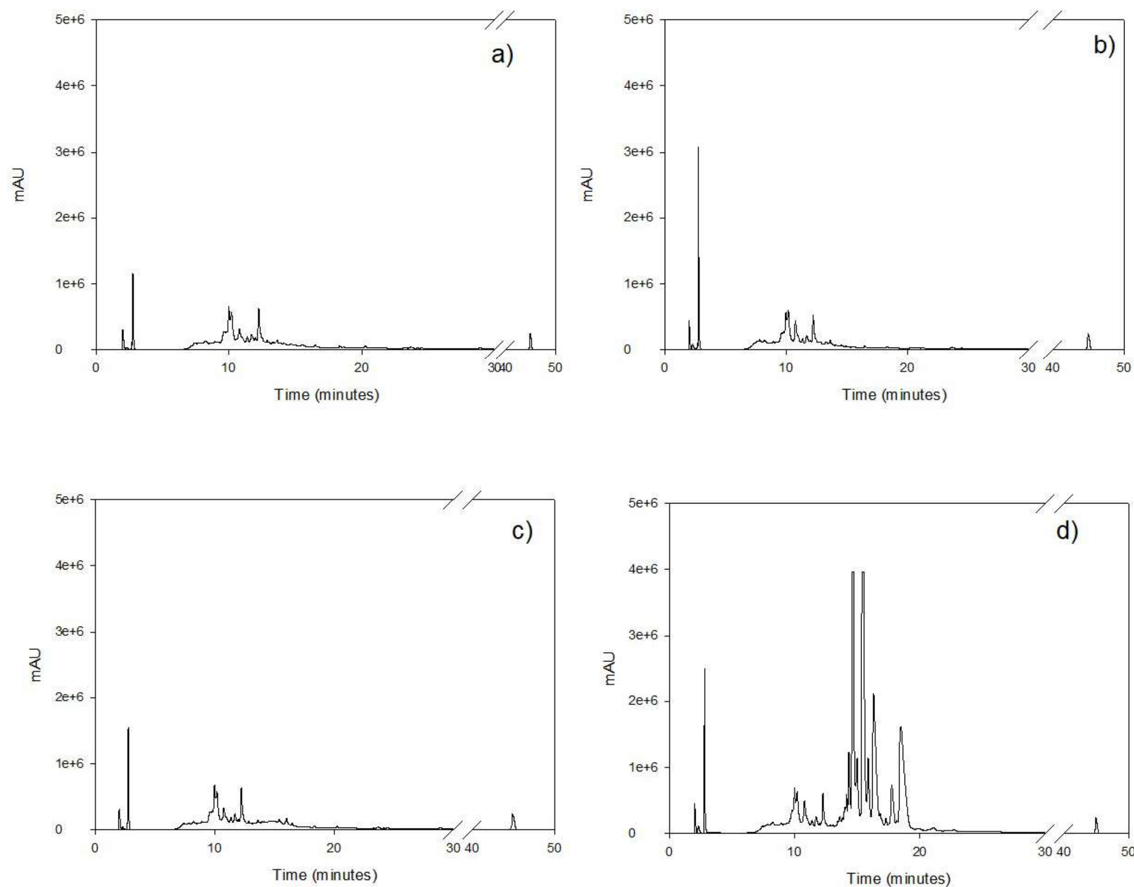

Figure 8 - HPLC chromatograms resulting from the separation of the supernatants of a) *P. cryoconitis*, b) *P. hartonius*, c) *P. himalayensis*, and d) *P. lusitanus* grown in TSB25%. The UV detection was performed at 215nm.

During the first 30 mins. of the run, fractions of 1 mL (corresponding to one minute) were collected and tested against *B. cereus*, *M. luteus* and *S. enterica*. None of the *P. cryoconitis*, *P. hartonius* or *P. himalayensis* fractions showed activity (as expected). The fractions from the separation of the supernatant of *P. lusitanus* showed activity against all strains, from 15 through 20 mins. (equivalent to fractions 16 through 20), with the highest activity in fractions 17 (corresponding to 16 and 17 mins. of the run) and 19 (corresponding to 18 and 19 mins. of the run) (Table 10).

Table 10 - Bioassay results of the samples collected from HPLC runs resulting from the supernatant of the different *Pedobacter* spp. strains grown in TSB 25%. Activity was evaluated quantitatively: - stands for no activity, +/- stands for moderated activity, + and ++ stand for activity and high activity, respectively.

		Time of collection (mins.)									
		1 to 15	16	17	18	19	20	21	22	23	24 to 30
P. cryoconitis	*B. cereus*	-	-	-	-	-	-	-	-	-	-

	M. luteus	-	-	-	-	-	-	-	-	-	-
	S. enterica	-	-	-	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-	-	-	-
P. hartonius	*M. luteus*	-	-	-	-	-	-	-	-	-	-
	S. enterica	-	-	-	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-	-	-	-
P. himalayensis	*M. luteus*	-	-	-	-	-	-	-	-	-	-
	S. enterica	-	-	-	-	-	-	-	-	-	-
	B. cereus	-	+/-	++	+	++	+/-	-	-	-	-
P. lusitanus	*M. luteus*	-	+/-	+	+/-	++	-	-	-	-	-
	S. enterica	-	-	++	+	+	-	-	-	-	-

2.1. Discussion

P. himalayensis shows bioactivity against some indicator strains (all of them Gram positive) prior to extraction with butanol. Yet, this activity was almost completely lost after compound extraction with butanol. This may be due to the low concentration of the compounds in the supernatant. During extraction with butanol, the compounds are not fully extracted from the supernatant and, although they are present, their concentration is lower than that needed to inhibit growth of the indicators. During the separation by HPLC, the compounds are further diluted and, therefore, the activity against *B. cereus* was lost as well.

The comparison of the chromatograms of *P. lusitanus* with those obtained for the other strains, added to the fact that the activity of the fractions of the HPLC separation of this bacterium is concentrated from 15 through 20 mins. (equivalent to fractions 16 through 20), with the highest activity in fractions 17 and 19, shows that the compounds responsible for the activity of *P. lusitanus* are being eluted at that time interval.

A comparison between the chromatograms resulting from the separation of the supernatants of *P. lusitanus* and that of the other strains, shows that the compounds responsible for the activity of *P. lusitanus* have a t_R between 15 and 20 mins..

3. Study of the compounds produced by *P. lusitanus* in different liquid media

Based on the results obtained from the first round of HPLC, the following HPLC studies were focused on *P. lusitanus* antibacterial compounds. Also, to improve the production of antibacterial compounds, the growth conditions of the producer strain were improved by increasing the growth temperature to 26 ºC, which is the optimum production temperature, and in which *P. lusitanus* reaches higher cell density in a short period of time.

P. lusitanus was grown in TSB25%, NB and R2V, the activity of the extracts was tested before HPLC and after HPLC separation the collected fractions were tested against all indicator strains. After butanol extraction of the compound, all the supernatants exhibit activity against the 12 of the indicator strains, except for the supernatant from the R2V, which had no activity against *S. aureus* (this was to be expected because, as seen in section 1.2, at 48 hours, *P. lusitanus* grown in R2V does not inhibit growth of all the indicator strains). Also, when *P. lusitanus is* grown in R2V the activity against most indicator strains is very poor (Table 11).

Table 11 - Bioassay results of *P. lusitanus* supernatant, grown in different liquid media, before the HPLC run. Activity was evaluated quantitatively: - stands for no activity, +/- stands for moderated activity, + and ++ stand for activity and high activity, respectively.

	TSB 25%	NB	R2V
A. hydrophila	++	++	+
B. cereus	++	++	++
E. coli	+	+	+
E. faecalis	+/-	+/-	+/-
E. faecium	+	+	+
H. influenzae	+	+	+
K. pneumoniae	+	+/-	+/-
L. monocytogenes	+/-	+/-	+/-
M. luteus	+	+	+
MRSA	+	+	+/-
P. aeruginosa	+/-	+/-	-
S. enterica	+	+	+
S. aureus	+/-	+/-	-

The chromatograms obtained from the HPLC separation of *P. lusitanus* supernatant grown in TSB25% (Figure 9) and NB (Figure 10) are very similar, meaning that the compounds produced by *P. lusitanus* in these media are probably the same and are produced roughly in the same amount. The chromatogram of the HPLC separation of the R2V supernatant (Figure 11) is very different from the other two, with no evident peaks along the separation. After HPLC, the fractions collected were tested against all indicator strains. The compounds

present on the HPLC fractions resulting from the separation of *P. lusitanus* supernatant grown in R2V were unable to inhibit growth of the indicator strains. Only the fractions, resulting from the separation of the supernatant of *P. lusitanus* grown in TSB25% and NB and with a t_R between 15 min and 20 min. showed activity against the indicator strains (Figures 9 and 10, Table 12). The fraction with more bioactivity have a t_R of 17 and 19 min., as previously reported in section 2 of Results and Discussion. Also, when comparing the activity of the collected fractions it is possible to see that fraction with the t_R=17 min. has a stronger activity against Gram negative bacteria (*A. hydrophyla*, *E. coli*, *H. influenzae*, *K. pneumoniae*, *M. luteus*, and *S. enteritidis*) whereas the fraction with the t_R= 19 min. has stronger activity against the Gram positive bacteria (*B. cereus*, *E. faecalis*, *E. faecium*, *L. monocytogenes*, MRSA and *S. aureus*).

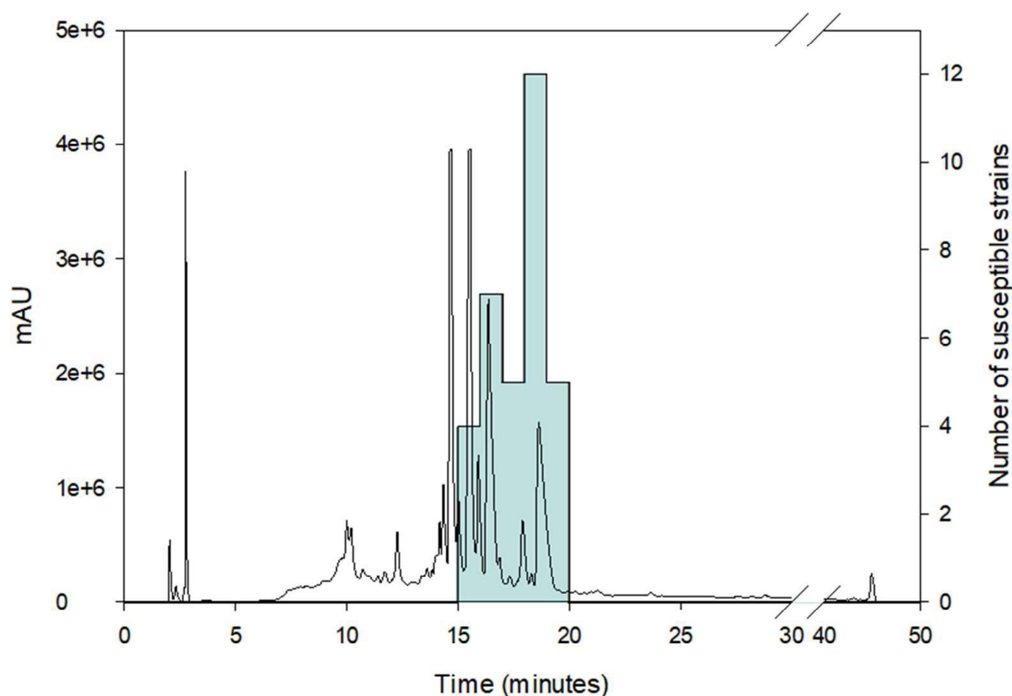

Figure 9 - Chromatogram resulting from the HPLC separation of the supernatant of *P. lusitanus* grown in TSB25%. Absorbance= 215nm (black line). The bioactivity of each collected fraction is represented in the blue area. The number of susceptible strains (out of 13) is represented.

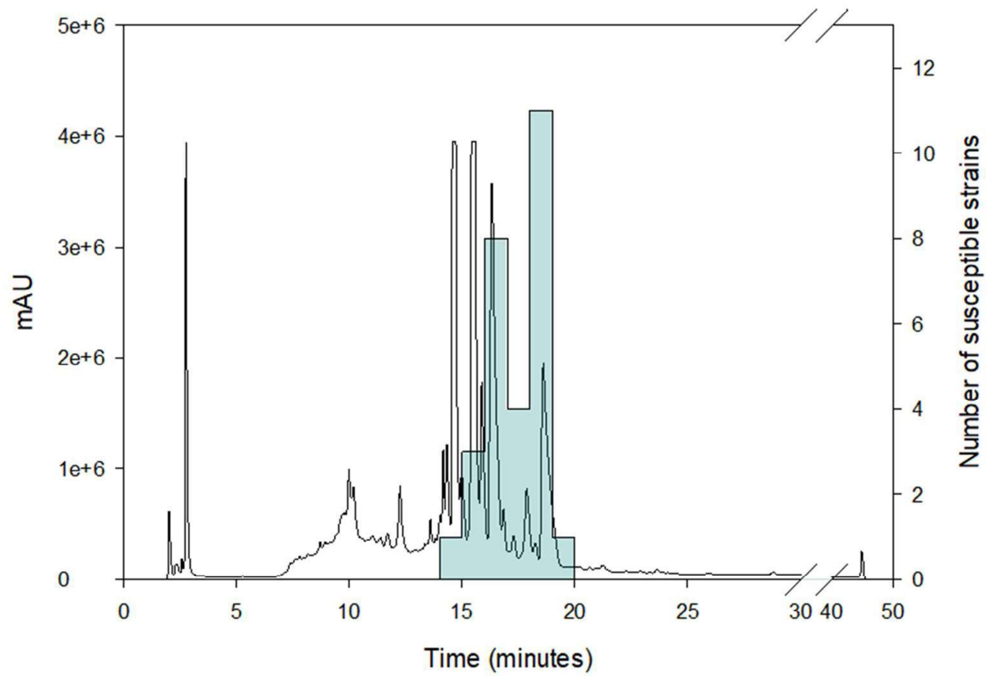

Figure 10 - Chromatogram resulting from the HPLC separation of the supernatant of *P. lusitanus* grown in NB Absorbance= 215nm (black line). The bioactivity of each collected fraction is represented in the blue area. The number of susceptible strains (out of 13) is represented.

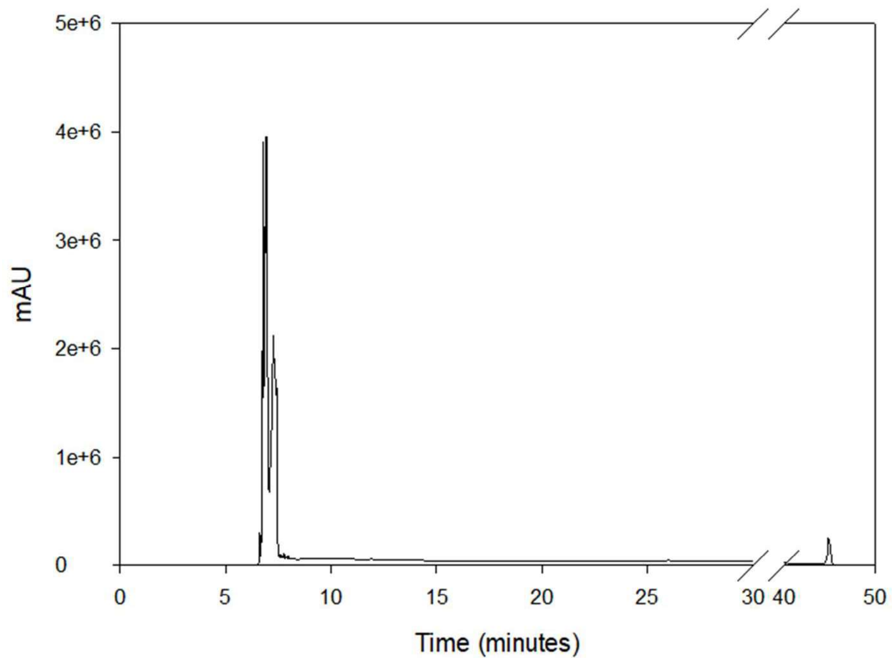

Figure 11 - Chromatogram resulting from the HPLC separation of the supernatant of *P. lusitanus* grown in R2V. Absorbance= 215nm (black line). None of the fractions collected presented antibacterial activity against the 13 studied indicator strains.

Table 12 - Bioassay results with the fractions collected from HPLC separation of the supernatant of *P. lusitanus* grown in different media. At each minute, fractions were collected and tested against all the indicator strains. Because activity was concentrated between t_R=15 and 21 mins. of the run only these results are presented. Activity was evaluated quantitatively: - stands for no activity, +/- stands for moderated activity, + and ++ stand for activity and high activity respectively.

	TSB 25%							**NB**						
	15	**16**	**17**	**18**	**19**	**20**	**21**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
A. hydrophila	-	+	++	+	+	+	-	-	-	++	-	+	-	-
B. cereus	-	+	+	+	++	+	-	-	+	+	+	+	+/-	-
E. coli	-	+	++	+	+	+	-	-	+	++	+	+	-	-
E. faecalis	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-
E. faecium	-	-	-	-	+/-	-	-	-	-	+/-	-	+/-	-	-
H. influenzae	-	-	+	-	+/-	-	-	-	+	++	+	+	-	-
K. pneumoniae	-	-	+	-	+/-	-	-	+	-	++	+	+	-	-
L. monocytogenes	-	-	-	-	+/-	-	-	-	-	-	-	+/-	-	-
M. luteus	-	+	+	+	++	+	-	-	-	++	-	+	-	-
MRSA	-	-	-	-	+/-	-	-	-	-	-	-	+/-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. enterica	-	+	++	+	+	+	-	-	-	++	-	+	-	-
S. aureus	-	-	-	-	+/-	-	-	-	-	-	-	+/-	-	-

3.1. Discussion

The comparison of the HPLC chromatograms resulting from the separation of the supernatant of *P. lusitanus* grown in TSB25% and NB with that resulting from the growth in R2V allows to identify peaks that are very clear in the first two conditions and are absent in the third. This indicates that the antibacterial compounds are being produced in much lower quantities in R2V. These differences were expected because the activity of the samples analysed by HPLC was also low. After HPLC separation, none of the fractions collected from the HPLC separation of the R2V supernatant showed antibacterial activity against the indicator strains. This may be due to the same reason previously mentioned for *P. himalayensis*: although there is activity before separation, during the separation the compounds are further diluted, reducing their concentration to a level at which it is no longer able to inhibit growth of the indicator strains.

The activity of *P. lusitanus* is concentrated in the fractions collected between 15 and 20 mins., especially those between 16 and 17 mins., and 18 and 19 mins.. In *P. lusitanus*, the compounds present in those fractions are responsible for the bioactivity observed. These

results are further confirmed since no activity is observed when the peaks are not detected in these fractions.

4. Identification of the compounds responsible for the antibacterial activity of *P. lusitanus*

The antibacterial activity of *P. lusitanus* was identified in the same peaks for all the growth media that showed activity, between t_R=15min. and 20 mins. (Figure 12). The absence of these peaks coincides with lack of activity. To identify the compounds responsible for the bioactivity of *P. lusitanus,* the peaks were collected and analysed by mass spectrometry (MS). Based on our previous results, the bioactive compounds were secreted to the supernatant when *P. lusitanus* was grown in TSB25%. These compounds were further separated by HPLC. The peaks collected within the region were bioactivity was detected were numbered from 1 to 9 (Figure 12). Their activity was investigated against *B. cereus*, *M. luteus* and *S. enterica*. Peaks 4, 5, 6, 7, 8 and 9 showed activity (Table 13). Peaks 6 and 9 exhibited more bioactivity. Even so, all the peaks were concentrated and suspended before MALDI-ToF analysis. The corresponding fractions (based on the retention time) of the supernatant of *P. lusitanus* grown in TSB100% were also collected (Figure 13) and analysed by MS. This consisted in a control sample since *P. lusitanus* does not produce antibacterial activity when grown in TSB100%.

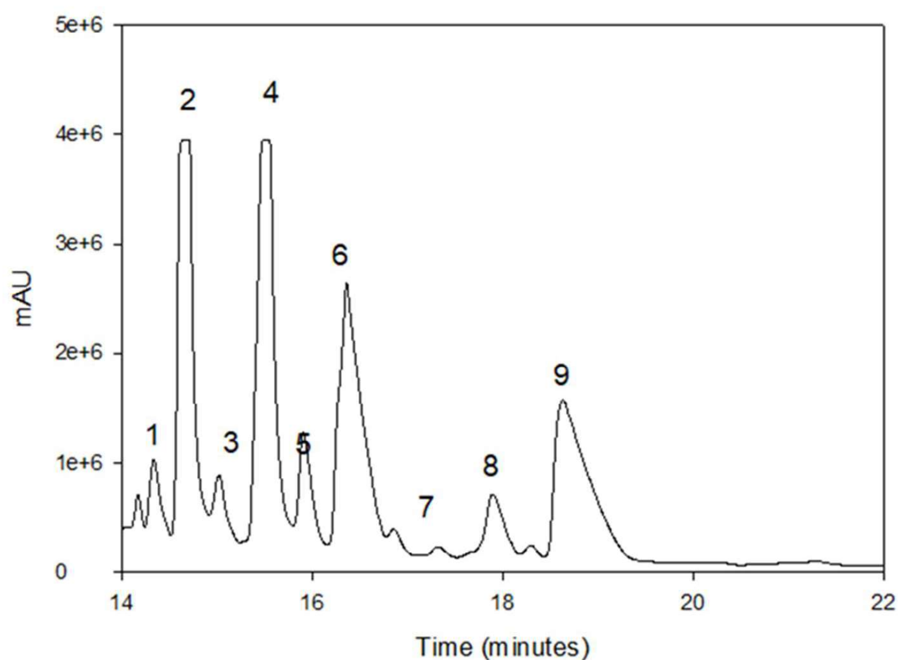

Figure 12 - Fractions collected from the HPLC separation of the supernatant from *P. lusitanus* grown in TSB25%. The peaks 1 through 9 were collected and their activity was tested against *B. cereus*, *M. luteus* and *S. enterica*

Table 13 - Antibacterial activity of the fractions (peaks) collected from the HPLC separation of the supernatant of *P. lusitanus* grown in TSB 25%. Activity was evaluated quantitatively: - stands for no activity, +/- stands for moderated activity, + and ++ stand for activity and high activity respectively.

	Peaks								
	1	2	3	4	5	6	7	8	9
B. cereus	-	-	-	+/-	+	+	+	+	++
M. luteus	-	-	-	+/-	+	+	+	+	++
S. enterica	-	-	-	+/-	+	++	+	+	+

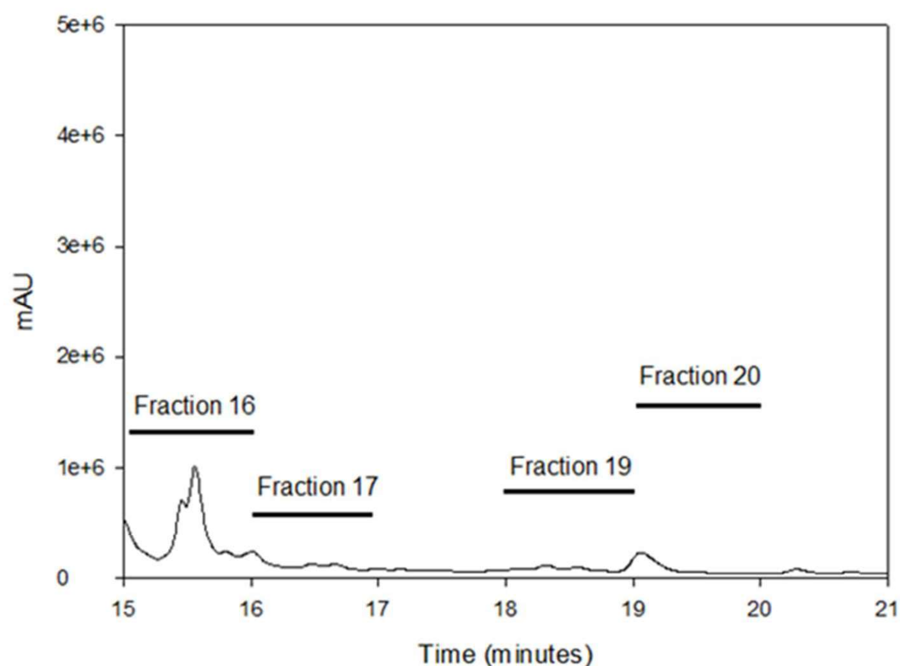

Figure 13 - Fractions collected from the HPLC separation of the supernatant from *P. lusitanus* grown in TSB100%. Because the peaks were not as intense as in the supernatant from *P. lusitanus* grown in TSB25%, the criterion for collection was based on the retention time of the peaks collected from the TSB25% supernatant. Their bioactivity of each fraction was evaluated against *B. cereus*, *M. luteus* and *S. enterica*. None of the fractions showed antibacterial activity.

The MS analysis revealed the presence of three compounds with molecular masses characteristic of pedopeptins in the peaks that showed antibacterial activity (Figure 12; Table 14). Pedopeptins are antibacterial peptides produced by *Pedobacter* sp. SANK 72003, that were identified as lipopolysaccharide (LPS) inhibitors (Hirota-Takahata et al. 2014). Based on their molecular structure, pedopeptins are divided in pedopeptin A, B and C (Hirota-Takahata et al. 2014). A description of each of the pedopeptins can be seen in Table 15.

Table 14 - Molecular masses of the most intense ions in each peaks/fraction, defined by their mass (m/z) and intensity of the peak. The peaks referring to pedopeptins A, B and C are identified as (A), (B) and (C), respectively.

Peaks	TSB25%		TSB100%		Fractions
	HR-MS (m/z)	Intensity	HR-MS (m/z)	Intensity	
4	713.47 752.479	9172 6569	752.444	8334	16
5	713.463 1101.652 (C) 1115.666 (A)	24911 4305 883	713.439	21259	16
6	1115.616 (A)	17022	747.4 1115.622 (A) 909.533	5429 4106 2116	17
7	909.518 1099.601 (B) 1101.584 (C) 1115.595 (A)	6554 584 357 2815			17
8	1085.639 1099.657 (B) 1115.637 (A)	22679 4679 397	1137.586 1099.628 (B) 1085.510	7115 9152 338	19
9	1099.65 (B) 1101.68 (C) 1115.645 (A)	13408 991 1264	1099.502 (B) 1121.483	2277 3172	20

Table 15 - Elucidation of the characteristics of the pedopeptins (Hirota-Takahata et al. 2014)

	Pedopeptin A	**Pedopeptin B**	**Pedopeptin C**
Chemical formula	$C_{53}H_{86}N_{12}O_{14}$	$C_{53}H_{86}N_{12}O_{13}$	$C_{52}H_{84}N_{12}O_{14}$
High-resolution mass spectra (m/z)	1115.6460 $(M+H)^+$	1099.6517 $(M+H)^+$	1101.6299 $(M+H)^+$
Structure	Dap-1/ Phe/ Dab/ Dhb/ Leu-1/ Dap-2/ Leu-2/ β-OH Val/ Asp/ Fatty acid	Dap-1/ Phe/ Dab/ Dhb/ Leu-1/ Dap-2/ Leu-2/ Val/ Asp/ Fatty acid	Dap-1/ Phe/ Dab/ Dhb/ Leu-1/ Dap-2/ Leu-2/ β-OH Val/ Asp/ Fatty acid

Peak 4

Peak 5

Peak 6

Peak 7

Peak 8

Peak 9

Figure 14 - High-resolution mass spectra of the peaks 4, 5, 6, 7, 8 and 9, collected from the HPLC separation of the supernatant of *P. lusitanus* grown in TSB25%.

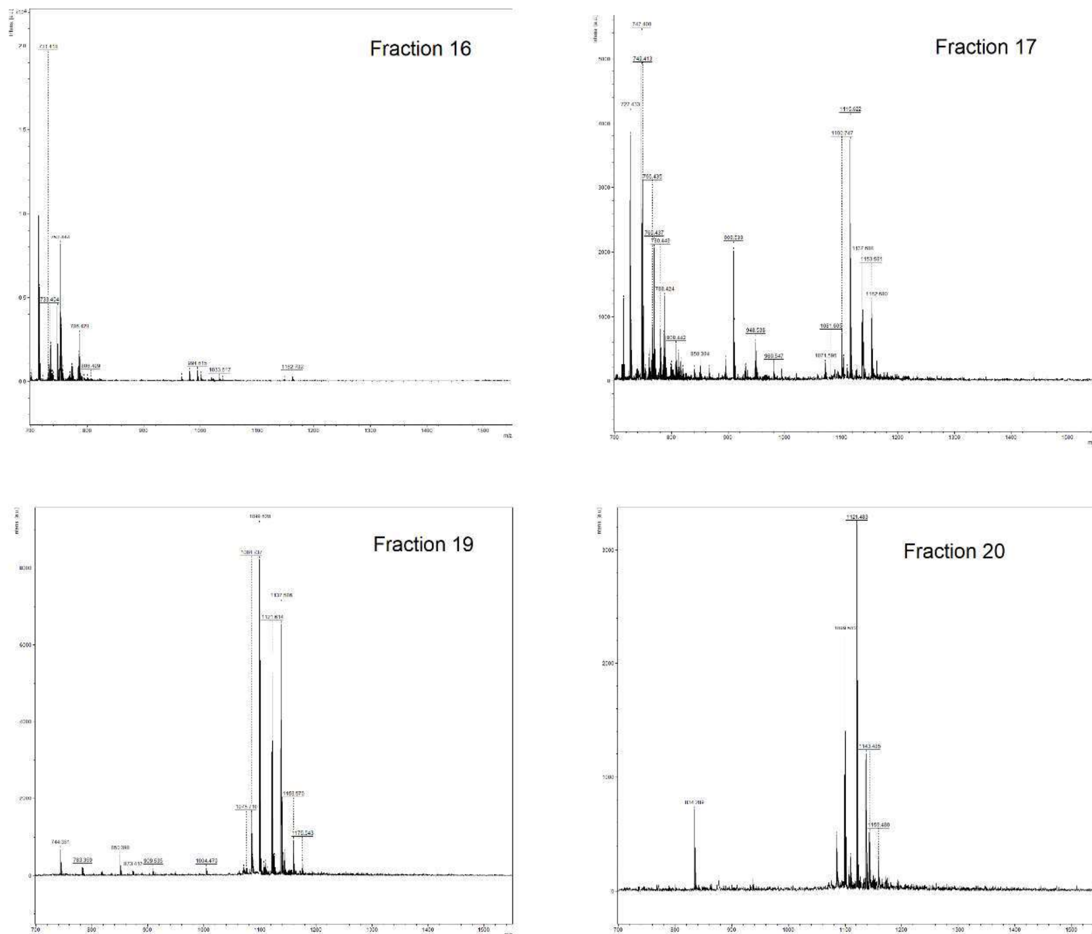

Figure 15 - High-resolution mass spectra of the fraction 16, 17, 19 and 20, collected from the HPLC separation of the supernatant of *P. lusitanus* grown in TSB100%.

Also, the fractions collected from the HPLC separation of the supernatant of *P. lusitanus* grown in TSB100% only show the presence of pedopeptins A and B (Figure 15). The only peak with activity that does not correspond to any pedopeptin is peak 4, whose most abundant compound has a mass of 713.47 Da. Yet, it is worth to mention that the activity of peak 4 was very weak (Table 13). A compound with the same mass is produced by *P. lusitanus* grown in TSB100%. The MS analysis performed is not quantitative. However, the counts obtained are indicative of the abundance of each compound in the sample. According to this, the production of the compound with a molecular mass of 713.47 Da seems to be similar in TSB25% and TSB100%. The same was not observed for pedopeptins. Albeit also detected in TSB100% fractions, their counts seem lower, especially for pedopeptin A (Table 14). Although the activity of the isolated pedopeptins has been

described (Hirota-Takahata et al. 2014), it is possible that their activity is synergistic and therefore increases when more than one type of pedopeptin is present. Apart from pedopeptin A and B another compound is present in peak 8.. This compound has a molecular mass of 1086 Da and it is also present in very low abundance in the fraction 19 collected from TSB100% (Table 14). Fraction 8 was not the fraction with higher antibacterial activity and also contains pedopeptin A and B. Thus, it is probable that the activity is due to these two peptides and not to the compound with 1086 Da. Yet, the production of this compound also decreased in TSB100%.

4.1. Discussion

The activity of the fractions with a t_R=17 min. (equivalent to peak 6; Table 12) and 19 min. (equivalent to peak 9; Table 12) showed that peak 6 is more active against Gram negative bacteria (*A. hydrophyla*, *E. coli*, *H. influenzae*, *K. pneumoniae*, *M. luteus*, and *S. enterica*) and peak 9 is more active against Gram positive bacteria (*B. cereus*, *E. faecalis*, *E. faecium*, *L. monocytogenes*, MRSA and *S. aureus*) (Table 12). MS analysis revealed that peak 6 has the highest concentration of pedopeptin A and peak 9 the highest concentration of pedopeptin B. This is in accordance with the results obtained by Kozuma et al. (2014). That study described that pedopeptins A and C were more active against *E. coli*, but only pedopeptin B showed a decent antibacterial activity against *S. aureus* (Kozuma et al. 2014). Even though, since no other indicator strains were tested in previous studies with pedopeptins, one can assume that these *E. coli* and *S. aureus* are representative of their Gram groups.
Peak 6 presents the highest activity against Gram negative bacteria. In this peak, the only pedopeptin present is pedopeptin A, in a very high abundance. Herein, we further suggest the hypothesis that pedopeptin B is active against Gram-positive bacteria. This is interesting because the described mechanism of pedopeptin action is based on the LPS inhibition, and the cell wall of Gram-positive bacteria doesn’t have LPS. The major difference between pedopeptin B and pedopeptins A and C is that, instead of having a β-OH Val, it has a Val, and that is probably the reason for that difference (Hirota-Takahata et al. 2014).
One interesting result of this study is the decreased activity of pedopeptin B against Gram negative bacteria, at least when comparing this activity with the activity against Gram

positive bacteria. Even though the MIC of this compound against the representative Gram positive and Gram negative strains was the same (4 $mgmL^{-1}$) (Kozuma et al. 2014), in the present study that was not observed.

5. Study of the effect of media compound concentration in the production of pedopeptins by *P. lusitanus*

5.1. Alterations in the concentration of media components

After verifying the differences between the antibacterial activity of *P. lusitanus* grown in TSB100% and TSB25%, an experiment was designed to understand the reason behind this difference. The concentration of each medium component was individually reduced to 25% (of its original value in TSB) and after 48 hr of growth in these modified media, the activity of *P. lusitanus* was evaluated against all the indicator strains by well diffusion assay. The results showed that the antibacterial activity of NaCl25% and PS25% was similar to that of TSB100%. On the contrary, the activity of PC25% was similar to that of TSB25%. Therefore, when peptone from casein (PC) is at its full concentration (5 g/L) in the growth media, the activity of compounds produced by *P. lusitanus* decreases drastically. Therefore, in this case, An high concentration of peptone from casein in the growth media is a disrupting factor in the bioactivity exhibited by *P. lusitanus* (Table 16).

Table 16 - Size (in mm) of the inhibition zones observed when the supernatant of *P. lusitanus* cultures was tested against different indicator bacteria. TSB25% has all its components reduced to 25% of their concentration in TSB100%. All the other media have their components referred (NaCl, PC and PS) at 25% of their concentration, and all other components of the medium were kept at their full concentration as in TSB100%. PC- peptone from casein and PS- peptone from soya.

	TSB25%	**TSB100%**	**NaCl25%**	**PS25%**	**PC25%**
A. hydrophila	13	0	7	8	13
B. cereus	14	7	9	10	14
E. coli	8	0	0	0	9
E. faecalis	10	0	0	0	8
E. faecium	10	0	7	0	10
H. influenzae	8	0	0	0	8
K. pneumoniae	9	0	0	0	10
L. monocytogenes	9	0	7	0	9

M. luteus	14	0	8	0	12
MRSA	9	0	0	0	8
P. aeruginosa	0	0	0	0	0
S. enterica	10	0	0	7	11
S. aureus	8	0	0	0	8

The supernatant of *P. lusitanus* grown in each of these media was separated by HPLC (Figure 16). Also, the bioactivity of the supernatant of *P. lusitanus* grown in each of the above referred media was studied before and after the compound extraction with butanol, against *B. cereus*, *M. luteus* and *S. enterica*. The supernatant of *P. lusitanus* grown in TSB100% showed very low activity, only before compound extraction and only against *B. cereus*. The supernatants of PS25% and NaCl25% showed very low activity against the studied indicator strains, before and after the butanol extraction, with similar values to those obtained in the previous bioassay (Table 16), except that a decrease in activity was observed after compound extraction. On the other hand, the supernatant of *P. lusitanus* grown in PC25% showed good activity against the three indicator strains before and after solvent extraction. The HPLC fractions were collected at each minute (corresponding to 1mL) and their activity was tested against *B. cereus*, *M. luteus* and *S. enterica*. The fractions corresponding to the separation of the TSB100%, NaCl25% and PS25% supernatants showed no activity against the indicator strains, which was expected because in TSB100% the activity was very low before the compound were extracted and it completely disappeared after that. The fractions from the PC25% and TSB25% supernatants showed an activity similar to that already described in the previous sections (Table 12, Section 3). Therefore, the fractions 16 to 20 (t_R between 15 and 20 mins. presented higher activity against *B. cereus* and *M. luteus* (especially fraction 19) and lower activity against *S. enterica* (especially fraction 17).

When comparing the HPLC profiles obtained, we see that the compounds produced in TSB100% are practically the same that are produced in NaCl25%. The higher peaks in this run were observed between t_R= 5 and 15 mins.. These compounds are also present in the profile of PC25% and TSB25% supernatants, but at a much lower concentration. In PC25% and TSB25%, as also referred in the last sections, the predominant compounds elute after 14/15 min. Moreover, the peaks corresponding to pedopeptins were also present in PC25%. Curiously, in PS25% the profile seems to be a hybrid between the conditions before mentioned. In PS25%, high peaks were identified within the range of t_R from 5 to 16/17 min

of the run. However, the peaks corresponding mainly to pedopeptins were very low in PS25%, which justifies the absence of antibacterial activity.

5.1.1. Discussion

Growth conditions (temperature, pH, medium components) are very important when evaluating the production of antibacterial compounds, because these compounds are products of secondary metabolism of bacteria, and this metabolism is a response to the external environment of the producer (Bode & Müller 2005). Changing the culture conditions, and, more specifically, the media components, has long been a tool for the production of new compounds by bacteria, or to increase the production of given compounds (Bode & Müller 2005; Singh et al. 2017). When even small changes in the media components, such as the carbon (C) and nitrogen (N) sources can lead to changes in the production of secondary metabolites, this change becomes an interesting and easy way to elicit the production of different compounds (Bode & Müller 2005; Singh et al. 2017; Sánchez et al. 2010). Herein, it was demonstrated that high concentrations of peptone from casein in the growth media of *P. lusitanus* tremendously reduces the production of pedopeptins. Peptone from casein is a nitrogen source that contains a high level of free amino acids, particularly tryptophan. For actinobacteria, especially *Streptomyces* spp., it was already shown that the nitrogen starvation changes the metabolism of the antibiotic producer strains that induces the production of secondary metabolites and causes a deceleration of the primary metabolism (Martín et al. 2017). Therefore, it is possible that the biosynthesis of pedopeptins in *P. lusitanus* is coupled with the nitrogen metabolism, being repressed when nitrogen is abundantly available.

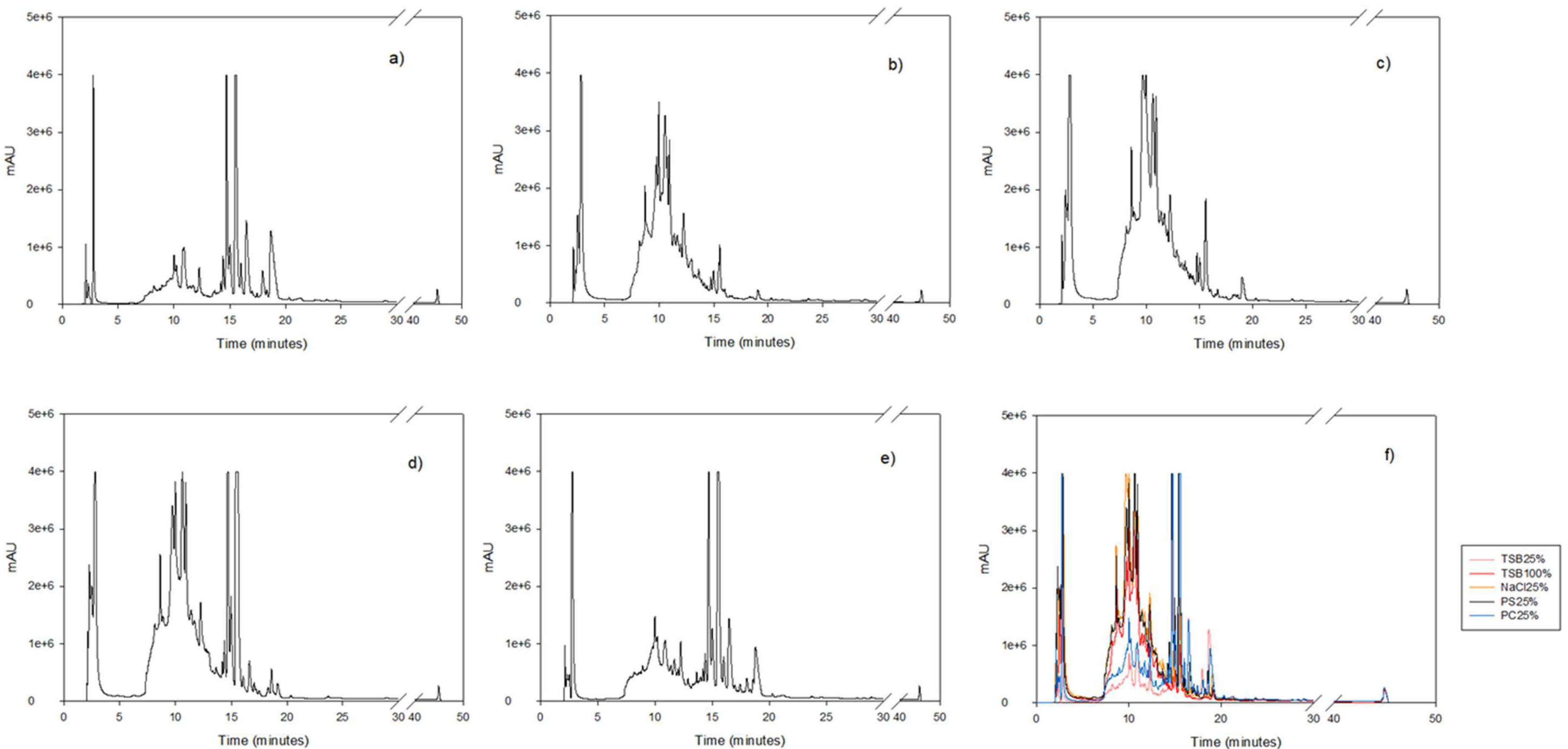

Figure 16 - Chromatograms resulting from the HPLC separation of the supernatants of *P. lusitanus* grown in TSB25% (a), TSB100% (b), NaCl25% (c), PS25% (d) and PC25% (e) media, and an overlap of all chromatograms (f). Detection was performed at 215nm.

5.2. Pedopeptin production: transcriptional analysis

5.2.1. Identification of the pedopeptin biosynthetic cluster

The pedopeptins were characterized by (Hirota-Takahata et al. 2014). However, their biosynthetic cluster was never described. Therefore, to confirm that the concentration of peptone from casein (PC) was repressing the production of pedopeptins, at the level of gene transcription, we proceeded to the identification of the genes that can be involved in their biosynthesis. With this purpose, the draft genome of *P. lusitanus* (Santos et al. 2015) was analysed with the antiSMASH (Medema et al. 2011) webtool and 18 biosynthetic clusters were identified (Table 17). This analysis led to the identification of two siderophore clusters, four lanthipeptide clusters, four NRPS clusters, one bacteriocin cluster and three polyketide clusters.

Table 17 - antiSMASH results of the draft genome of *P. lusitanus*

Cluster	Type	Contig	Location
1	Siderophore	4	20167-35828
2	T1pks-NRPS	6	30460-81104
3	T1pks-Otherks	6	90729-142079
4	Terpene-NRPS	7	1-54709
5	Siderophore	14	13004-27348
6	T3pks	16	1-40995
7	Lanthipeptide	17	10452-34741
8	NRPS	28	1-92230
9	Bacteriocin	30	1-10138
10	NRPS	31	18353-89241
11	NRPS	33	1-55660
12	Lanthipeptide	34	7417-43410
13	Lanthipeptide	36	15180-33038
14	Linaridin	48	30037-50585
15	Lanthipeptide	56	8696-24957
16	Other	81	1103-28956
17	NRPS	92	1-3284
18	NRPS	127	15756-85967

The NRPS of cluster 10 is located in contig 31 and has two subunits (NRPS1 and NRPS2), encoded by the genes TH53_08205 and TH53_08200, respectively (Figure 17, Table 18). Using the PKS/NRPS Analysis Web-site (Bachmann & Ravel 2009), it is possible to determine which amino acids are probably activated by each of the NRPS subunits. The subunit NRPS1 should code for the peptide sequence X-Phe-X-Thr-Leu-X-Leu and the subunit NRPS2 for X-Asp (Figure 13), where X represents a "no hit" result. As previously described, the following amino acids are present in the structure of the pedopeptins: Dap-Phe-Dab-Dhb-Leu-Dap-Leu-(β-OH)-Val-Asp (Figure 17). That being said, we can identify these NRPS subunits as those responsible for the production of pedopeptins, because the unidentified fragments are those of altered amino acids. The genetic environment of these NRPS was also studied, and a study of the transcriptome of *P. lusitanus* was also performed in parallel, but using the same samples as those that were used in the present study (Table 18).

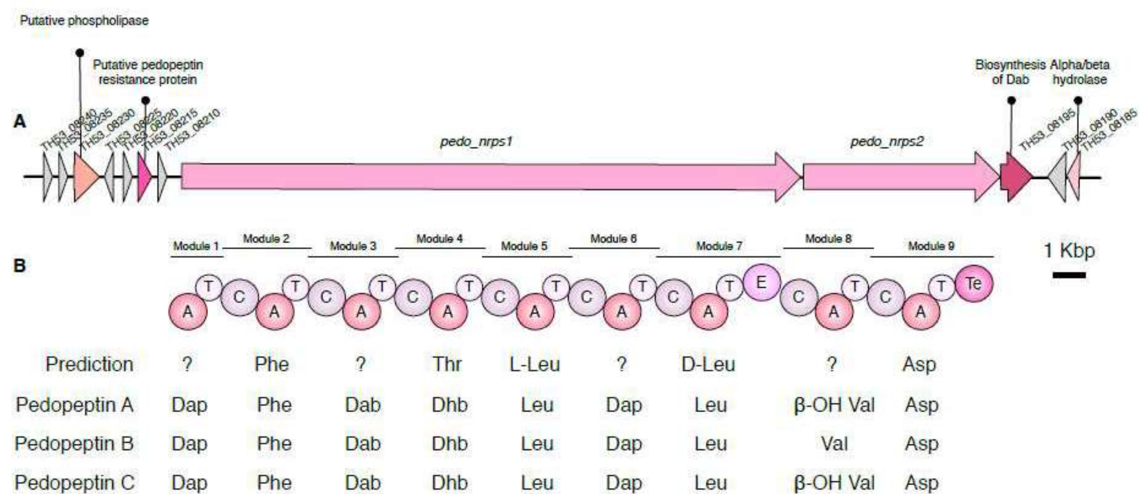

Figure 17 - A) Schematic representation of the biosynthetic cluster responsible for the production of the pedopeptins in *P. lusitanus*. Pedopeptins are produced by two NRPS subunits encoded by the *pedo_nrps1* and *pedo_nrps2* genes (TH53_08205 and TH53_08200 genes of the contig 31 of the draft genome of *P. lusitanus*, respectively). The other genes in this cluster have different activities, including the biosynthesis of the diaminopropionic acid (Dap) present in the pedopeptin, and phospholipase activity, possibly responsible for the addition of the fatty acid to the peptide. A further analysis of this cluster can be seen in Table 18.
B) Schematic representation of the NRPS modules responsible for the production of pedopeptins, and prediction of the amino acids encoded by them.

Table 18 - Description of the genes of the biosynthetic cluster of the pedopeptins in *P. lusitanus*. This cluster includes several genes that encode proteins involved in the synthesis of the pedopeptins. Also, transcriptome analysis of the RNA extracted from *P. lusitanus* grown in PC25% and TSB100% shows a differential expression of certain genes in this cluster, including a 23-fold higher expression of *pedo_nrps1*, and 9-fold higher expression of *pedo_nrps2*, in PC25% (unpublished).

Gene	Protein	Putative function	RNA-seq
TH53_08240	Hypothetical protein	No conserved domains detected	
TH53_08235	Patatin-like phospholipase	Putative cleavage of fatty acids from membrane lipids	
TH53_08230	Hypothetical protein	No conserved domains detected	
TH53_08225	Hypothetical protein	No conserved domains detected	
TH53_08220	Hypothetical protein	Disrupted – it is possible that TH53_08220 and TH53_08215 encode a unique protein	-2
TH53_08215	Glyoxalase/Vicinal oxygen chelate family	Pedopeptin resistance protein	
TH53_08210	Hypothetical protein	No conserved domains detected; Due to the proximity to *pedo_nrps* genes, it should be involved in the biosynthesis of pedopeptins	-19
TH53_08205	Nonribosomal peptide synthetase	Synthesis of pedopeptins	-23
TH53_08200			-9
TH53_08195	L-diaminobutyric acid transaminase	Putative transamination of aspartate semialdehyde (primary metabolite) to form 2,4-Dab; Synthesis of the pedopeptin monomer Dab	-5
TH53_08190	Hypothetical protein	No conserved domains detected	
TH53_08185	Alpha/beta hydrolase	The alpha/beta hydrolase fold includes proteases, lipases, peroxidases, esterases, epoxide hydrolases and dehalogenases.	

5.2.2. Influence of the concentration of PC in the expression of *pedo_nrps* genes

As seen in section 5.1, the antibacterial activity of *P. lusitanus* is very low when the bacterium is grown in TSB100% and it is higher when cells are grown in PC25%. To understand if the effect of the PC concentration was in the expression of pedopeptins, the expression of the

pedo_nrps genes in both these conditions was determined by RT-qPCR absolute quantification.

After studying the genetic expression of both *pedo_nrps* genes by RT-qPCR and calculating their copy number using the absolute quantification method described by Whelan *et al.* (Whelan et al. 2003) it was found that both genes are expressed in low quantity when *P. lusitanus* is grown in TSB100%, when compared with the values of the bacterium grown in PC25%. For *pedo_nrps1* the copy number in PC25% is more than 10 times larger than in TSB100% and for *pedo_nrps2* this difference is smaller but the copy number in PC25% is still almost three times higher than in TSB100% (Table 19).

Table 19 - Results of the RT-qPCR absolute quantification of the NRPS genes *pedo_nrps1* and *pedo_nrps2* presented as gene copy number.

		Gene Copy Number	
		PC 25%	TSB 100%
Genes	*pedo_nrps1*	$1,75 \times 10^4$	$1,78 \times 10^3$
	pedo_nrps2	$4,68 \times 10^4$	$1,71 \times 10^4$

5.3. Discussion

The antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) webtool allows for the identification of biosynthetic clusters that include all known secondary metabolite classes (PKs, NRPs, terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, nucleosides, beta-lactams, butyrolactones, siderophores, melanins and others) (Medema et al. 2011).

During the identification of the biosynthetic clusters of *P. lusitanus* we observed a total of 18 clusters, which reveals the high potential of this bacterium to produce secondary metabolites.

A comparison of the results of the PKS/NRPS Analysis Web-site (Bachmann & Ravel 2009) with the structure of the pedopeptins already studied (Hirota-Takahata et al. 2014), allows us to conclude that pedopeptins are non-ribosomal peptides, synthetized in *P. lusitanus* by a NRPS made up of two subunits expressed by the *pedo_nrps* genes, *pedo_nrps1* (gene

TH53_08205 of the contig 31 of the draft genome of *P. lusitanus*) and *pedo_nrps2* (gene TH53_08200 of the contig 31 of the draft genome of *P. lusitanus*). This is the first time pedopeptins are described as NRPs.

The higher expression of the *pedo_nrps* genes in PC25% grown *P.* lusitanus proves that the concentration of PC is critical for the production of pedopeptins by this bacterium. There can be a number of reason for this effect, including the high amino acid content of peptone from casein, the changes in nitrogen concentration or the alterations to the pH due to these changes.

The differences are not very pronounced, but that is one factor that may be very important in this difference. The RNA used in the study is extracted when the OD_{600} of *P. lusitanus* is at 0.500. This value is very low, and at this point the bacterium is still at a very early growth phase, and, as showed in the present work (section 1.2 of Results and Discussion) not at its full antibacterial activity.

These values are further confirmed by the transcriptome analysis of *P. lusitanus* grown in PC25% and TSB100%, as shown in Table 18. In this analysis (data not published), the expression of the *pedo_nrps* genes in PC25% was found to be higher than in *P. lusitanus* grown in TSB100%, especially for the *pedo_nrps1* gene.

Conclusions and Future Perspectives

This study described for the first time the antibacterial activity of *P. himalayensis* MTCC 6384^T, *P. cryoconitis* DSM14825 and *P. hartonius* DSM 19033^T in agar media. However, using the equivalent broth media, no antibacterial activity was observed, or, for *P. himalayensis* MTCC 6384^T, that activity was very low. In future studies, it would also be interesting to investigate this activity further, especially by testing other temperatures, different broth media, and identify the compounds responsible for it.

The antibacterial activity of *P. lusitanus* NL19 was further described, and compared between different solid and liquid media. Bioactivity was found to be mainly due to the production of pedopeptins. Pedopeptins are a mixture of three structurally identical peptides produced by a *Pedobacter* spp. that are lipopolysaccharide (LPS) inhibitors. Here, we suggest, for the first time, that pedopeptins are nonribosomal peptides. The nonribosomal peptide synthetase genes putatively involved in their biosynthesis were identified in the genome of *P. lusitanus* NL19 (*pedo_nrps1* and *pedo_nrps2*). As described before, *P. lusitanus* NL19 encodes a high number of clusters associated with the production of various natural products. Therefore, it would be interesting to test different media components and culture conditions to i) potentiate the production of pedopeptins and ii) elicit the production of other compounds of interest.

When investigating the antibacterial activity of NL19, it was found that antibacterial activity is detected when *P. lusitanus* NL19 grows in TSA media, but not in TSB (TSB100%). Curiously, dilution of this broth resulted in the production of antibacterial compounds. We further analysed the consequences of the reduction of the TSB components to 25% and found out that the high concentration of peptone from casein (PC; 15 g/L) in the growth media represses the production of pedopeptins. Nevertheless, when this concentration was decreased to 3.75 g/L (25% of the initial concentration) production was restored. This effect was proved to be due, at least, to the decrease in the expression levels of the pedopeptins synthetases genes (*pedo_nrps1* and *pedo_nrps2)*. Since PC is mainly a nitrogen source, it is possible that the synthesis of pedopeptins production is metabolically coordinated with the uptake of nitrogen. This can be further confirmed by studying the production of pedopeptins in minimal medium with different concentrations of nitrogen.

Finally, it would also be interesting to further investigate and explore other bioactivities of *P. lusitanus* NL19, such as, for instance, its antifungal and antitumor activity. Moreover, given the characteristics of the environment from which it was isolated, it would be it will be worth analysing its bioactivity in media supplemented with metals.

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