Universidade de Aveiro
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Departamento de Química

Liliana de Almeida Dias

Pesquisa de novos lantibióticos

Search for new lantibiotics

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, Ramo de Biotecnologia Molecular, realizada sob a orientação científica da Doutora Sónia Mendo, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e da Doutora Tânia Caetano, Investigadora em Pós-Doutoramento no Departamento de Biologia da Universidade de Aveiro

Dedicada aos meus queridos pais, Graça e Henrique, pelo constante apoio e motivação.

o júri

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palavras-chave

Lantipéptidos, lantibióticos, bacteriocinas, péptidos antibacterianos, minas de urânio, minas de ferro, grutas, *genome mining*

resumo

A procura de microrganismos produtores de novos compostos antimicrobianos é primordial no combate ao fenómeno mundial de resistência microbiana face aos antimicrobianos atualmente disponíveis. A natureza é uma fonte de diversos produtos, entre os quais se podem destacar os antimicrobianos. Os péptidos naturais podem ser sintetizados *in vivo*, por bactérias, através de vias metabólicas ribossomais ou não ribossomais. De destaque entre os péptidos antimicrobianos ribossomais produzidos por bactérias são as bacteriocinas. As bacteriocinas de classe I incluem os péptidos com modificações pós-traducionais, os lantipéptidos, que são caracterizados pela presença dos aminoácidos lantionina e metil-lantionina. Os lantipéptidos com atividade antimicrobiana designam-se lantibióticos. Estes, são capazes de inibir o crescimento de várias bactérias de Gram-positivo clinicamente relevantes como, por exemplo, *Staphylococcus aureus* resistente à meticilina. O trabalho desenvolvido teve como objectivo a procura/ pesquisa de novos antibacterianos, produzidos por bactérias isoladas em ambientes pouco comuns, nomeadamente, grutas e locais muito contaminados por metais pesados - minas de urânio e de ferro.

Para tal, testou-se a atividade antibacteriana de 76 bactérias isoladas nos diferentes locais contra 12 estirpes indicadoras, de Gram-positivo e de Gram-negativo. Todos os isolados que apresentaram atividade foram classificados por sequenciação do gene 16S rRNA. Dentro deste grupo, foram selecionadas bactérias pertencentes aos filos Firmicutes e Actinobacteria para a realização de ensaios de produção de antimicrobianos em cultura líquida. De seguida, determinou-se a estabilidade proteolítica e térmica dos antimicrobianos produzidos por três estirpes de *B. amyloliquefaciens* (SL8, Sma1 e MO15). Atualmente, existem e estão depositados nas bases de dados 13 genomas de *B. amyloliquefaciens* totalmente sequenciados. Assim, e utilizando a plataforma antiSMASH 2.0 procedeu-se à identificação de possíveis clusters de lantibióticos nesses genomas. Os genes biossintéticos de lantibióticos foram identificados apenas em três desses genomas; em dois desses genomas detectou-se o cluster de genes do lantibiótico mersacidina e no outro detectou-se a presença de um cluster ainda não caracterizado. Com base nessa informação, investigou-se a presença de genes característicos de operões biossintéticos de lantipéptidos nas estirpes SL8, Sma1 e MO15. O gene estrutural da mersacidina foi detectado nos três isolados. No entanto, o gene *mrsM*, que codifica para a enzima de modificação da mersacidina, apenas foi identificado nas estirpes SL8 e Sma1. Por outro lado, a amplificação de outra *lanM* não foi possível no isolado MO15. Foi ainda pesquisada a presença do péptido mersacidina, nos sobrenadantes das culturas líquidas destas estirpes, por MALDI-TOF/MS. Contudo, este lantibiótico não foi detectado em nenhum dos sobrenadantes.

O presente estudo abre perspectivas para a identificação de lantibióticos nas estirpes em estudo. Por outro lado, outros estudos serão realizados envolvendo a pesquisa e caracterização dos compostos produzidos pelas restantes estirpes da coleção e que não foram exploradas nesta tese.

keywords

Lanthipeptides, lantibiotics, bacteriocins, antibacterial peptides, uranium mines, iron mines, caverns, *genome mining*

abstract

Searching for new antimicrobials is crucial to address the phenomenon of microbial resistance to the most common antibiotics. Nature is a source of several products, namely antimicrobials. Natural peptides can be synthetized *in vivo* by bacteria through a nonribosomal or a ribosomal pathway. Among the ribosomal antimicrobial peptides produced by bacteria, the so-called bacteriocins are worth attention. Among these, the class I bacteriocins comprise the post-translationally modified peptides, designated lanthipeptides, which are characterized by the uncommon amino acids lanthionine and methyllanthionine. The lanthipeptides with antimicrobial activity are referred as lantibiotics. The lantibiotics are able to inhibit the growth of several clinically relevant Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus*. This work aimed to search for new antibacterials produced by microorganisms isolated from uncommon sources, namely, caverns and heavy metal contaminated sites – uranium and iron mines.

To that end, the antibacterial potential of 76 bacterial isolates from the different origins was investigated against 12 indicator strains, both Gram positive and Gram negative. All the isolates with antibacterial activity were affiliated by 16S rRNA gene sequence. Among these, bacteria belonging to the phyla Firmicutes and Actinobacteria were selected and included three *Bacillus amyloliquefaciens* - SL8, Sma1 and MO15. These isolates were investigated for antibacterial production assays in liquid culture. Supernatants with bioactivity were further investigated for proteolytic and temperature stability of the antimicrobial compounds produced. Presently, 13 genomes *B. amyloliquefaciens* are fully sequenced and assembled. Using the antiSMASH 2.0 platform putative clusters for lanthipeptides were surveyed in all of these genomes and were found on three of the genomes only. Two of these clusters corresponded to the mersacidin and the other was uncharacterized. Thus, the presence of genes involved in the biosynthesis of lantibiotics was investigated in the three strains selected. It was found that SL8, Sma1 and MO15 isolates contain the mersacidin structural gene. However, the *mrsM* gene, which encodes the mersacidin modification enzyme, was only amplified in SL8 and Sma1 strains. Moreover, no other *lanM* was identified in the MO15 isolate. Therefore, the presence of the mersacidin peptide in culture supernatants was investigated by MALDI-TOF/MS. However, this lantibiotic was not detected in any of the three supernatants.

The present work opens perspectives for the identification of lantibiotics produced by the strains studied. Also, other studies will be carried out to characterize the peptides produced by all the isolates of the bacterial culture collection constructed in the present study.

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1 Introduction

1.1 Natural products as a source of new antimicrobials

Natural products are molecules derived from living organisms/natural sources that since early had incited investigators attention due to its useful applications in human welfare (Clardy and Walsh, 2004; Koehn and Carter, 2005). These biomolecules produced by plants, bacteria and fungi, have played important roles over the past century on medicine and front-line drug development, as they are a substantial source of bioactive compounds with pharmaceutical properties (Arnison *et al.*, 2013; Koehn and Carter, 2005; Pickens *et al.*, 2011). More specifically, natural products represent a pharmaceutically important class of compounds, that are sources of new therapeutic agents for bacterial and fungal infectious diseases, cancer and lipid disorders (lowering cholesterol levels) (Clardy and Walsh, 2004; Newman and Cragg, 2012).

All the natural products are secondary metabolites that are produced by different species (not essential for growth) showing a wide range of chemical structures. Moreover, secondary metabolites are related to the ecology of the producing organisms, encompassing the interactions between the local communities, thus they can be considered “molecules of adaptation” (Pickens *et al.*, 2011; Verpoorte, 1998).

Antibiotics are low-molecular-weight organic molecules produced by microorganisms that are particularly important for humankind (Demain, 1999). It is assumed that the antibiotic era started in 1928 with the discovery of penicillin by Alexander Fleming, when the first step for one of the great discoveries of the 20th century was given (Hassan *et al.*, 2012; Stokes and Gillings, 2011). However, antibiotics’ golden era lasted less than 50 years (Jan, 2003; Overbye and Barrett, 2005). During this period, antibiotics were used massively in a global scale. Antibiotics’ targets are bacteria, which are known to adopt different strategies of antibiotic resistance, triggered by natural selection (Högberg *et al.*, 2010; Livermore, 2003). Some of these mechanisms are the horizontal gene transfer, responsible for de acquisition of heterologous resistance (Lupo *et al.*, 2012); and spontaneous mutations in the gene encoding the antibiotics’ target protein, which decreases antibiotics affinity (Hassan *et al.*, 2012; Overbye and Barrett, 2005). Antibiotic-resistant genes can also degrade the antibiotic enzymatically or exclude it out of the cell by a mechanism of transport (Hassan *et al.*, 2012). The fact that these genes can be located in integrons and in mobile genetic elements such as plasmids and transposons, also enhance their dissemination (Livermore, 2003).

Owing to the emergence of this bacterial multiresistance phenomenon, bacterial infections considered controlled in the past, do not respond now to the typical treatments (Levy and Marshall, 2004). It has been considered a serious worldwide public health problem, leading scientific efforts to the discovery of new antimicrobials efficient against clinically relevant pathogenic microorganisms (Overbye and Barrett, 2005; Shenkarev *et al.*, 2010).

During the 20th century, many classes of natural products were discovered (Arnison *et al.*, 2013). Among them, it is important to highlight a class of natural products produced by bacteria: the peptides with antimicrobial activity (Hassan *et al.*, 2012). The biosynthesis of these peptides can be ribosomal (in which they can undergo post-translational modifications by biosynthetic enzymes) or independent of ribosomal translation (Figure 1). The bacterial peptides originated by the first pathway are called bacteriocins and the last ones are referred as nonribosomal peptides (NRP) (Nolan and Walsh, 2009).

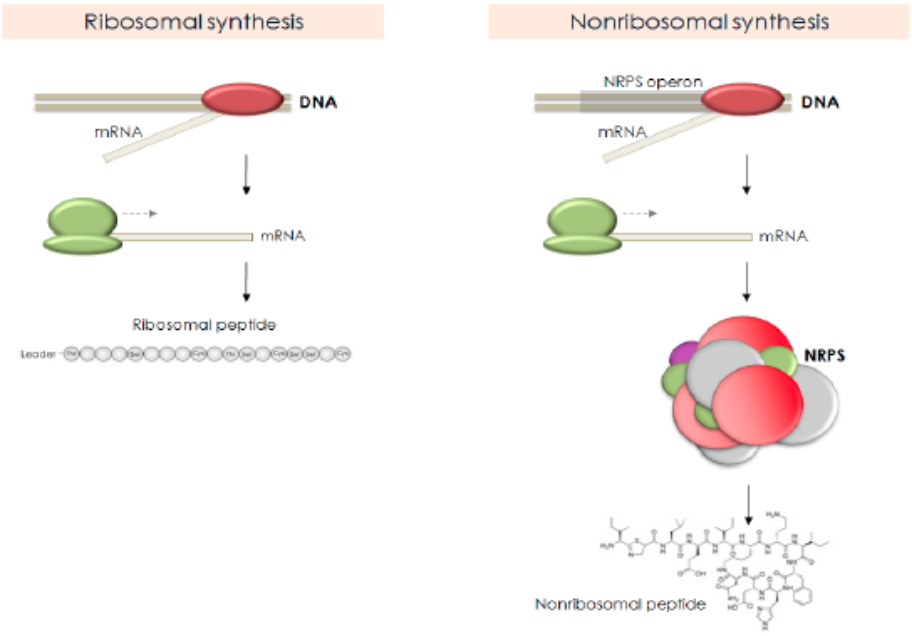

Figure 1 – Schematic representation of the ribosomal and nonribosomal biosynthetic peptide pathways (Caetano, 2011).

1.2 Nonribosomal peptides produced by bacteria

The traditional peptide antibiotics are produced by the nonribosomal biosynthetic pathway through the action of multi-enzyme complexes designated as nonribosomal peptide synthetases (NRPS) (Nes *et al.*, 2007). NRPS have a modular organization in which each module is responsible for the incorporation of one amino acid into the growing polypeptide chain. The modules are organized in domains, which are the catalytic enzymatic units of the individual NRP synthesis step. At least, each NRPS module comprises three different domains: the adenylation (A) domain that selects and activates the cognate amino acid as aminoacyl adenylate with ATP consumption; the peptidyl carrier protein (PCP) domain, which accepts the previously activated amino acid and transports it through the NRPS catalytic units; the condensation (C) domain, which catalyzes the formation of the peptide bond. A thioesterase (TE) domain is present at the termination module, which is responsible for releasing the final peptide from the NRPS (Figure 2) (Caetano, 2011; Finking and Marahiel, 2004; Schwarzer *et al.*, 2003). The NRPS domains can be identified at the protein level by characteristic, highly conserved sequence motifs designated as core-motifs (Schwarzer *et al.*, 2003).

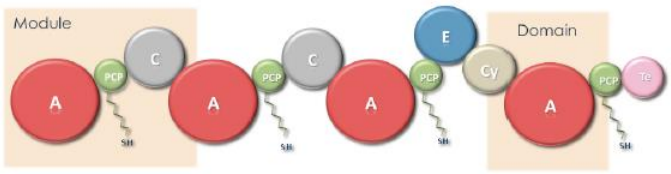

Figure 2 – Schematic representation of the NRPS' modular organization (Caetano, 2011).

The NRPS are used concurrently as template (since the determination of the amino acid to be incorporated is performed by the module) and biosynthetic machinery (all necessary catalytic functions are ensured by the module) (Finking and Marahiel, 2004). The NRP can be modified by tailoring enzymes, including glycosyl transferases, halogenases and hydroxylases, which are usually encoded in the same biosynthetic gene cluster of the NRPS enzymes. This remarkable structural modifications lead to potent antibiotic activity, characteristic of the NRP (Nolan and Walsh, 2009; Walsh *et al.*, 2001).

Some NRP antibiotics can be used in the treatment of bacterial infections in humans, such as: penicillin, produced by the fungus *Penicillium chrysogenum* and *Emericella nidulan* (Brakhage *et al.*, 2005); cephalosporin, produced by the fungus *Acremonium chrysogenum* (cephalosporin C) and by some Gram negative and positive

bacteria (Brakhage *et al.*, 2005); vancomycin, produced by *Amycolatopsis orientalis* (Moellering and Ferraro, 2012) and daptomycin, from *Streptomyces roseosporus* (Figure 3) (Steenbergen *et al.*, 2005). Furthermore, the NRPS gene cluster can be manipulated to allow the generation of new drugs (Fortman and Sherman, 2005; Zhang *et al.*, 2009).

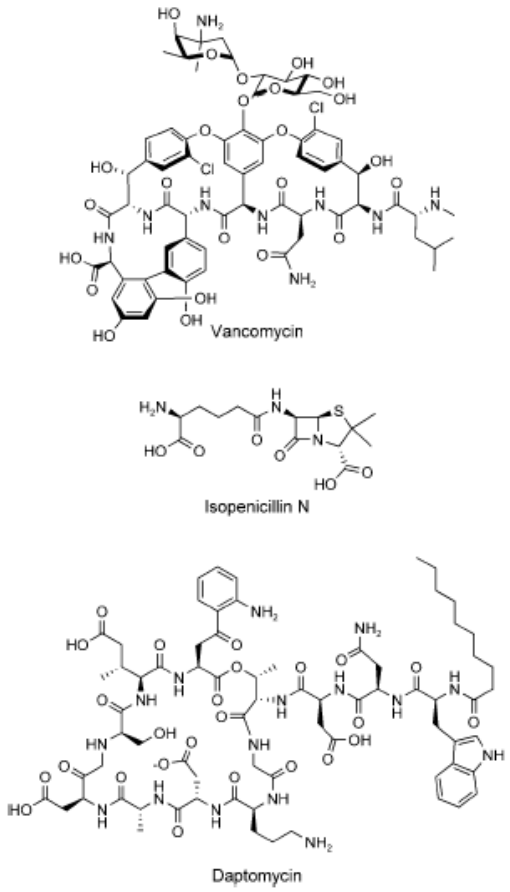

Figure 3 – Representation of examples of nonribosomal synthesized antibiotic peptides (Nolan and Walsh, 2009).

1.3 Bacteriocins: ribosomally synthesized antimicrobial peptides from bacteria

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various bacteria, which inhibit closely related or more distant species, acting as a bactericide or bacteriostatic agent (Begley *et al.*, 2009; de Jong *et al.*, 2006; Teng *et al.*, 2012). The mechanism of action of Gram-positive bacteriocins is quite effective since they are potent at pico- to nanomolar concentration (though its action spectra is limited at these concentrations) and specific, usually consisting on pore formation and/or inhibition of cell wall synthesis in target cells (Nissen-Meyer and Nes, 1997). These characteristics make bacteriocins promising antimicrobial agents suitable for being used as food preservative and infection treatment due to its competence in controlling antibiotic-resistant pathogens (Hassan *et al.*, 2012). Gram-positive bacteria produce bacteriocins smaller than 6 kDa, while bacteriocins produced by Gram-negative bacteria are larger than 20 kDa, being colcidin V and microcins exceptions, since they are smaller than 10 kDa (Nissen-Meyer and Nes, 1997). It is also known that these antimicrobial peptides produced by Gram-positive bacteria are non-toxic to eukaryotic cells and have broader spectra of action when compared to bacteriocins of Gram-negative bacteria (Nes *et al.*, 2007).

Initially, bacteriocins were classified into two main classes according to their post-translational modifications: the class I, that comprises the modified bacteriocins or lantibiotics and the class II, that represents the unmodified bacteriocins (Nissen-Meyer and Nes, 1997). Currently, the bacteriocins are classified into five different classes based on various characteristics such as the producer microorganisms, mode of action, molecular weight, physical proprieties, chemical structures and specific antibacterial activity (de Jong *et al.*, 2006):

Class I: post-translationally modified bacteriocins, the lantibiotics;

Class II: non-modified heat stable bacteriocins;

Class III: non-modified large heat-labile bacteriocins;

Class IV: non-modified complex bacteriocins carrying lipid or carbohydrate moieties;

Class V: non-modified circular bacteriocins.

This thesis will focus on class I, bacteriocins highly post-translationally modified and containing unusual amino acids in their composition - the lantibiotics (Guder *et al.*, 2000).

1.4 Lantibiotics

The interest on lantibiotics started in 1928, when nisin was first reported by presenting activity against *Streptococcus lactis* (now designated as *Lactococcus lactis*) in fementation cultures (Rogers and Whittier, 1928). During the last 60 years, nisin has been used commercially as food preservative (Cotter, 2012), since it presents activity against many pathogenic bacteria. Probably, the success of nisin in this area, along with its low stability at physiological pH, restricted the use of other lantibiotics in other fields such as the clinical setting (Cotter *et al.*, 2005). However, the urgent need to find new and potent antibiotics has renewed the interest on these compounds as antibacterial drugs with broad clinical potential (Müller *et al.*, 2012). Lantibiotics can be potently active against several clinically relevant Gram-positive bacteria, such as antibiotic-resistant strains of *Staphylococcus,* including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus*, *Enterococcus*, including vancomycin-resistant enterococci (VRE), *Clostridium* and oxacillin-resistant Gram positives, as well as against Gram-negative pathogens, such as *Neisseria* and *Helicobacter* (Cotter *et al.*, 2005; Piper *et al.*, 2009).

Lantibiotics have various advantages when compared to the classical antibiotics: they are far more effective since their usual target is the essential cell wall precursor lipid II; their ribosomal (gene encoded) origin makes them more liable to bioengineering, leading to the creation of new lantibiotics derivatives with improved competences (Breukink and de Kruijff, 2006; Field *et al.*, 2010). Although the development of resistance to lantibiotics is still unknown and the emergence of resistant bacteria is still a possibility, that might be minimized by peptide engineering and by a comprehensive study about lantibiotic mechanisms (Cotter *et al.*, 2012; Shenkarev *et al.*, 2010).

Nowadays, there are various members of this family in clinical development, including duramycin for the treatment of cystic fibrosis (Grasemann *et al.*, 2007) and a derivative of actagardine for *Clostridium difficile* infections (Knerr and van der Donk, 2012). Moreover, the lantibiotic mutacin 1140 is presently in preclinical development for the treatment of infections caused by Gram-positive bacteria (Ghobrial *et al.*, 2010). Some other lantibiotics' applications are being developed, including in agriculture and veterinary medicine (Cotter *et al.*, 2012) and in molecular imaging (Zhao *et al.*, 2008).

The term lantibiotics derived from lanthionine (Lan)-containing antibiotics and this is a promising class of new antibacterial agents, which are small (< 5 kDa) bioactive polycyclic peptides characterized by the presence of uncommon amino acids such as thioether-linkage-containing *meso*-lanthionine (Lan) and *(2S,3S,6R)*-3-methyllanthionine (MeLan), as well as by various unsaturated amino acids such as 2,3-didehydroalanine

(Dha) and (*Z*)-2,3-didehydrobutyrine (Dhb) (Figure 4) (Nolan and Walsh, 2009; Teng *et al.*, 2012).

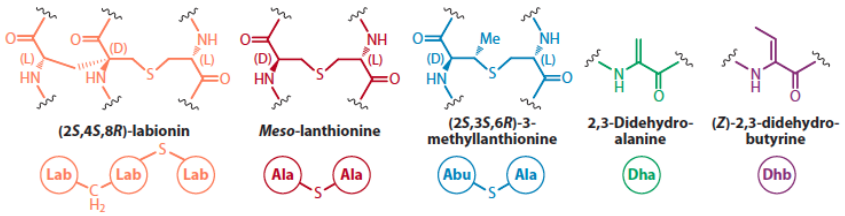

Figure 4 – Representation of the usual lantibiotic post-translational modifications. Abbreviations: Abu, 2-aminobutyric acid; Dha, 2,3-didehydroalanine; Dhb, (*Z*)-2,3-didehydrobutyrineM Lab, labionin (Willey & van der Donk, 2007).

Lantibiotics undergo post-translational modifications performed by lantibiotic synthetases, which are enzymes responsible for the formation of the uncommon amino acids mentioned before. These enzymes are notable catalysts that use macrocyclization as a critical strategy that allows an increased proteolytic and heat denaturation stability and also a better affinity of the peptides to their targets (Knerr and van der Donk, 2012; Willey and van der Donk, 2007). The first post-translational side-chain modifications are performed through the dehydration of serine and threonine residues. In this step, phosphorylation of the side-chain hydroxyl moieties of those amino acids occurs, which allows the cleavage of the Cβ−O bonds by the elimination of the phosphate group (Figure 5). This results in the conversion of serine and threonine residues into Dha and Dhb, respectively. Subsequently, a cyclase catalyses the intermolecular cyclization via a Michael-type addition: the dehydrate Dha and Dhb residues are intramolecularly and regioselectively captured by the side-chain thiol groups of the cysteine residues, establishing the thioether bridges Lan and Melan, respectively (Figure 6) (Knerr and van der Donk, 2012; Nolan and Walsh, 2009).

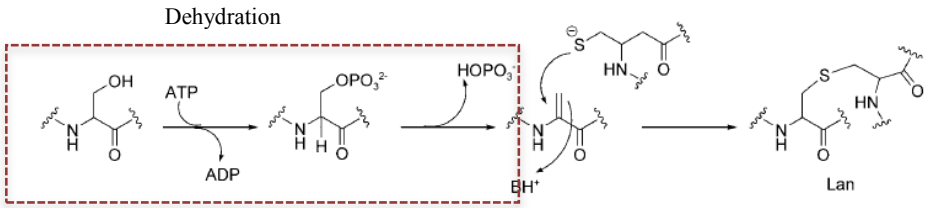

Figure 5 - Representation the mechanism of dehydration in detail. Adapted from (Nolan and Walsh, 2009).

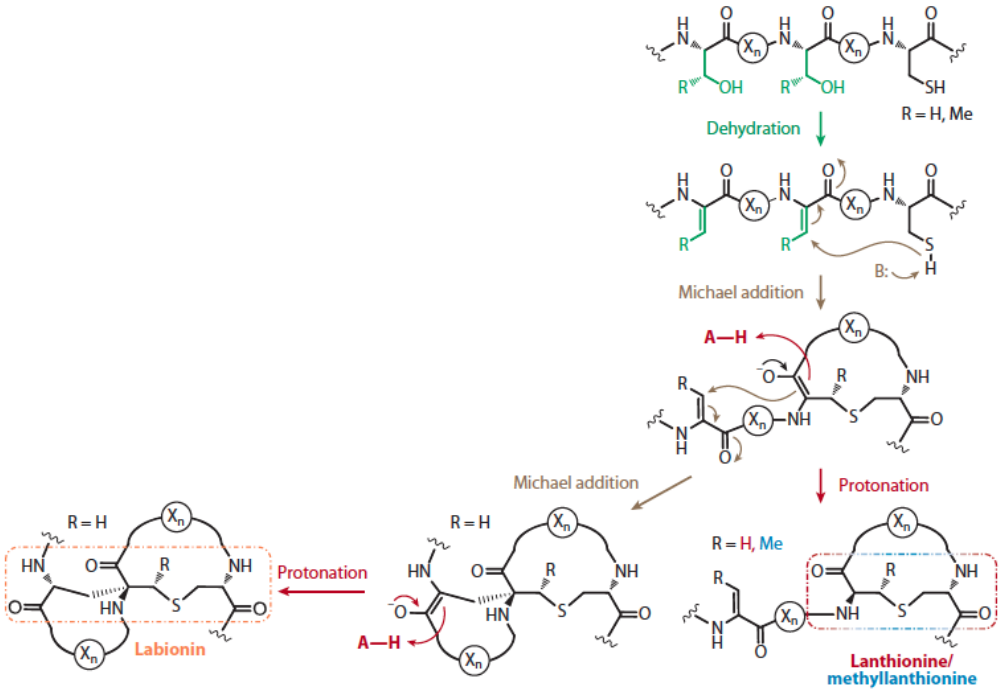

Figure 6 - Representation of the mechanism of Lan, MeLan and Lab (labionin) thioether cross-link formation, which are usual in mature lantibiotics (Willey and van der Donk, 2007).

In general, lantibiotics are synthesized as a precursor peptide, the biologically inactive linear peptide with no amino acid modification that will yield the mature and active peptide. The precursor peptide comprises two regions: the N-terminal leader sequence (with 23-59 amino acids) and the C-terminal core peptide (Figure 7). The post-translational modifications occur only in the core peptide of the precursor peptide (Nolan and Walsh, 2009; Teng *et al.*, 2012; Willey and van der Donk, 2007). The leader peptide is an essential recognition element for the post-translational modification enzymes, immunity (as a self-protective mechanism) and export signaling (Oman and van der Donk, 2010). Usually, the leader peptide is cleaved off by a specific protease during or after the exportation process through an ATP-binding cassette (ABC) transporter and the lantibiotic becomes biologically active (Figure 7) (Bierbaum and Sahl, 2009).

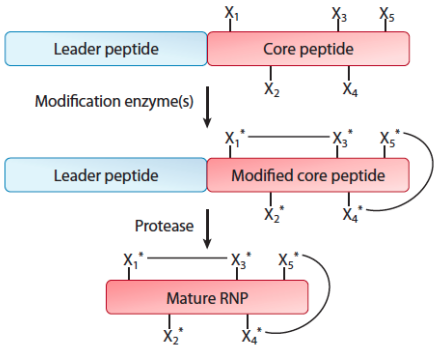

Figure 7 – General representation of ribosomally synthesized natural products (RNP) biosynthesis, common in lantibiotics biosynthesis. X_n represents a modified residue (Knerr and van der Donk, 2012).

The genes involved in their biosynthesis are organized in clusters and encode information for the precursor peptide and all the enzymes responsible for modification (proteases and ABC transporters). Immunity factors and regulatory proteins might also be present in the lantibiotic gene cluster (Sahl and Bierbaum, 1998). These genes were first attributed with the generic designation *lan* followed by a capital letter. However, this designation can be replaced by a more specific nomenclature according to each lantibiotic genotype (e.g. *nis* for nisin, *ltn* for lacticin 3147, *mrs* for mersacidin, *cin* for cinnamycin, *lch* for lichenicidin). Considering the general nomenclature, the precursor peptide is encoded by the structural gene *lanA*, common to all gene clusters; the genes *lanB*, *lanC*, *lanD*, *lanM* and *lanJ* codify for the enzymes required for the post-translational modifications, *lanP* and *lanT* are responsible for proteolytic processing and *lanT* for the peptide transportation. Genes involved in regulation (*lanR*, *lanK*, *lanQ* and *lanX*) and in immunity (*lanI*, *lanH* and *lanEFG*) (Figure 8) (Guder *et al.*, 2000; Nagao *et al.*, 2006) are also present in the gene cluster. These gene clusters can be located on the chromosome of the producer (e.g. subtilin, mersacidin) or on mobile elementes such as conjugative transposons (e.g. nisin) or plasmids (*e.g.* epidermin, lacticin 481, lacticin 3147) (Chatterjee *et al.*, 2005b; Nagao *et al.*, 2006).

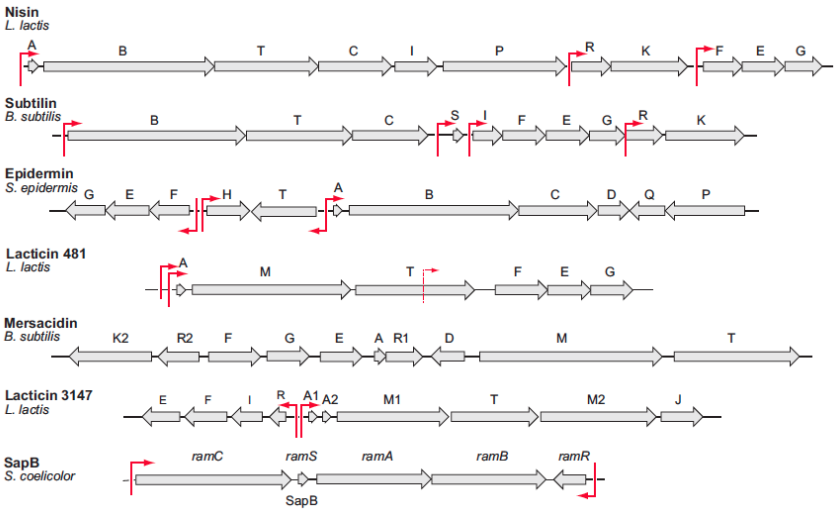

Figure 8 – Representative biosynthetic gene clusters of lantibiotics (Willey and van der Donk, 2007).

In the last years, peptides without antibiotic activity, but possessing a biosynthetic strategy and structure similar to lantibiotics were discovered. As so, lantibiotics were integrated into a broader family of compounds named lanthipeptides (Goto *et al.*, 2010). The non-lantibiotic lanthipeptides have different functions, including biosurfactant activity by decreasing the surface tension at the colony-air interface. This promotes the growth of vegetative hyphae during the sporulation of the producer bacteria (Knerr and van der Donk, 2012; Kodani *et al.*, 2004; Kodani *et al.*, 2005; Willey and van der Donk, 2007). Other example is labyrinthopeptin A2, which demonstrated a notable efficacy against neurophatic pain in an *in vivo* mouse model, being the first lanthipeptide discovered with this propriety (Knerr and van der Donk, 2012; Müller *et al.*, 2010). This lanthipeptide is also characterized by the presence of labionin (Lab) (Figure 4, Figure 6, Figure 9), which is a carbacyclic, post-translationally modified amino acid resultant from the activity of the enzyme LabKC, in the motifs Ser-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Cys existent in the corresponding core peptides (Field *et al.*, 2010; Meindl *et al.*, 2010; Müller *et al.*, 2010).

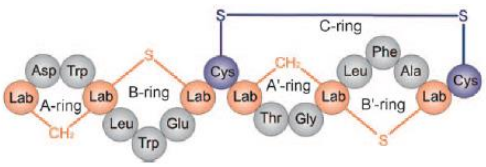

Figure 9 – Structure of the labyrinthopeptin A2, a type-III lantibiotic (Müller *et al.*, 2010).

1.4.1 **Classification of lantibiotics**

Lanthipeptides are classified into four different classes based on their biosynthetic machinery (Figure 10) (Knerr and van der Donk, 2012). However, only the lanthipeptides of classes I and II display antibacterial activity (the lantibiotics), which are the main focus of this thesis. Thus, these two classes will be discussed in more detail in the following sections.

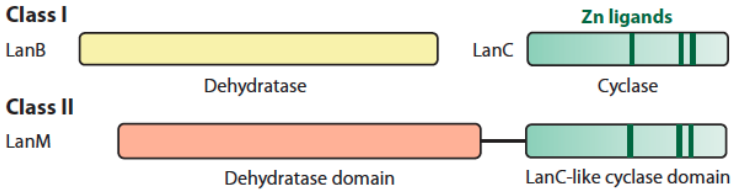

Figure 10 - Representation of the two classes of lantibiotics based on the lanthionine-generating enzymes (Knerr and van der Donk, 2012).

1.4.1.1 Class I

The lantibiotics of class I are post-translationally modified by two different enzymes: the dehydratase LanB and the cyclase LanC (Figure 10) (Knerr and van der Donk, 2012). The most intensively studied class I lantibiotic is nisin, which has been used as food preservative during the last 60 years (Cotter, 2012). Nisin has important physiochemical properties such as the resistance to an acidic pH and high temperatures (Nagao, 2009). The study of its structure, biosynthetic gene cluster and mechanism of action provided a paradigm for the elucidation of these features on others lantibiotics (Nolan and Walsh, 2009), promoting protein engineering (Müller *et al.*, 2012).

The lantibiotic nisin is a flexible molecule with two amphiphilic domains, which consist of three lanthionine rings on its N-terminal (the A, B and C rings), two lanthionine rings on the C-terminal (the D and E rings) (Figure 11) and a flexible hinge region (between the C and D rings) that includes the residues Asn^{20}, Met^{21} and Lys^{22} (Christ *et al.*, 2007; Van Den Hooven *et al.*, 1996). There are known four distinct forms of nisin, being three of them produced by *Lactococcus lactis*. Those three are nisin A (the prototype), nisin Z (differs one amino acid from the first one) and nisin Q (differs at four positions), having all of them 34 residues in length (Delves-Broughton *et al.*, 1996). The other variant of nisin is nisin U (produced by *Streptococcus uberis*), which lacks the three residues at the C-terminal, sharing 78% of sequence similarity with nisin A (Wirawan *et al.*, 2006).

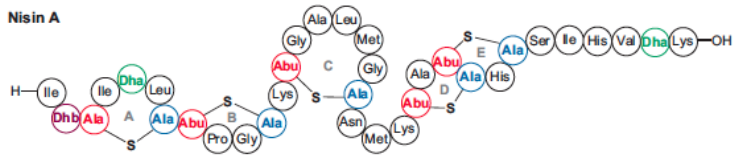

Figure 11 - Structure of the class I lantibiotic nisin A (Willey and van der Donk, 2007).

The nisin biosynthetic gene cluster is usually representative of class I lantibiotics. It has about 14 Kbp (Nolan and Walsh, 2009) and is located on a large conjugative transposon. This cluster is composed by eleven genes, which are involved in nisin biosynthesis, regulation and immunity (Nolan and Walsh, 2009; Trmčić *et al.*, 2011). The first operon consists of *nisA*, the structural gene of nisin A, which encodes the nisin precursor peptide, NisA, with 57 residues-long and includes the leader peptide of 23 residues-long (Nolan and Walsh, 2009). This gene is separated from the following genes by an inverted repeat. A weak promoter precedes the immunity gene, which allows the expression of the immunity protein before the production of mature nisin. The fourth and last operon encodes the immunity proteins genes *nisFEG* (Figure 12) (Lubelski *et al.*, 2008).

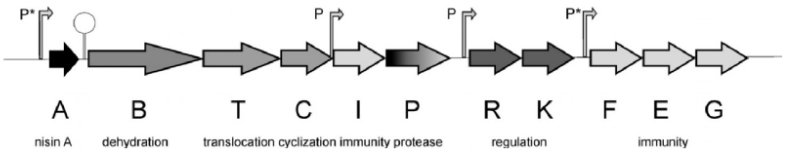

Figure 12 - Representation of the transcriptional organization of the biosynthetic gene cluster of nisin, a class I lanthipeptide. There are represented four separate transcriptional units. P* is referred to promoters controller by the two-component system NisRK and P corresponds to constitutive transcription (Lubelski*, et al.*, 2008).

The translation products of the *lanB* genes (*nisB* for nisin) are proteins of approximately 1000 amino acid residues that possess about 120 kDa of molecular weight, which do not show homology with any other known proteins (Chatterjee *et al.*, 2005b; McAuliffe *et al.*, 2001). Besides, the LanB family only shares 30% of sequence identity. The fact that those enzymes are rather hydrophobic in nature, associated with the presence of some hydrophobic domains, suggest they are associated with the cytoplasmic membrane (Chatterjee *et al.*, 2005b).

The LanC cyclases (NisC for nisin) are enzymes responsible for the cyclization reactions. They are about 400 residues long that share no sequence similarity with known proteins, having only 20%-30% sequence homology across the family (Knerr and van der

Donk, 2012). Nevertheless, they share some structural motifs in which the amino acids cysteine and histidine are conserved (McAuliffe *et al.*, 2001). They are zinc metalloproteins with an active site composed by a Cys-Cys-His triad that function as ligands for zinc binding. This suggests a mechanism of action where zinc activates the thiol group of cysteine in order to proceed at the intramolecular nucleophilic attack to Dha/Dhb in the core peptide (Knerr and van der Donk, 2012; Okeley *et al.*, 2003). It was also suggested a membrane location for these proteins (McAuliffe *et al.*, 2001).

After the modification of class I lantibiotics, an ABC transporter, LanT (NisT for nisin), exports the modified precursor peptide through the membrane and the maturation is complete when the leader peptide is cleaved in the extracellular medium by a subtilisin-like serine protease, LanP (NisP for nisin) (Willey and van der Donk, 2007). The characteristics of these proteins are discussed below.

The LanP proteases diverge in size due to the presence or absence of an N-terminal sec-signal sequence and a C-terminal cell wall anchor sequence. However, all of them share homology with the serine protease subtilisin, mainly in the proximal sequence of the catalytic triad Ser-His-Asp and in the asparagine involved in oxyanion hole formation (Chatterjee *et al.*, 2005b). Regarding the nisin protease (NisP), these residues were predicted to be Asp^{259}, His^{306}, Ser^{512} (van der Meer *et al.*, 1993). The LanP proteins can be found either anchored to the cytoplasmic membrane or at the cytoplasmic side of the membrane. Though the proteolytic removal of the leader peptide is required for all lantibiotics biosynthesis, the genes encoding those proteases are not always present on the lantibiotics' gene cluster, suggesting that the producer strain secretes other proteases that can also play this role (Chatterjee *et al.*, 2005b).

The LanT proteins (NisT for nisin) encoded in the biosynthetic gene clusters of class I lantibiotics are transmembrane ABC-transporters possessing 600 residues in length. The LanT transporters have the function to export the modified peptide of the cell (Knerr and van der Donk, 2012). These proteins share significant homology with hemolysin B-like ATP-dependent transport proteins, founded in a wide variety of organisms. They usually present three domains: a hydrophobic N-terminal domain, a six-helix crossing the cytoplasmic membrane and a C-terminal ATP-binding domain (Chatterjee *et al.*, 2005b). However, as a typical ABC transporter includes four modules (two transmembrane segments and two nucleotide-binding domains), LanT is normally considered an ABC half-transporter, which will most likely require another half-transporter to form an active unit. In the case of the lantibiotic nisin, two molecules of NisT (the ABC half-transporter for nisin) work together to form an active homodimer (Lubelski *et al.*, 2008). Nevertheless, they

show relaxed substrate specificity, since NisT is competent on exporting unmodified nisin precursor peptide as well as unrelated peptides fused to the NisA leader sequence (van den Berg van Saparoea *et al.*, 2008). This substrate promiscuity empowered the use of nisin biosynthetic machinery for *in vivo* bioengineering of various peptides with Lan and MeLan residues (Moll *et al.*, 2010). The scheme of nisin's biosynthesis is represented in Figure 13.

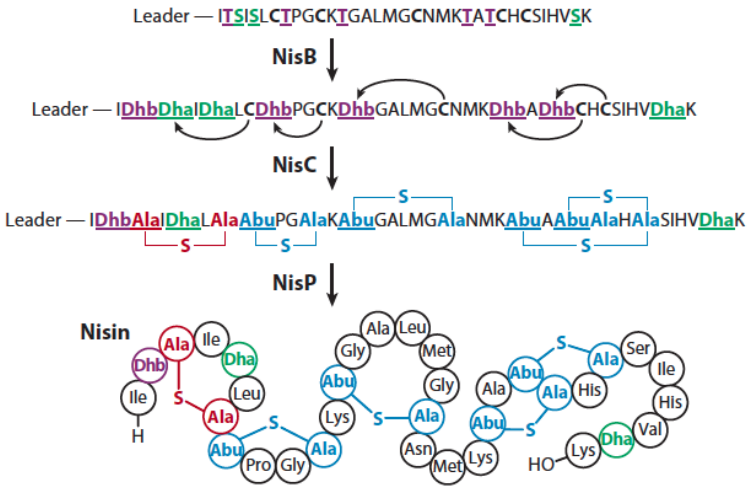

Figure 13 – Post-translational modifications of the lantibiotic nisin (Knerr & van der Donk, 2012).

Apart from nisin, the lantibiotics subtilin (produced by *Bacillus subtilis*), epidermin (produced by *Staphylococcus epidermidis*) and Pep5 (produced by *Staphylococcus epidermidis* strain 5) are also representative examples of class I (Figure 14) (Willey and van der Donk, 2007). Generally, these peptides have a more linear structure than those of class II.

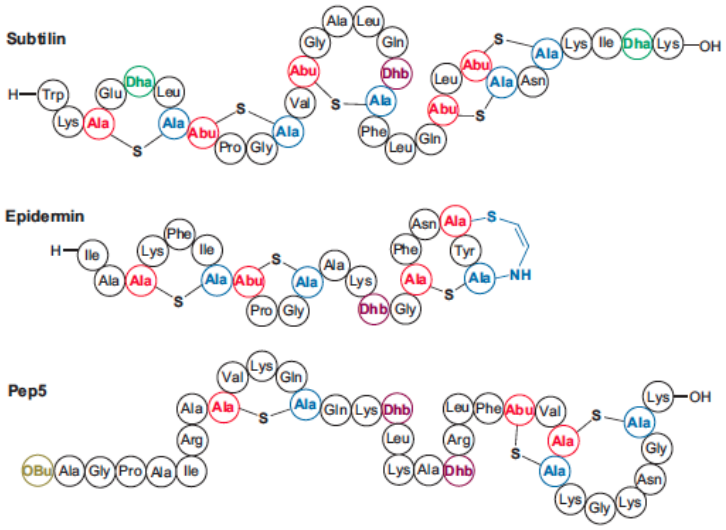

Figure 14 – Structure of three representatives class I lantibiotics: subtilin, epidermin and Pep5 (Willey & van der Donk, 2007).

1.4.1.2 Class II

In class II, the precursor peptide is modified by a single bifunctional lantibiotic synthetase, LanM, which catalyzes both dehydration and cyclization reactions (Figure 10). The lacticin 481 (Figure 15), produced by several strains of *Lactococcus lactis* is usually used as an example of class II lantibiotic. Its biosynthesis begins with the transcription of the structural gene *lctA* (Figure 8), which encodes for a LctA precursor peptide with 51 amino acids long (Xie *et al.*, 2004).

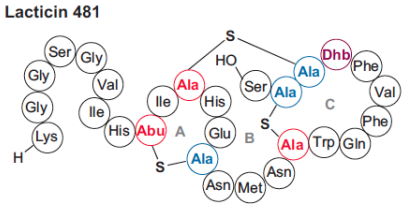

Figure 15 - Structure of the class II lanthipeptide lacticin 481 (Willey and van der Donk, 2007).

The LanM proteins (LctM for lacticin 481) are large polypeptides with usually 900-1200 residues in length and about 120 kDa (Knerr and van der Donk, 2012; You and van der Donk, 2007). These enzymes contain two domains: the N-terminal dehydratase (sharing no homology with LanB) and the C-terminal LanC-like cyclase (having about 25% of sequence similarity to LanC). The C-terminal (more specifically the LanC-like cyclase

domain) also presents the zinc-binding residues essential for NisC cyclase activity (Figure 10). As it occurs with LanB and LanC, LanM proteins do not share high sequence identity across their own family (Knerr and van der Donk, 2012; Li and van der Donk, 2007; Willey and van der Donk, 2007). A mutation of the cysteine ligands to the zinc in LctM eradicates the correct macrocyclization reactions, though the dehydration reactions are not affected (Knerr and van der Donk, 2012; Paul *et al.*, 2007).

The *in vitro* reconstitution of the LctM revealed that this enzyme requires ATP and Mg^{2+} to perform the phosphorylation reaction of serine and threonine residues. Subsequently, LctM eliminates the resulting phosphate ester, forming Dha and Dhb, respectively (Figure 16 a) (Chatterjee *et al.*, 2005a). It was also known that ADP and Mg^{2+} must be bound to the active site during the phosphate elimination reaction (You and van der Donk, 2007).

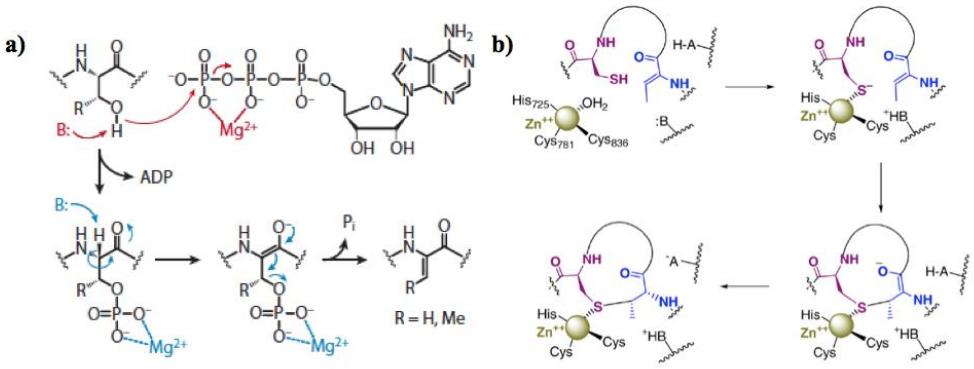

Figure 16 – Proposed mechanisms of serine/threonine dehydration (a) (Knerr and van der Donk, 2012) and cyclization (b) (Paul *et al.*, 2007) by the lacticin 481 synthetase, LctM.

The dehydration and cyclization reactions of LctM are independent. A proposed model defends that the cyclization begins when the zinc activates the thiol groups of cysteine residues for nucleophilic attack. It was also reported the existence of an active site base and acid involved in deprotonation of the thiol groups and protonation of the enolate intermediate, respectively (Figure 16 b). The conserved His^{725} is important but not mandatory for the correct cyclization of the precursor peptide (Paul *et al.*, 2007).The preference of performing the intrinsic cyclization of Dha over Dhb demonstrates the notable regioselectivity of lanthipeptide cyclases (Knerr and van der Donk, 2012; Zhang *et al.*, 2007).

The LanT enzymes of class II lantibiotics present about 700 residues long (they are 100 residues longer than class I) and, besides the membrane spanning and C-terminal

ATP-binding domains, they contain a conserved N-terminal domain: the papain-like cysteine protease domain (Chatterjee *et al.*, 2005b). These multifunctional enzymes perform the secretion and the leader processing (Willey and van der Donk, 2007). The protease domain of LanT is able to cleave both unmodified and LctM-modified LctA, which demonstrates a relaxed substrate specify. However, the leader cleavage site seems constant: the double-glycine-type cleavage site is conserved in class II lantibiotics, where processing occurs (Chatterjee *et al.*, 2005a; Furgerson Ihnken *et al.*, 2008). The ABC transporter domain is not necessary for LanT protease activity, being proposed to play a role in a more specific substrate recognition (Knerr and van der Donk, 2012). A general scheme of the biosynthesis of lantibiotic lacticin 481 is represented in Figure 17.

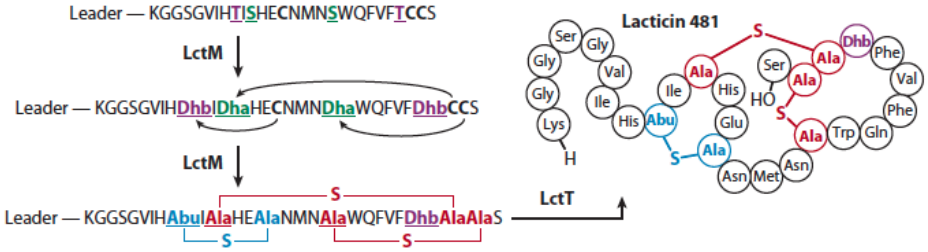

Figure 17 – Representation of post-translational modifications involved on the biosynthesis of the lantibiotic lacticin 481 (Knerr and van der Donk, 2012).

Some other representative class II lantibiotics include cinnamycin (produced by *Streptomyces cinnamoneus*) (Widdick *et al.*, 2003), mersacidin (produced by *Bacillus* sp. strain HIL Y-85,54728) (Altena *et al.*, 2000) (Figure 18), duramycin (produced by *Streptomyces cinnamoneus*), duramicin B (produced by *Streptoverticillium sp.*) and duramycin C (produced by *Streptomyces griseoluteus*) (Guder *et al.*, 2000; Willey and van der Donk, 2007).

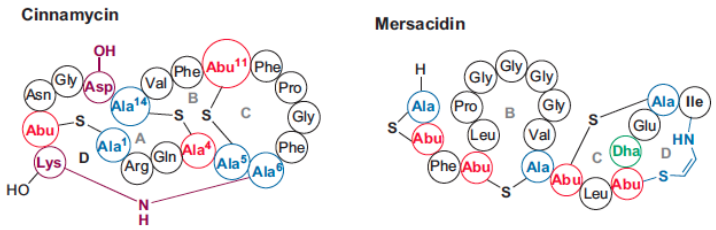

Figure 18 – Structures of representative examples of Class II lantibiotics: cinnamycin, lacticin 481 and mersacidin (Willey and van der Donk, 2007).

The two component lantibiotics (e.g. haloduracin, lichenicidin) are a special group of this class (Willey and van der Donk, 2007) that will be described in the next section.

1.4.1.3 Two-component lantibiotics

The biosynthesis of the two-component lantibiotics follows the general mechanism of those from class II (Caetano *et al.*, 2011). However, they are constituted by two structurally different peptides that show broader biological activity when acting in synergy (Shenkarev *et al.*, 2010). This group comprises cytolysin L, haloduracin, lacticin 3147 and lichenicidin, among others (Figure 19) (Knerr and van der Donk, 2012; Willey and van der Donk, 2007).

The unmodified precursor peptides (LanA1 and LanA2) are encoded by individual structural genes (*lanA1* and *lanA2*) and are enzymatically modified to their mature and bioactive forms (Lanα and Lanβ, respectively) by separate LanM enzymes (LanM1 and LanM2, respectively) (Oman and van der Donk, 2009; Willey and van der Donk, 2007). The structures of the mature α-peptides resemble the lantibiotic mersacidin, because of its globular structure, with several thiother rings, while the β-peptides are usually more elongated and flexible (Figure 19) (Martin *et al.*, 2004; Oman and van der Donk, 2009). The sequence homology among α-peptides and mersacidin, more specifically the three C-terminal rings, suggest they are important motifs for lipid II binding (Willey and van der Donk, 2007). The modification process is complete when a single multifunctional protein, LanT, which contains an ABC transporter and a protease domain, removes the leader sequence and secretes both biologically active peptides (Caetano *et al.*, 2011; Oman and van der Donk, 2009; Willey and van der Donk, 2007).

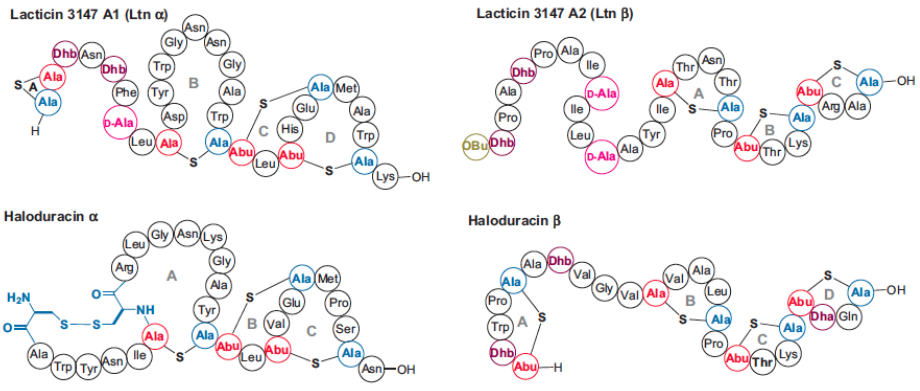

Figure 19 - Structures of representative examples of two-component lantibiotics, a class II lantibiotics' group (Willey and van der Donk, 2007).

As an example of the two-component lantibiotics is lichenicidin, that is produced by the Gram positive endospore-forming *Bacillus licheniformis*, and inhibits growth of other Gram positive bacteria (Mendo *et al.*, 2004). This lantibiotic was discovered in *Bacillus licheniformis* ATCC 14580 through a genome mining approach, using the bioinformatic tool BAGEL2 (Begley *et al.*, 2009; de Jong *et al.*, 2010). Its biosynthetic gene cluster comprises all the genes necessary for its synthesis, regulation and immunity of the producer (Figure 20) (Caetano *et al.*, 2011; Dischinger *et al.*, 2009).

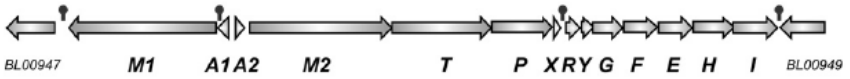

Figure 20 - Representation of the lichenicidin biosynthetic gene cluster, according to the genome annotation for *Bacillus licheniformis* ATCC 14760 (Caetano *et al.*, 2011).

Lichenicidin presents antimicrobial activity against MRSA and *Listeria monocytogenes* when the two mature peptides (Bliα and Bliβ) are produced (Figure 21). However, a study proved that, if only one of the peptides is produced, the antibacterial activity can be re-established by external supply through cross-feeding with the complementary missing peptide (Caetano *et al.*, 2011).

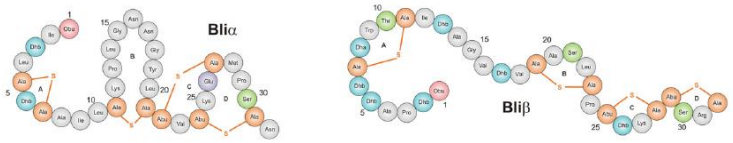

Figure 21 - Structure of the mature lichenicidin peptides, Bliα and Bliβ (Caetano *et al.*, 2011).

1.4.2 **Mode of action of lantibiotics**

The lantibiotics' mode of action consists mainly in two possibly cell target: the cytoplasmic cell membrane and the essential cell wall precursor lipid II (Bierbaum and Sahl, 2009). For instance, the class I lantibiotic nisin is characterized by a dual mode of action, employing its activity on both targets mentioned by interfering with the cell wall biosynthesis and affecting the integrity of the cytoplasmic membrane (Wiedemann *et al.*, 2001).

The cell wall is an important barrier for bacteria survival, which is composed by a strong extracellular peptidoglycan matrix, resistant at the high osmotic pressure of the cytoplasm. The peptidoglycan is constituted by monomeric units that are the building

blocks of the cell wall. The monomeric peptidoglycan consists of two amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), and a pentapeptide usually L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala attached to the carboxyl group of MurNAc. These subunits are founded attached with an undecaprenyl phosphate carrier (a membrane-anchoring) and all together form the lipid II (Figure 22). The lipid II is transported for the extracellular domain where it accomplishes the polymerization of the peptidoglycan moiety (Hsu *et al.*, 2004).

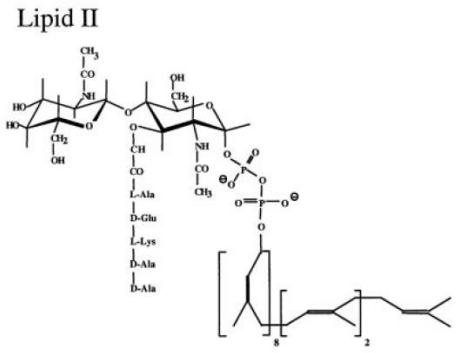

Figure 22 - Structure of the cell wall precursor Lipid II (Wiedemann *et al.*, 2001).

Studies demonstrated that nisin binds first to a lipid II, which serves as a docking molecule for this lantibiotic's activity: the two N-terminal rings of nisin form a binding pocket (also called the pyrophosphate cage) that encloses the undecaprenyl pyrophosphate moieties of lipid II (Bierbaum and Sahl, 2009). The contact between the nisin and lipid II is performed through the A and B ring of nisin, which are conserved in other class I lantibiotics, including microbisporicin and mutacin 1140 (Knerr and van der Donk, 2012). This binding pocket is stabilized by hydrogen bonds involving the residues Dhb^2 Ala^3 Ile^4 Dhb^5 and Abu^8 (Hsu *et al.*, 2004). The next step involves the C-terminus of nisin, which inserts into the membrane, oligomerizes and forms a pore constituted by eight molecules of nisin and four molecules of lipid II (Figure 23) (Breukink *et al.*, 2003; Hasper *et al.*, 2004). When nisin binds to lipid II, the peptidoglycan biosynthesis is inhibited by physical sequestration, preventing the actions of transglycosylases (enzymes that polymerize lipid II) and transpeptidases (enzymes that crosslink the glycan chains of the emerging cell wall) (Breukink and de Kruijff, 2006; Oman and van der Donk, 2009).

The flexible hinge region of nisin (residues 20-22) is fundamental for the pore formation. This causes a collapse of the vital ion gradients across the membrane and also induces a rapid efflux of cytoplasmic solutes such as amino acids and nucleotides. The simultaneous cytoplasmic membrane depolarization results in the immediate terminus of

all biosynthetic processes, including the cell-wall synthesis (Wiedemann *et al.*, 2001). This unique mode of action makes nisin a vey potent bactericidal agent at nanomolar concentrations, against closely strains related to its producers, including *Streptococci*, *Staphylococci*, *Enterococci* and drug-resistant bacteria such as MRSA and VRE (Nagao, 2009).

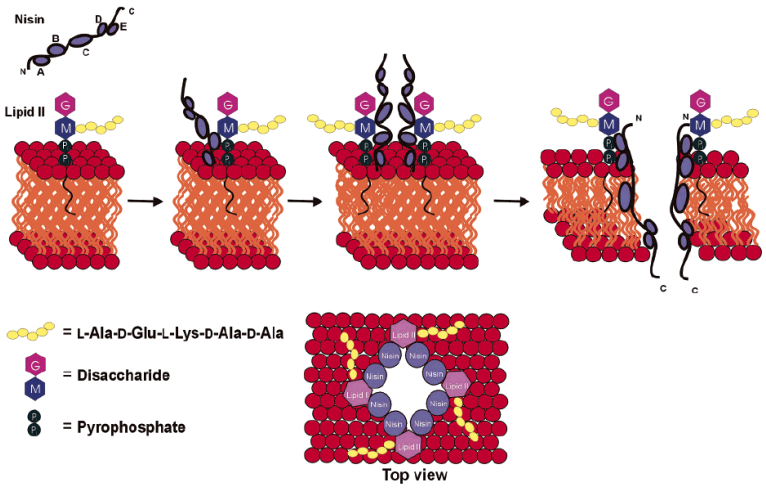

Figure 23 – Representation of a proposed model for lipid-II mediated pore formation by the lantibiotic nisin. The present arrangement of the pore structure is speculative (Chatterjee *et al.*, 2005b).

Some class I lantibiotics, including the lantbiotic epidermim, produced by *Staphylococcus epidermidis* Tü 3298 and the lantibiotic gallidermin, produced by *Staphylococcus gallinarum* Tü 3928 (Bonelli *et al.*, 2006) bind with high affinity to lipid II, through their N-terminal, and kill bacteria without permeabilizing the membrane, meaning they act without the formation of pores. This is explained by the existence of another lipid II mediated mechanism of cell inactivation (Hasper *et al.*, 2006; Islam *et al.*, 2012). These lantibiotics have the ability to sequester the lipid II from its functional to non-functional locations, conducting to the terminus of the cell wall synthesis (Islam *et al.*, 2012; de Kruijff *et al.*, 2008).

The class II lantibiotic mersacidin exerts its biological functions by binding to lipid II and inhibiting transglycosylation (and, consequently, the cell wall biosynthesis) through a Ca^{2+}-dependent mechanism, without forming pores (Böttiger *et al.*, 2009; Knerr and van der Donk, 2012).

Considering the class II two-component lantibiotics, such as lacticin 3147, lichenicidin and halorudacin, their two structural peptides display low activity individually, but when they interact synergistically, they show potent antibiotic activity at nanomolar concentrations (Knerr and van der Donk, 2012; Shenkarev *et al.*, 2010). Their mode of

action is usually exemplified by the lacticin 3147 (Ltnα and Ltnβ peptides), which has a dual mode of action. The α-peptide binds to the lipid II (inhibiting peptidoglycan biosynthesis) and a part of this peptide is partially inserted into the membrane bilayer. Subsequently, a conformational change in the Ltnα occurs, Ltnβ recognizes and binds to the complex previously formed and inserts into the cytoplasmic membrane, leading to an efficient pore-formation and cell death (Figure 24) (Piper *et al.*, 2009; Wiedemann *et al.*, 2006). It is also known that the α-peptide of lacticin 3147 and halorudacin shares the mersacidin-lipid II binding motif (Knerr and van der Donk, 2012).

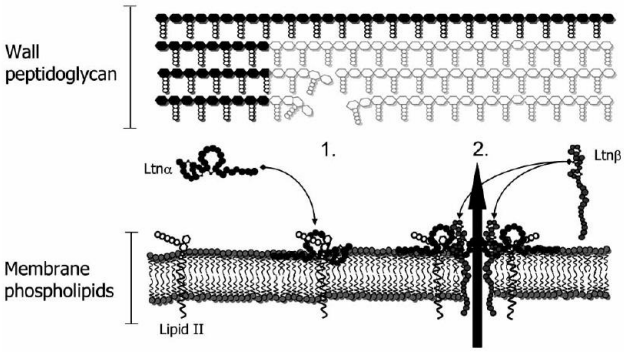

Figure 24 - The dual mode of action of the two-peptide lantibiotic lacticin 3147. The α peptide (Ltnα) binds to the lipid II and is partially inserted into the membrane (**1.**). This triggers a conformational change in the Ltnα and the β peptide (Ltnβ) binds to the complex formed, allowing pore formation and rapid cell death (**2.**) (Piper *et al.*, 2009).

Nevertheless, not all lantibiotics act exclusively as antimicrobial agents. For instance, cinnamycin and related duramycins are potent inhibitors of phospholipase A2 (Märki *et al.*, 1991; Willey and van der Donk, 2007). Besides, duramycin can also increase the chloride secretion in lung epithelium (Cloutier *et al.*, 1990); hence, as mentioned before, duramycin has been clinically tested in order to evaluate its efficacy in clearing mucus secretions from the lungs, state associated with cystic fibrosis and other airway diseases (Grasemann *et al.*, 2007; Willey and van der Donk, 2007). Recently, it was discovered that duramycin may exhibit antiproliferative properties and induces apoptosis in tumoral cells (Yates *et al.*, 2012).

1.5 *In silico* analysis in the discovery of new natural products

Natural products or secondary metabolites have radically improved human life quality. With the increased of longevity combined with the emerging antibiotic resistant pathogens and infectious diseases, there is an urgent need to discover new therapeutic agents (Winter *et al.*, 2011).

The classical approach for natural products discovery usually includes rigorous screening tests of crude extracts naturally produced by fungi and bacteria. Subsequently, a bioassay-guided fractionation or chemical screenings is performed for the final structure elucidation. Despite being favorable in the past, this approach is now unfeasible due to the high discovery rates of new natural products (Winter *et al.*, 2011).

The research in natural products field has experienced a significant decline during the last decades. However, many efforts have been undertaken in order to controvert that situation. These efforts, including whole-genome sequencing projects, metagenomic approaches and genome studies of microorganisms from extreme and unusual habitats, proved that the natural products field was initially undervalued, presenting instead an immensity of unexplored therapeutic potential (Winter *et al.*, 2011). The genome sequencing projects provided an impressive genetic data available, which can be deeply exploited by genome mining. The *in silico* genome mining approach associated with the bioinformatics tools, allows the detection of cryptic or silent biosynthetic pathways for metabolites never detected before, resulting in the discovery of new natural products. Based on known paradigms of secondary metabolite biosynthesis, it is also possible to discover new natural products and isolate the respective biosynthetic gene clusters. Furthermore, the heterologous expression of genetic data from difficult to manipulate strains or uncultured organisms has also contributed to the discovery of novel natural products, either by the identification of gene clusters, or by simplifying genetic manipulation of the target gene. Finally, and probably most importantly, the constant discovery of novel biosynthetic enzymes contributes to a constant update of the features inherent in natural product biosynthesis (Van Lanen and Shen, 2006).

According to the main theme of this work, the lantibiotics, automated *in silico* genome mining and annotation software approaches have been extremely useful for the discovery of novel compounds of this family (Knerr and van der Donk, 2012; Marsh *et al.*, 2010). The availability of this genomic information has already led to the identification of the class I epidermin-like lantibiotic, Bsa (produced by *Staphylococcus aureus*) (Daly *et al.*, 2010), the class II lantibiotics haloduracin (produced by *Bacillus halodurans* C-125)

(McClerren *et al.*, 2006) and lichenicidin as well as various cyanobacteria-associated lanthipeptides (Li *et al.*, 2010; Marsh *et al.*, 2010).

In this context, it is important to highlight three bioinformatics tools for *in silico* identification of secondary metabolites, which have already been used in lantibiotic-context: BAGEL (de Jong *et al.*, 2006), BACTIBASE (Hammami *et al.*, 2007) and antiSMASH (Medema *et al.*, 2011).

The BAGEL (BActeriocin GEnome mining tooL) is a specific bioinformatics tool that enables the identification of bacteriocins and respective biosynthetic gene clusters. The ORF (open reading frames) detection makes BAGEL independent of GenBank annotations, allowing the access of bacteriocin genes (usually, small genes) that probably would be omitted in the annotation process of bacterial genomes deposited on GenBank. The BAGEL results are also based in motifs databases. All of these innovations turn BAGEL into a promising bacteriocin cluster detector in (new) bacterial genomes (de Jong *et al.*, 2006). An improved version was recently released, BAGEL2, which allows high-throughput screening capable of dealing with the increasing genomic data sets and also a large input data set (>1000 genomes), in order to optimize bacteriocin identification (de Jong *et al.*, 2010).

The BAGEL lacks information based on protein properties. Thus, a new and original database named BACTIBASE appeared in 2007. This database contains calculated or predicted physicochemical properties of bacteriocins produced by both Gram positive and Gram negative bacteria, allowing a rapid and comprehensive structural and functional analysis (Hammami *et al.*, 2007). An improved version was released on 2010, which contains an expanded number of entries and diverse tools, including multiple sequence alignment and homology search (Hammami *et al.*, 2010).

The bioinformatic tool antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) allows the search of natural product biosynthetic gene cluster from the whole genomes deposited. Then, it is possible to predict both the substrate selection and the structure of the associated product (Winter *et al.*, 2011). In fact, this database is capable of identifying biosynthetic loci, covering all known secondary metabolites classes, including the lantibiotics' family. In this context, antiSMASH is an advantageous tool that encompasses diverse bioinformatics analysis, providing a very high accuracy in its individual cluster annotations (Medema *et al.*, 2011). The three bioinformatic tools mentioned might be used in consortium in order to obtain a more complete and detailed information.

The genome mining approaches usually take advantage of the highly conserved nature of lantibiotic biosynthetic enzymes. For instance, an *in silico* screen for class II lantibiotics (*lanM genes*) resulted in the identification of 61 novel lantibiotic (Begley *et al.*, 2009). Additionally, an *in silico* screen for *lanC* genes allowed the identification of 49 novel class I lantibiotic gene clusters associated with species, genera and even phyla of bacteria which have never been associated with the production of this compounds, including the thermophilic *Geobacillus*, a source of thermostable enzymes with industrial interest. All of these new gene clusters are extremely valuable as they can yield an immensity of novel antimicrobials and biosynthetic enzymes (Marsh *et al.*, 2010), enhancing the impact of the bioinformatics tools alongside with *in silico* approaches.

1.6 Objectives

The emergence of new multiple drug-resistant pathogenic bacteria has been a global health problem. So, the discovery of new and highly active molecules against these bacteria represents a major goal.

Thus, the main objectives of this thesis, were:

- Screening for antibacterial activity of bacterial isolates from different environments;
- Identification and selection of the strains with an interesting spectrum of activity;
- Identification of potential biosynthetic clusters encoding for lantibiotics.

2 Results and Discussion

2.1 Selection of bacteria producing antibacterial compounds

The bacterial isolates of this study were firstly characterized by performing a phenotypic screening for antimicrobial activity against relevant indicator bacterial strains. In this screening, 76 bacteria were tested against 12 indicator strains (Table 4) using colony bioassay methodologies. With this approach, a total of 58 bacteria (76,3 % of the isolates) showed inhibitory activity at least against one indicator strain. The sequence of the 16S rRNA gene of these isolates was analyzed, allowing their division into three main groups: 27,6% belong to the phylum Firmicutes, (Figure 27), 22,4% to the phylum Actinobacteria (Figure 26) and 50,0 % were Gram-negative bacteria (Figure 25) of the phyla Proteobacteria (44,8%) and Bacteroidetes (5,2%).

In a general overview, the Gram-negative bacteria were the less affected by the isolates tested. For instance, it was not possible to detect a clear antibacterial activity against *Pseudomonas aeruginosa* or *Klebsiella pneumonia* indicator strains. In fact, Gram-negative bacteria are normally less permeable to antibiotics than Gram-positive microorganisms. This is mostly due to its outer membrane, which function as a barrier to the entrance of compounds (Sr and Rao, 2012). Besides that, Gram-negative bacteria can repeal, for instance, cationic peptides with antimicrobial activity by reducing the net negative charge of this outer membrane (Koprivnjak and Peschel, 2011).

The Gram-positive isolates under study yield antibacterial products effective against a major range of Gram-positive and Gram-negative indicator strains than Gram-negatives. Thus, only isolates belonging to Actinobacteria and Firmicutes phylum were selected for further discussion. Generally, the Actinobacteria isolates showed activity mostly after 72 h and 1 week of inoculation in solid medium trypic soy agar (TSA), whereas the Firmicutes were mainly active after 24 h and 48 h of incubation (Figure 26 and Figure 27). Though, the results obtained for these two phyla were discussed in more detail in the following sections.

Figure 25 – Spectrum of antibacterial activity of Gram-negative bacteria identified by 16S rRNA gene sequencing, using agar-based bioassays.

Sample	Identification	*Enterococcus faecalis* ATCC 29212 45	*Enterococcus faecium* 547261	*Haemophilus influenzae* 121642	*Listeria monocytogenes* 71	*Micrococcus luteus* ATCC 9341 100	*Staphylococcus aureus* ATCC 29213	*Bacillus cereus* Av2	*Escherichia coli* ATCC 35218	*Klebsiella pneumoniae* 100603	*Pseudomonas aeruginosa*	*Aeromonas hydrophila* ATCC 7966	*Salmonella enteretidis* ATCC 13076
POR1	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
POR3	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
POR4	*Acinetobacter* sp.	-	-	-	±	±	-	-	±	-	-	-	-
POR9	*Acinetobacter* sp	-	-	-	-	±	-	-	-	-	-	-	-
POR15(2)	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
POR17	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
POR18	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
POR19	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
POR20	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
VT10	*Stenotrophomonas* sp.	-	-	-	-	±	-	-	-	-	-	-	-
VT12	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
VT13	*Ensifer* sp.	±	-	-	-	±	-	-	-	-	-	-	-
VT17	*Stenotrophomonas* sp.	-	-	-	-	±	-	-	-	-	-	-	-
AR7	*Acinetobacter* sp.	-	-	-	-	±	-	-	-	-	-	-	-
NM9	*Acinetobacter* sp.	+	-	-	-	-	±	-	-	-	-	-	-
NM11	*Pseudomonas* sp.	-	-	-	-	-	±	-	-	-	-	-	-
MO1	*Pantoea* sp.	-	-	-	±	±	-	-	-	-	-	±	-
MO2	*Pantoea* sp.	-	-	-	+	-	-	-	-	-	-	-	-
MO3	*Pantoea* sp.	+	-	+	+	-	+	-	±	±	-	±	+
MO4	*Myroides* sp.	-	-	-	-	±	-	-	-	-	-	-	-

Figure 25 (continuation) - Spectrum of antibacterial activity of Gram-negative bacteria identified by 16S rRNA.

Sample	Identification	*Enterococcus faecalis* ATCC 29212 45	*Enterococcus faecium* 547261	*Haemophilus influenzae* 121642	*Listeria monocytogenes* 71	*Micrococcus luteus* ATCC 9341 100	*Staphylococcus aureus* ATCC 29213	*Bacillus cereus* Av2	*Escherichia coli* ATCC 35218	*Klebsiella pneumoniae* 100603	*Pseudomonas aeruginosa*	*Aeromonas hydrophila* ATCC 7966	*Salmonella enteretidis* ATCC 13076
MO6	*Myroides* sp.	-	-	-	-	±	-	-	-	-	-	-	-
MO7	*Pantoea* sp.	+	-	-	-	-	-	-	-	-	-	-	-
MO9	*Myroides* sp.	-	-	-	-	±	-	-	-	-	-	-	-
MO13	*Serratia* sp.	-	-	-	+	±	-	-	-	-	-	-	-
MO18	*Pantoea* sp.	-	-	-	+	±	-	-	-	-	-	-	-
MO19	*Enterobacter* sp.	-	-	-	+	±	-	-	-	-	-	-	-
MO20	*Pantoea* sp.	-	-	-	+	±	-	-	-	-	-	-	-
MO21	*Enterobacter* sp.	-	-	-	+	-	-	-	-	-	-	-	-
MO29	*Pantoea* sp.	-	-	-	+	±	-	-	-	-	-	-	-

- no inhibition; + Inhibition halo; ± partial inhibition halo (inhibition zone was not totally clear).

+ and ±: in black, after 24 h; in pink, after 48h.

Figure 26 – Spectrum of antibacterial activity of Actinobacteria identified by 16S rRNA gene sequencing, using agar-based bioassays.

Sample	Identification	*Enterococcus faecalis* ATCC 29212 45	*Enterococcus faecium* 547261	*Haemophilus influenzae* 121642	*Listeria monocytogenes* 71	*Micrococcus luteus* ATCC 9341 100	*Staphylococcus aureus* ATCC 29213	*Bacillus cereus* Av2	*Escherichia coli* ATCC 35218	*Klebsiella pneumoniae* 100603	*Pseudomonas aeruginosa*	*Aeromonas hydrophila* ATCC 7966	*Salmonella enteretidis* ATCC 13076
POR6	*Rhodococcus* sp.	-	-	±	-	+	-	±	-	-	-	-	-
POR15(1)	Streptomyces sp.	±	-	-	±	±	-	±	-	-	-	-	-
VT1	*Streptomyces* sp.	+	-	+	+	+	+	+	-	-	±	-	+
VT4	*Arthrobacter* sp.	-	-	±	-	±	-	±	-	-	-	-	-
AR1	*Streptomyces* sp.	±	-	+	+	+	±	-	-	-	-	-	±
AR9	*Oerskovia* sp.	-	-	±	-	±	-	±	-	-	-	-	±
LA1	*Rhodococcus* sp.	-	-	±	-	±	-	±	-	-	-	-	-
αA	*Microbacterium* sp.	±	-	±	+	±	±	+	-	-	-	±	±
M1	*Streptomyces* sp.	+	+	±	+	+	+	+	-	-	-	-	±
NL9	*Microbacterium* sp.	±	-	±	+	±	±	-	-	-	±	-	±
NL10	*Rhodococcus* sp.	-	-	±	-	±	-	±	-	-	-	-	-
NL11	*Rhodococcus* sp.	-	-	-	-	±	-	±	-	-	±	-	-
MO23	*Curtobacterium* sp.	-	-	+	-	-	-	-	±	±	-	-	-

- no inhibition; + Inhibition halo; ± partial inhibition halo (inhibition zone was not totally clear).

+ and ±: in black, after 24 h; in pink, after 48h; in orange, after 72 h; in blue, after 1 week.

Figure 27 - Spectrum of antibacterial activity of Firmicutes identified by 16S rRNA gene sequencing, using agar-based bioassays

Sample	Identification	*Enterococcus faecalis* ATCC 29212 45	*Enterococcus faecium* 547261	*Haemophilus influenzae* 121642	*Listeria monocytogenes* 71	*Micrococcus luteus* ATCC 9341 100	*Staphylococcus aureus* ATCC 29213	*Bacillus cereus* Av2	*Escherichia coli* ATCC 35218	*Klebsiella pneumoniae* 100603	*Pseudomonas aeruginosa*	*Aeromonas hydrophila* ATCC 7966	*Salmonella enteretidis* ATCC 13076
SL1	Bacillus sp.	-	-	-	-	+	-	-	-	-	-	-	-
SL3	*Bacillus* sp.	-	-	-	+	+	+	+	-	-	-	-	-
SL8	*Bacillus* sp.	-	+	+	+	+	+	+	+	-	-	+	-
SL9	*Bacillus* sp.	-	+	+	+	+	+	+	+	-	-	+	-
SL10	*Bacillus* sp.	-	+	+	+	+	+	+	+	-	-	+	-
SL11	*Caryophanon* sp.	-	-	-	-	+	-	-	-	-	-	-	-
SL12	*Bacillus* sp.	-	-	-	+	+	+	+	-	-	-	-	-
αB	*Paenibacillus* sp.	-	-	+	+	+	-	-	±	±	±	-	±
Sma1	*Bacillus* sp.	+	+	+	+	+	-	+	+	-	±	+	+
NL8	Bacillus sp.	-	-	-	-	+	-	-	-	-	-	-	-
MO10	*Bacillus* sp.	-	-	-	+	+	-	+	-	-	-	+	-
MO15	Bacillus sp.	-	-	+	+	-	-	+	-	-	-	+	-
MO22	*Bacillus* sp.	-	-	-	+	+	-	+	-	-	-	+	-
MO27	*Bacillus* sp.	-	-	-	-	+	-	-	-	-	-	-	-
MO28	*Bacillus* sp.	-	-	-	+	+	+	+	-	-	-	+	-
MO31	*Bacillus* sp.	-	-	-	-	+	-	±	-	-	-	-	-

- no inhibition; + Inhibition halo; ± partial inhibition halo (inhibition zone was not totally clear).

+ and ±: in black, after 24 h; in pink, after 48h; in orange, after 72 h; in blue, after 1 week, in green, after 11 days.

2.1.1 **Antibacterial activity of Actinobacteria isolates**

The phylum Actinobacteria comprises most of the Gram-positive organisms with a high G+C content (Gao and Gupta, 2012). This phylum has been intensely studied due to its biotechnological potential. It is an important class of microorganisms for drug discovering, as approximately 70% of antibiotics available in market were obtained from soil Actinobacteria (Monaghan and Barrett, 2006; Sr and Rao, 2012). Moreover, Actinobacteria members produce an immensity of other secondary/specialized metabolites with important biological activities such as antitumor and anti-infection agents, plant-growth promoters and enzymes (Qin *et al.*, 2011). In this study, the most active Actinobacteria isolates were VT1, AR1, αA, M1 and NL9, as they inhibited (clearly or less clearly) at least 6 indicator strains, including Gram-negatives (Figure 26).

The isolates VT1, AR1 (both from caves in Portimão) and M1 (from Cunha Baixa) were identified as belonging to *Streptomyces* genus and they had a similar inhibition spectrum. All of them were effective against the Gram-positives *Enterococcus faecalis* ATCC 29212, *Haemophilus influenzae* 121642, *Listeria monocytogenes* 71, *Micrococcus luteus* ATCC 9341 and *Staphylococcus aureus* ATCC 29213. Moreover, they also showed antibacterial activity (although reduced) against the Gram-negative *Salmonella enteretidis* ATCC 13076. Some differences were detected among the inhibition spectra of these 3 isolates: M1 was the only Actinobacteria isolate that inhibited *Enterococcus faecium* 547261; VT1 and M1 had the capability of *Bacillus cereus* Av2 inhibition and VT1 was able to partially inhibit *Pseudomonas aeruginosa* PAO1 (Figure 26).

Streptomyces are soil filamentous bacteria, which experience a complex process of morphological differentiation. It is known that this genus can produce an immensity of secondary products, many of which with antibiotic or other pharmacologically useful activities (Pang *et al.*, 2004). Moreover, they are also lanthipeptide-producers, being SapB (produced by *Streptomyces coelicolor*) the best well characterized (Kodani *et al.*, 2004).

The bacterial isolates αA and NL9 were classified as belonging to the genus *Microbacterium*. The *Microbacterium* organisms are Gram-positive, aerobic and rod-shaped bacteria. They can be isolated from diverse environments, such as environmental sources, clinical patients (Funke *et al.*, 1995), pork sausage (associated with its flavor deterioration) (McLean and Sulzbacher, 1953) and radioactive sites (Bendiks *et al.*, 2013; Nedelkova *et al.*, 2007). Although αA and NL9 presented a broader inhibition spectrum, most of the results were detected as a partial inhibition. In this way, αA had a clear effect against the Gram-positives *L. monocytogenes* and *B. cereus*. The partial inhibitions

corresponded to the Gram-positives *E. faecalis*, *H. influenzae*, *M. luteus* and *S. aureus*; and to the Gram-negatives *Aeromonas hydrophila* ATCC 7966 and *S. enteretidis*. The NL9 isolate presented the same activity against the Gram-positive indicators as αA, though its antimicrobials were partially effective against the Gram-negatives *P. aeruginosa* and *A. hydrophila* (Figure 26). So far, there are no descriptions regarding the identification of antibacterial compounds produced by *Microbacterium* spp. isolates. Studies involving this genus are normally focus to identification and description of species.

2.1.2 **Antibacterial activity of Firmicutes isolates**

Firmicutes are known as Gram-positive bacteria with low G + C content and some of them are characterized by endospore formation (Onyenwoke *et al.*, 2004). The most active isolates belonging to this genus were αB, SL8, SL9, SL10, Sma1 and MO28, as they inhibit the greater number of Gram-positive and Gram-negative indicator strains.

The isolate αB belong to the *Paenibacillus* genus, a Gram-positive endospore-forming bacteria (Lorentz *et al.*, 2006). Strains of *Paenibacillus* have been identified in several environments, including soils (Berge *et al.*, 2002), water, rhizosphere and hospital clinical material (Bosshard *et al.*, 2002; Lorentz *et al.*, 2006). In this way, was not surprising the isolation of this strain from soil samples. The αB isolated was able to inhibit the Gram-positives *H. influenzae, L. monocytogenes* and *M. luteus*; and, with lower intensity, the Gram-negatives *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Salmonella enteritidis* (Figure 27).

The bacteria SL8, SL9, SL10, Sma1 and MO28 were identified as *Bacillus* spp. isolates. The elements of this genus are Gram-positive, aerobic and endospore-forming bacteria with a ubiquitous distribution. In fact, they can be found in soils, rocks, dust, aquatic environments, vegetation, food as well as in the gastrointestinal track of several insects and animals (Abriouel *et al.*, 2011; Nicholson, 2002). They are normally an interesting source of secondary metabolites with distinct activities and structures. These metabolites include antibiotics, antifungals, growth promoters for animals and plants, enzyme inhibitors and other bioactive compounds that constitute an evolutive advantage for their survival in its natural environment (Stein, 2005). For instance, *Bacillus subtilis* can produce more than 24 antibiotics with different structures, being the peptide antibiotics the most prevalent class (Chaabouni *et al.*, 2012; Stein, 2005). Some of this peptides are lantibiotics, the class I and class II lanthipeptides possessing antibacterial activity (Abriouel *et al.*, 2011). Based on that, the genus *Bacillus* has been widely investigated due to the potential application of its products in the agriculture, food and pharmaceutical

industries, in order to prevent/control spoilage and pathogenic microorganisms (Chaabouni *et al.*, 2012; Motta *et al.*, 2007). Similarly to metabolites from lactic acid bacteria, several products from *Bacillus* are generally recognized as safe (GRAS) (O'Sullivan *et al.*, 2002). This status was firstly approved for *B. coagulans* strain GanedenBC^{30}. Actually, some *Bacillus* are used as probiotics for human, veterinary and aquaculture use (Cutting, 2011). Moreover, *Bacillus*-based probiotics are also being developed for use as plant growth promoters (Chaabouni *et al.*, 2012).

The bacteria SL8, SL9 and SL10 were isolated from the same sampling point (Cunha Baixa) and were effective against the same indicator strains: *E. faecium*, *H. influenzae*, *L. monocytogenes*, *M. luteus*, *S. aureus* and *B. cereus*; and the Gram-negative *E. coli* and *A. hydrophila*, however when incubated for different times (Figure 27). Among all Firmicutes, the Sma1 bacteria showed the most interesting spectrum of inhibition. This isolate was partially active against *P. aeruginosa* and totally active against all the other indicator strains, except *S. aureus* and *K. pneumoniae*.

2.2 Selection of bacteria for production of antibacterial compounds in liquid medium

It is known that the production of antibacterial compounds in agar-based medium is not always accomplished using the equivalent liquid medium (Compaoré *et al.*, 2013). Thus, in this present study, the bacteria with the most interesting spectrum of activity were selected to investigate the production of these secondary metabolites also in liquid medium. In this context, only the Actinobacteria and Firmicutes isolates were considered. However, the number of strains to be tested in liquid medium was reduced, based on the genus, similar spectrum of activity and finally rep-PCR fingerprinting (by ERIC and BOX-PCR). In this molecular analysis, isolates differing at least in one band with either of the two reactions performed were considered different strains. Thus, the Firmicutes tested in liquid medium were SL1, SL3, SL8, SL11, αB, Sma1, NL8, MO15, MO22, MO27 and MO31 isolates, whereas the Actinobacteria isolates tested were POR6, POR15(1), VT1, VT4, AR1, AR9, LA1, αA, M1, NL9, NL11 and MO23.

2.2.1 **Production of antibacterial compounds in liquid medium**

In this bioassay, Firmicutes and Actinobacteria isolates were grown in trypic soy broth (TSB) liquid medium over a week period at 26 ºC. This assay aimed to verify if the antibacterial agents produced by agar-based colony bioassay were released into the culture supernatant and to determine the corresponding inhibitory spectra. Thus, the activity of the cell-free supernatants (CFSs) from 22 different isolates was tested against all the 12 indicator strains used in this study. The production of antibacterial compounds was tested after 24 h, 48 h, 72 h and 1 week (168 h) of incubation, using three replicas. The results showed that the CFSs of 13 isolates (αA, αB, NL11, MO27, AR9, NL9, SL11, NL8, POR6, LA1, VT4, AR1 and MO23) did not inhibit any of the indicator strains. Then, only 9 strains were effective against at least one indicator strain (Figure 28 and Figure 29).

Overall, higher inhibition areas were observed with Gram-positive indicator strains (*E. faecalis*, *E. faecium*, *H. influenzae*, *L. monocytogenes*, *M. luteus* and *S. aureus*), as it was also observed for colony bioassay (Figure 28). This could be due to the composition of the cell wall of Gram-positive bacteria, which lacks an outer membrane. Regarding the Gram-negative indicators, *P. aeruginosa* was not inhibited by any of the tested CFSs, whereas *A. hydrophila* was the indicator with most prominent inhibition halos (about 4 mm) (Figure 29). Moreover, none of the CFSs from Actinobacteria (POR15(1), VT1 and M1) were able to affect the growth of the Gram-negative bacteria.

Based on all information from CFSs bioassay, SL8, Sma1 and MO15 had the broadest spectra of inhibition as, when cultivated in liquid medium, they were active against 11 from a total of 12 different indicator bacteria, including Gram-positives and Gram-negatives (*E. faecalis*, *E. faecium*, *H. influenzae*, *L. monocytogenes*, *M. luteus*, *S. aureus*, *B. cereus*, *E. coli*, *K. pneumonae*, *A. hydrophila* and *S. entereditis*). For these isolates, the maximum inhibition was generally observed after 48 h of incubation (Figure 28). On the other hand, the CFSs collected after 72 h were completely inactive against all the Gram-negatives, except *A. hydrophila* (Figure 28 and Figure 29). It is important to highlight that CFS from MO15 corresponded to maximum inhibition radius (12,7 mm) against *S. aureus*, when compared to the inhibitions against the remaining indicator bacteria (Figure 28).

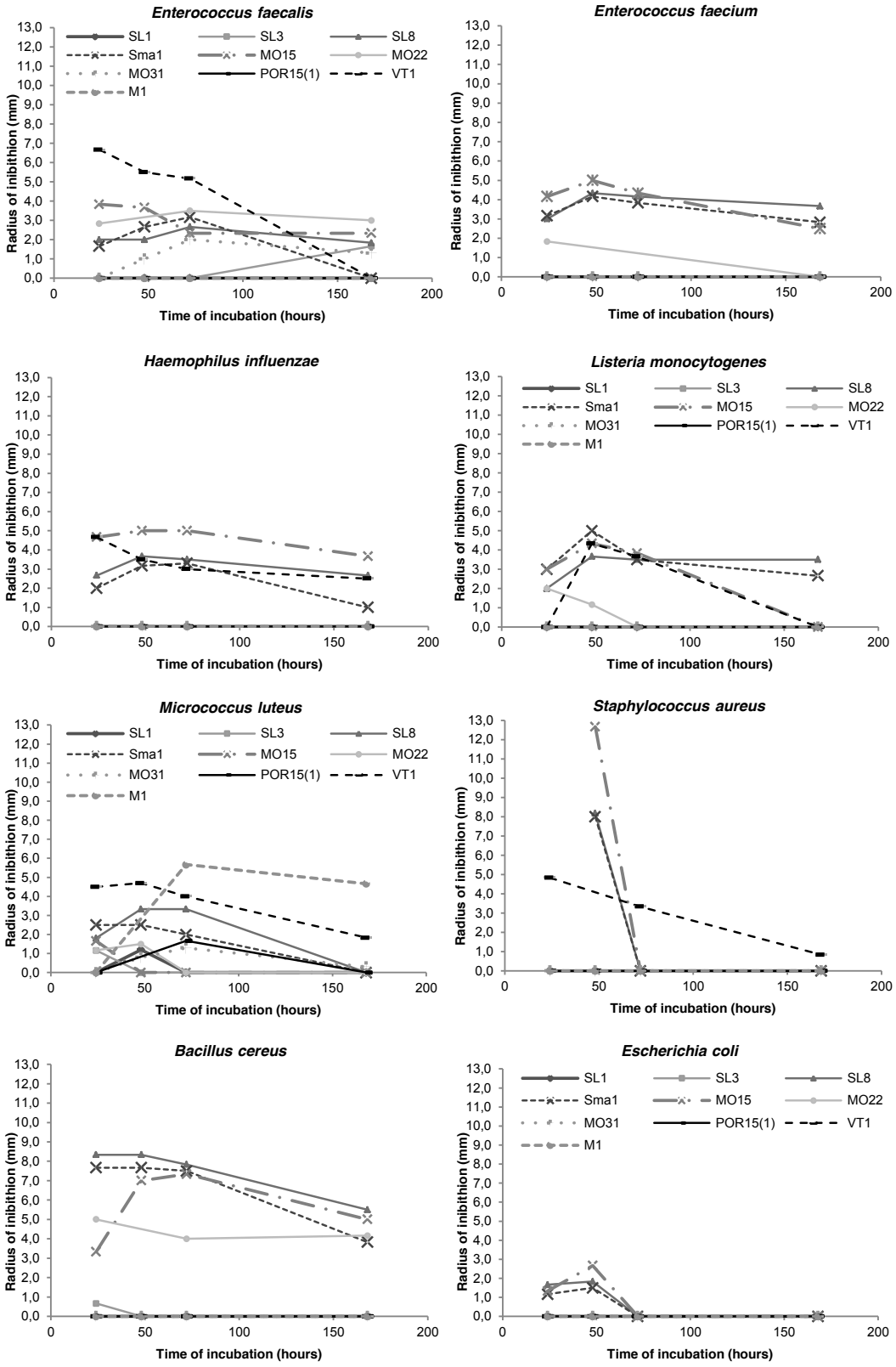

Figure 28 - Bioactivity results of cell-free supernatants (CFS) determined by agar-well diffusion assay (AWDA) against the Gram-positive and Gram-negative indicator bacteria (these results continue on the next figure). Each point represents the measurement mean of 3 biological replicas halos from the same experience.

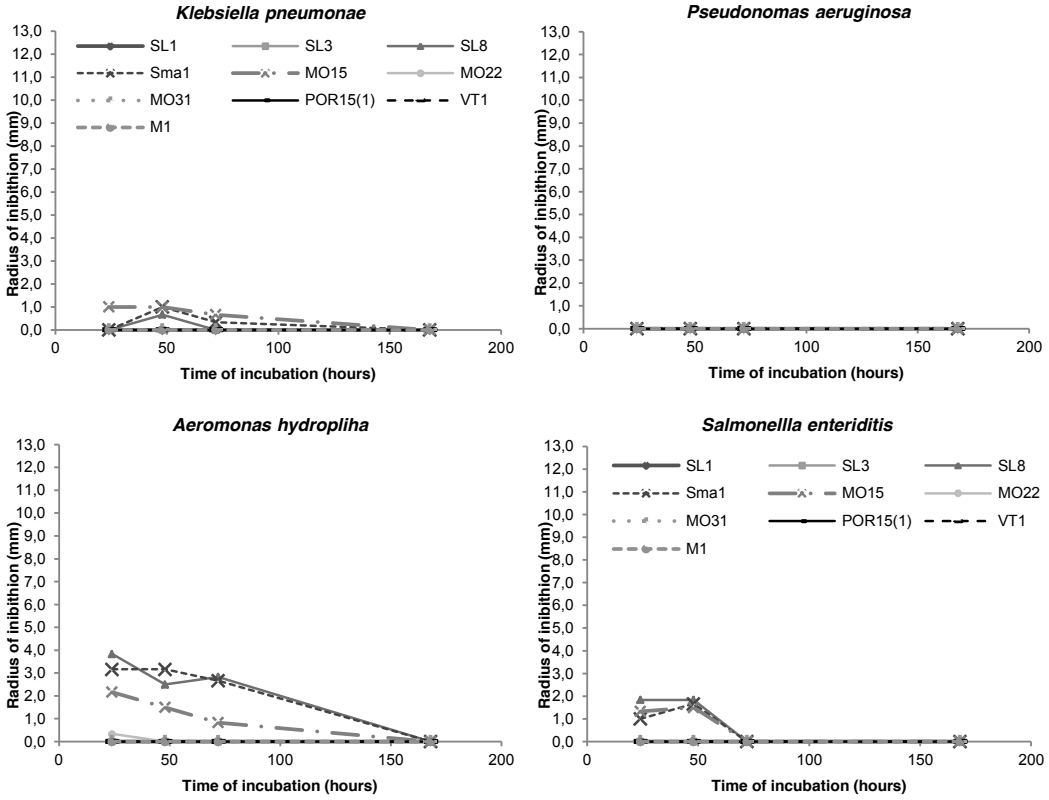

Figure 29 – Continuation: bioactivity results of cell-free supernatants (CFS) determined by agar-well diffusion assay (AWDA) against the Gram-negative indicator bacteria. Each point represents the measurement mean of 3 biological replicas halos from the same experience.

Comparing the bioassay results obtained with colony and liquid cultures (Figure 30), it was notorious that, in the majority of the cases, the antimicrobials produced in solid medium were not detected in CFSs using the agar-well diffusion assay. The lack of activity can be due to the absence or insufficient production of the compounds in the conditions tested and these differences have also been noticed on previous studies (Compaoré *et al.*, 2013; Phelan *et al.*, 2013; Prieto *et al.*, 2012). In order to improve or stimulate the antimicrobial production in liquid media, alternative and more specific media can be further tested. For instance, the media YEME and KM4 can be used in *Streptomyces* isolates (POR15(1), VT1, AR1 and M1) (Kieser *et al.*, 2000). Nevertheless, when the production in liquid media is not successful, the extraction of antimicrobial compounds from solid media for additional characterization can be optimized (Völler *et al.*, 2012). Still, for some *Bacillus* spp. isolates (SL8, Sma1, MO15 and MO22), the spectrum of activity of the CFSs was even wider than that detected in the colony bioassay.

Based on these results, the isolates SL8, Sma1 and MO15 were selected for further characterization, since they were able to inhibit almost all the indicator strains tested. Even more importantly, their products were active against Gram-negative bacteria, which are normally more difficult to identify. All of these three isolates showed activity against bacteria with impact in food industry, such as *L. monocytogenes* and *S. enteritidis*. For instance, the food-spoiler *L. monocytogenes* is reported in several dairy products, meat products and sorts of seafood, being implicated in listeriosis cases in humans and other animals (Farbed and Peterkin, 1991; Rossmanith *et al.*, 2006). Regarding *Salmonella* spp., they usually colonize all of the major livestock species (poultry, cattle and pigs), resulting in contaminated meat and other food products of animal origin. Thus, this bacterium is mostly associated with salmonellosis in humans, mainly due to the improper handling of animal-based food (Newell *et al.*, 2010). On the other hand, *Aeromonas hydrophila* is a bacterium that can infect various hosts, including important fish species at a commercial level, such as eels. These infection diseases caused by *A. hydrophila* has been a major problem resulting in serious economic losses in aquaculture sector (Cao *et al.*, 2011; Fang *et al.*, 2004). The SL8, Sma1 and MO15 isolates also inhibit the growth of indicator bacteria with clinical relevance, such as enterococci, staphylococci and *E. coli*. Thus, the secondary metabolites of SL8, Sma1 and MO15 isolates can have potential as natural biopreservatives for control of potential pathogenic and spoilage microorganisms in food industry, in aquaculture sector and they can also be applied in clinical area due to their notable antimicrobials' competence.

Figure 30 - Comparison of the results obtained using colony (C) and cell-free supernatants (L) bioassay.

	Sample	*Enterococcus faecalis* ATCC 29212 45		*Enterococcus faecium* 547261		*Haemophilus influenzae* 121642		*Listeria monocytogenes* 71		*Micrococcus luteus* ATCC 9341 100		*Staphylococcus aureus* ATCC 29213		*Bacillus cereus* Av2		*Escherichia coli* ATCC 35218		*Klebsiella pneumoniae* 100603		*Pseudomonas aeruginosa*		*Aeromonas hydrophila* ATCC 7966		*Salmonella enteretidis* ATCC 13076	
		C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L
Actinobacteria	POR6	-	-	-	-	±	-	-	-	+	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-
	POR15 (1)	±	-	-	-	-	-	±	-	±	+	-	-	±	-	-	-	-	-	-	-	-	-	-	-
	VT1	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	±	-	-	-	+	-
	VT4	-	-	-	-	±	-	-	-	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-
	AR1	±	-	-	-	+	-	+	-	+	-	±	-	-	-	-	-	-	-	-	-	-	-	±	-
	AR9	-	-	-	-	±	-	-	-	±	-	-	-	±	-	-	-	-	-	-	-	-	-	±	-
	LA1	-	-	-	-	±	-	-	-	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-
	αA	±	-	-	-	±	-	+	-	±	-	±	-	+	-	-	-	-	-	-	-	±	-	±	-
	M1	±	-	±	-	±	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	±	-
	NL9	±	-	-	-	±	-	+	-	±	-	±	-	-	-	-	-	-	-	±	-	-	-	±	-
	NL11	-	-	-	-	-	-	-	-	±	-	-	-	±	-	-	-	-	-	±	-	-	-	-	-
	MO23	-	-	-	-	+	-	-	-	-	-	-	-	-	-	±	-	±	-	-	-	-	-	-	-
Firmicutes	αB	-	-	-	-	+	-	+	-	+	-	-	-	-	-	±	-	±	-	+	-	-	-	±	-
	SL1	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SL3	-	+	-	-	-	-	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
	SL8	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	+
	SL11	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NL8	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SMA1	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	±	-	+	+	+	+
	MO15	-	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-	+
	MO22	-	+	-	+	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-
	MO27	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MO31	-	+	-	-	-	-	-	-	+	+	-	-	±	-	-	-	-	-	-	-	-	-	-	-

Note: in this table, the incubation times were not considered.

2.3 Effect of temperature and proteolysis on the antibacterial activity of SL8, Sma1 and MO15 isolates

The stability of antimicrobials produced by SL8, Sma1 and MO15 isolates to increasing temperature (Figure 31) and proteolytic activity (Figure 32) was tested. This was accomplished using the CFSs from these three isolates collected after 48 h of incubation. *B. cereus* and *A. hydrophila* were chosen as indicator strains, once they were the most affected Gram-positive and Gram-negative bacteria, respectively, by the SL8, Sma1 and MO15 CFSs.

The thermal bioassay has been used to determine the nature of an antimicrobial, which, in turn, influences its possible applications. Relatively to bacteriocins, this bioassay has been used not only to prove its presence as antimicrobial, since they are characterized to be thermo-resistant, but also to determine, for instance, their possible applications as food preservatives, where they have to remain stable during processing operations (Ben Belgacem *et al.*, 2012). To complement this information, the study of proteolysis effect on antimicrobial CFSs is also widely used. This leads to some information regarding the nature of the antimicrobials, more specifically if they have any peptidic moiety available to the enzymes' action. Thus, several studies included these two assays, in order to characterize a new natural antimicrobial (Cladera-Olivera *et al.*, 2004; Lisboa *et al.*, 2006; Phelan *et al.*, 2013; Prieto *et al.*, 2012).

2.3.1 **Effect of temperature**

In a general perspective, the exposition of CFSs to increasing temperatures led to the decrease of activity (Figure 31). Relatively to *B. cereus* inhibition results (Figure 31A), it was found that the antimicrobials produced by SL8 and Sma1 had a similar behavior in this bioassay: approximately 50 % of their activity was lost after the incubation at 80 ºC, whereas some activity was still observed after the treatment at 100 °C. Regarding the CFS of MO15 strain, it was found that the antibacterial(s) inhibiting *B. cereus* were relatively stable to all temperatures tested. Overall, the untreated samples were less active against *A. hydrophila* than *B. cereus*. Thus, different antimicrobial(s) can be involved in the inhibitory effect observed for these two strains. For instance, some of compounds active against *B. cereus* can be inactive towards *A. hydrophila,* due to the outer membrane protective barrier. The results also showed that half of the CFS activity against *A. hydrophila* was lost after incubation at temperatures between 70 ºC and 80 ºC.

Nevertheless, Sma1 and SL8 retained a little of its activity after being exposed to 100 °C, whereas MO15 CFS was totally ineffective when treated at 90 °C.

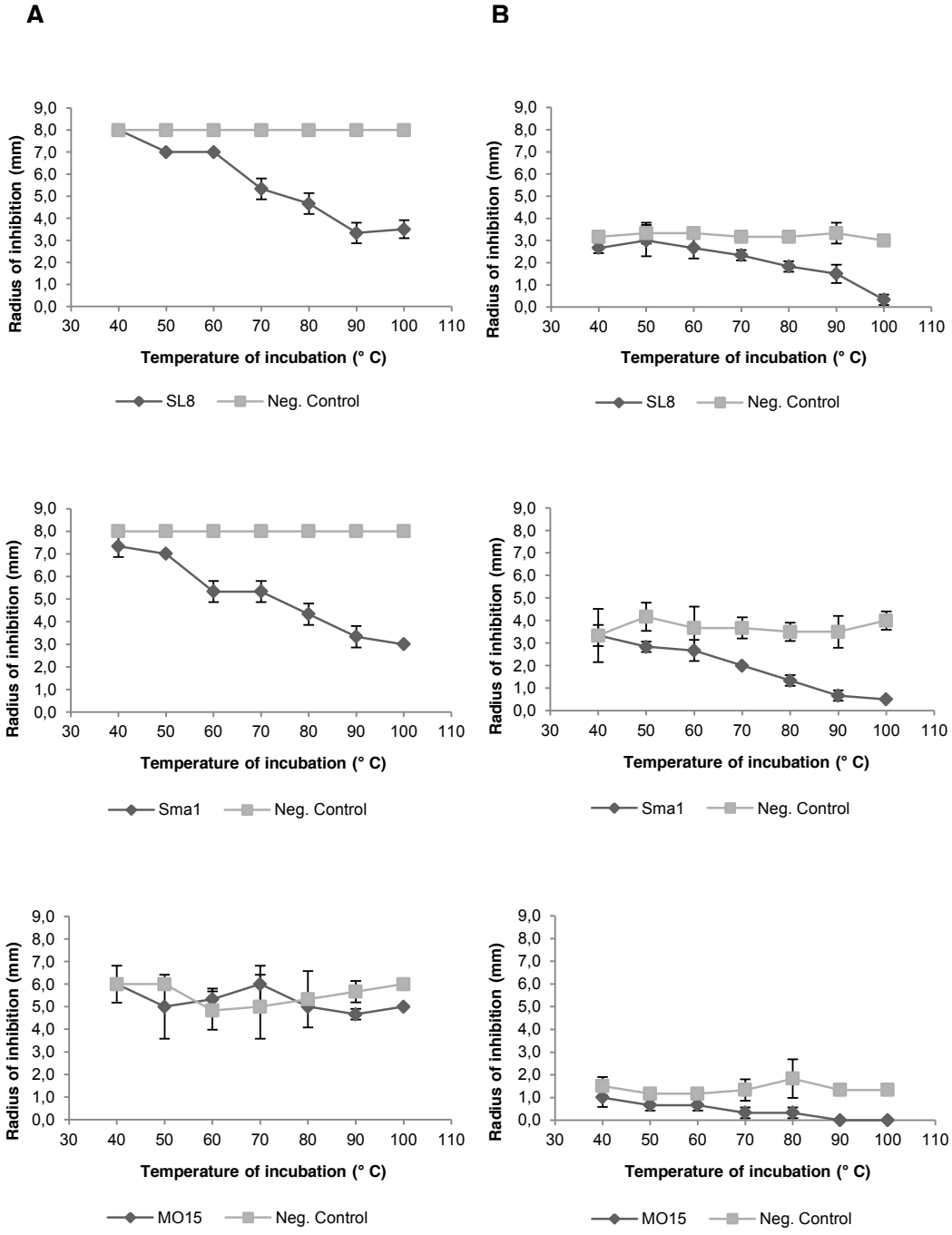

Figure 31 - Effect of temperature in the antimicrobial activity of SL8, Sma1 and MO15 cell-free supernatants (CFSs) against *B. cereus* **(A)** and *A. hydrophila* **(B)**. Each point represents the measurement mean of 3 biological replicas.

2.3.2 **Effect of proteolytic enzymes**

All the four enzymes tested in the present work were proteolytic enzymes belonging to hydrolase class (*Enzyme Commission* - EC 3), subclass of peptide hydrolases (EC 3.4).

The antimicrobial activity of the CFSs from SL8 and Sma1 against *B. cereus* was slightly reduced with the treatment with α-chymotrypsin and trypsin (Figure 32). However, these enzymes had no evident impact in MO15 activity against the same indicator. Considering the results obtained for *A. hydrophila* inhibition with the same two proteases, it was found that they had no effect in the antibacterial activity of SL8 and Sma1 CFSs. However they affected the ability of MO15 compounds to control the growth of this Gram-negative bacterium. Thus, it is expected that some of antimicrobials produced by MO15 and targeting *A. hydrophila* possess a peptidic moiety. This moiety should contain at least one tyrosine (Tyr), tryptophan (Trp), phenylalanine (Phe) or leucine (Leu) residue, since α-chymotrypsin cleaves the peptide bond after these hydrophobic amino acids. Additionally, they should also contain an arginine (Arg) and/or lysine (Lys) because trypsin cleaves exclusively the C-terminal of these positively charged residues (Olsen *et al.*, 2004).

The serine protease proteinase K cleaves preferentially peptide bonds adjacent to the carboxyl group of any aliphatic or aromatic amino acid residue. The inhibition halos of SL8 and MO15 did not suffer a noticeable reduction against the Gram-positive indicator, after exposition to this protease. However, in the same conditions, a slightly decrease of Sma1 CFS's activity was detected. Against the Gram-negative indicator bacterium, the proteolytic activity of proteinase K had impact exclusively in the CFS of MO15.

Pronase E treatment was responsible for the total loss of activity of the SL8 and MO15 CFSs against *A. hydrophila*. This protease also decreased appreciably the activity of SL8 CFS against *B. cereus* and the CFS from Sma1 against both of the indicator strains. On the contrary, the compounds produced by MO15 strain responsible for *B. cereus* inhibition were not affected by pronase E action (considering error bars overlapping from control and corresponding treated sample).

Overall, the pronase E was the protease with more impact in the activity of the tested CFSs. In fact, pronase E activity is not stringent, since it is a mixture of at least 10 proteases produced by *Streptomyces griseus* K-1 able to digest casein to the extent of >70% as mono-amino acids (Burrell, 1993). In fact, its preferential cleavage site is P6P5P4P3P2P1↓P1'P2'P3'P4', where P1 and P1' are hydrophobic amino acids (alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phy), tryptophan (Trp) and tyrosine (Tyr)). It is worth to mention that proteinase K and pronase E alone had

inhibitory activity against the Gram-positive *B. cereus*. Nevertheless, such effect was never superior to the untreated or treated CFSs (Figure 32). The other control constituted by the buffer alone had no effect in the indicator strains and therefore were not included in the results (Figure 32).

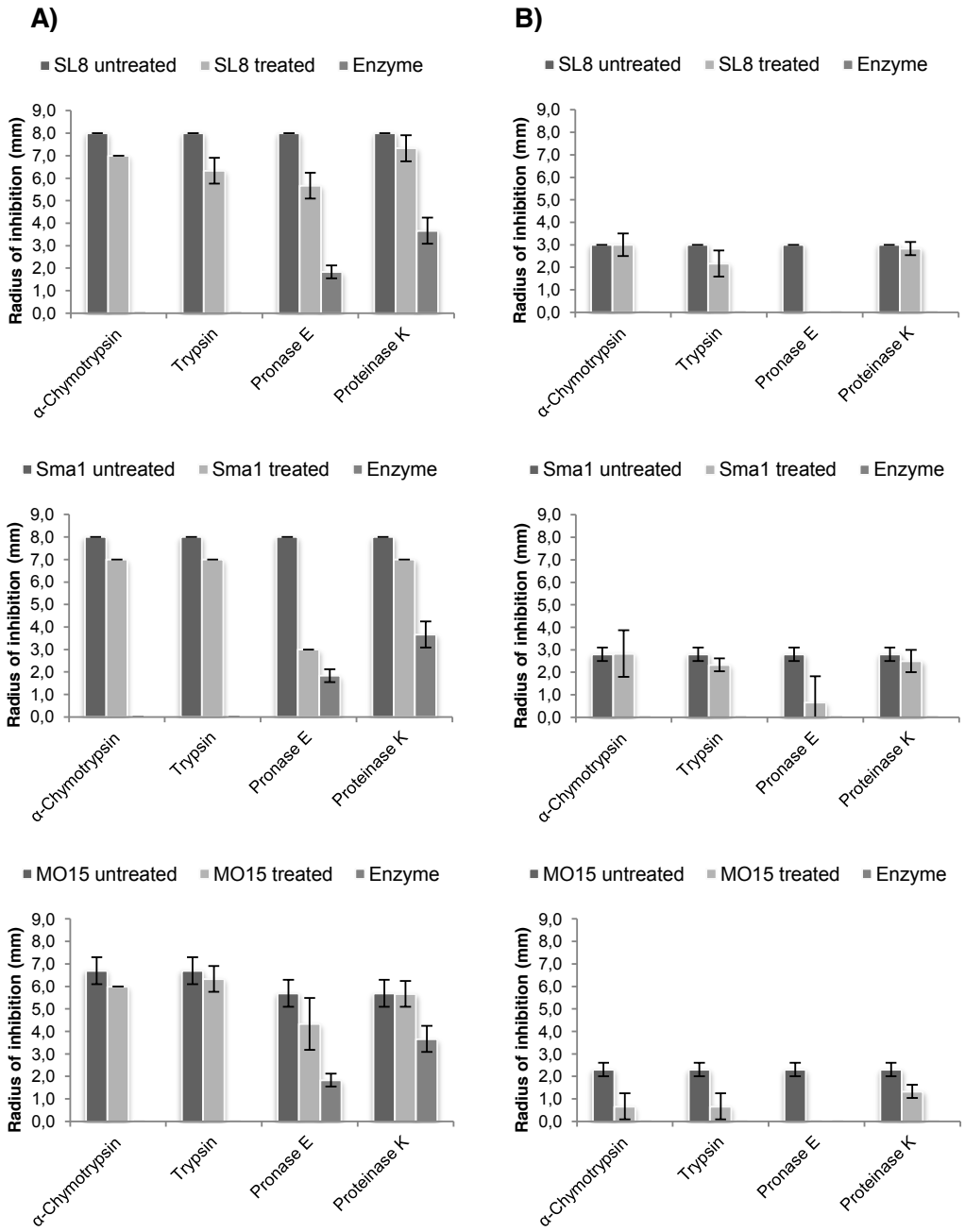

Figure 32 - Effect of proteolytic enzymes on SL8, Sma1 and MO15 cell-free supernatant (CFS) antimicrobial activity against *B. cereus* (**A**) and *A. hydrophila* **(B)**. Each point represents the measurement mean of 3 biological replicas.

These results point out that the antibacterial activity of SL8, Sma1 and MO15 strains against *B. cereus* and *A. hydrophyla* should result from a mixture of compounds, including those containing peptidic moieties. In fact, the activity of SL8 and MO15 against *A. hydrophyla* seems to be exerted mainly by peptidic products or molecules with a peptide moiety. The same can be proposed for the effect of Sma1 on *B. cereus*. However, against this Gram-positive bacterium, the majority of antimicrobials produced by SL8 and MO15 should not have a proteinaceous nature.

Nevertheless, the absence of an effect in activity after the treatment with proteases does not always exclude the possibility of antimicrobials containing proteinaceous structure. For instance, it is known that some antimicrobials produced by *Bacillus* spp. are cyclic peptides containing unusual amino acids, conferring them more resistance to proteases (von Dohren, 1995; Lisboa *et al.*, 2006). In the group of lantibiotics, their Lan and MeLan thioether bridges normally contribute to its lower sensitivity to proteolytic activity. For instance, it was shown that the removal of a Lan bridge (ring A of Lntα) resulted in thermal and proteolytic sensitivity of the lantibiotic Lacticin 3147 (Suda *et al.*, 2010). The native Lacticin 3147 is susceptible to proteinase K and α-Chymotrypsin and stable when treated with peptidase, pepsin, trypsin and pronase E (1 mg/mL at 37 °C for 1 to 3 h) (Gardiner *et al.*, 2007; Suda *et al.*, 2010). On the other hand, the lantibiotic subtilomycin was found to be susceptible to pronase E and insensitive to α-Chymotrypsin and proteinase K (1 mg/mL at 37 °C for 1 h) (Phelan *et al.*, 2013).

Moreover, in this study, the antimicrobials produced by SL8, Sma1 and MO15 in liquid cultures were tested using only one concentration of each protease. Then, it is still possible that all the proteases affect their antimicrobial activity, if used at a concentration superior to 5 mg/mL, as it was observed for some antimicrobials produced by *B. amyloliquefaciens* (Lisboa *et al.*, 2006).

2.4 Affiliation of SL8, Sma1 and MO15 isolates to the specie level

The amplification and sequencing of 16S rRNA gene did not allow the specie discrimination of the isolates SL8, SMA1 and MO15. The comparison of their 16S rRNA sequences with those available in databases was performed using BLAST®. The results revealed their 99% identity with different *Bacillus* spp. isolates classified as *B. amyloliquefaciens*, *B. subtilis*, *B. vallismortis* and *B. velezensis*.

Therefore, the sequence of the *gyrA* gene, encoding the subunit A of DNA gyrase was amplified and analyzed. The use of BLAST® tool revealed 99% of identity between

gyrA sequence of the three isolates and those of *B. amyloliquefaciens* and *B. velezensis* strains. However, it was found that *B. velezensis* is a later heterotypic synonym of *B. amyloliquefaciens* (Wang *et al.*, 2008). Moreover, the phylogenetic analysis of *gyrA* nucleotide sequences suggested that SL8, Sma1 and MO15 isolates are *B. amyloliquefaciens*, more specifically belonging to the subspecie *plantarum* (Figure 33).

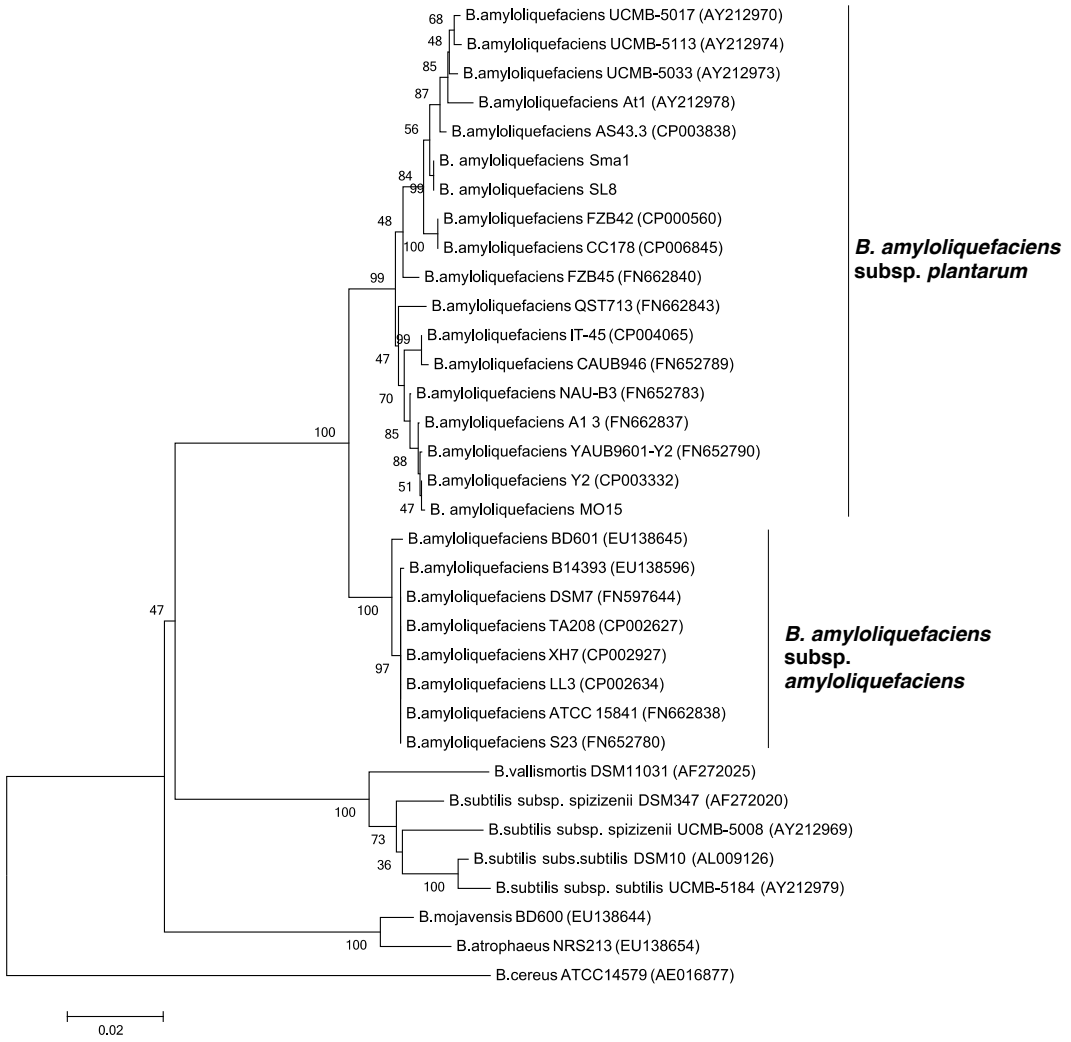

Figure 33 – Phylogenetic tree based on the *gyrA* nucleotide sequences of several *Bacillus* spp. isolates. The type strains for the two subspecies of *B. amyloliquefaciens* are in bold. The *B. cereus* ATCC 14579 sequence was included as an outgroup representative.

2.5 Identification of clusters of lanthipeptides in *B. amyloliquefaciens* genomes by genome mining

Currently, 13 *B. amyloliquefaciens* genome projects are finalized (data available at NCBI site on 20th of October, 2013). Therefore, the identification of gene clusters typically involved in the production of lanthipeptides was performed using the bioinformatics tool antiSMASH 2.0 in those 13 genomes. The antiSMASH 2.0 constitutes a platform for the genome mining of a vast diversity of secondary metabolites, including lanthipeptides (Medema *et al.*, 2011). The results revealed the presence of gene clusters encoding the biosynthesis of different families of natural compounds, most of them with antimicrobial activity, in all the 13 genomes. These included NRPS, PKS and terpenes. The biosynthetic clusters characteristic of lanthipeptides were also detected, however in only 3 of the analyzed strains: *B. amyloliquefaciens* Y2, *B. amyloliquefaciens* YAU B9601-Y2 and *B. amyloliquefaciens* IT-45. These three clusters will be discussed in more detail in the following sections.

2.5.1 *Bacillus amyloliquefaciens* Y2 and YAU B9601-Y2

The strains *B. amyloliquefaciens* subsp. *plantarum* Y2 (accession number NC_017912) and YAU B9601-Y2 (accession number NC_017061) were both isolated from wheat rhizosphere and are considered plant-growth promoters (Hao *et al.*, 2012). Using the platform antiSMASH 2.0, a typical cluster for the production of lanthipeptides was identified in these two strains (Figure 34). The BLASTX analysis of the *lanM* gene of the two clusters revealed a 100% identity with the MrsM enzyme, involved in the production of mersacidin. This lantibiotic is a class II lanthipeptide composed by 20 amino acids, which is active against MRSA (Bierbaum *et al.*, 1995; Herzner *et al.*, 2011; Motta *et al.*, 2007). In fact, the two clusters found by antiSMASH were identical to the full mersacidin cluster. However, two genes were not detected by this analysis: i) the mersacidin structural gene (*mrsA*), in the cluster of YAU B9601-Y2 strain and ii) the gene encoding the MrsD flavoprotein, in the cluster of Y2 strain. Nevertheless, using the graphical analysis tool ORF finder, it was possible to identify these two genes in the respective gene clusters (arrows with dashed lines; Figure 34). Thus, *B. amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2 and Y2 strains have the complete mersacidin biosynthetic gene cluster in their chromosomes.

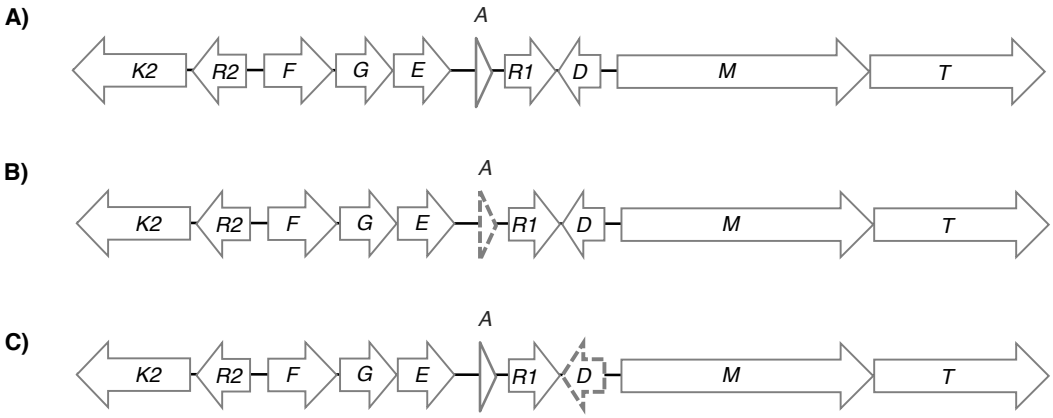

Figure 34 – Representation of mersacidin biosynthetic cluster already well-characterized (**A**) and the mersacidin clusters detected in *Bacillus amyloliquefaciens* subsp. *plantarum* Y2 (**B**) and YAU B9601-Y2 (**C**) by antiSMASH 2.0 analysis. The genes not detected by this platform are represented with dashed lines.

2.5.2 ***Bacillus amyloliquefaciens* IT-45**

The antiSMASH analysis of this bacterial genome (accession number NC_020272) identified a lanthipeptide's gene cluster containing 2 *lanM* (class II lanthipeptides) and 2 putative *lanA* genes. This cluster was designated as *bam* and its partial representation is present on Figure 35.

Figure 35 – Partial representation of lanthipeptide's cluster detected in the *Bacillus amyloliquefaciens* IT-45 chromosome by antiSMASH 2.0 analysis. *A1* is respectable to a possible lantibiotic structural gene (KSO_013315), *M1* and *M2* correspond to two different modification enzymes' genes, *T* to the gene that codifies the LanT transporter and *FEG* to putative immunity genes. *#1*, **#**2 and *#3* correspond to genes associated with two-component regulatory components. The dashed arrows (*A2*, *A3* and *A4*) correspond to putative *lanA* genes recognized after ORF finder search analysis.

Table 1 – Sequence of the precursor peptides encoded by the structural genes detected with antiSMASH 2.0 of the *B. amyloliquefaciens* IT-45 genome. ↓ represents the cleavage site proposed in the antiSMASH analysis. The underlined amino acid residues correspond to the most probable cleavage-site of the leader peptide, according to this study. The residues essential for the biosynthesis of lanthipeptides are shown in bold (Ser, Thr and Cys).

Gene Locus	Leader ↓ core peptide sequence
KSO_013260	MINEQRARSYIHWSYAAHEVLGG ↓ QHGDY**S**VMMQKPFLR**C**QNDDILPAVF
KSO_013315	MNEKMYRFAGDLREELEEISLS ↓ EF**S**GGGGAEQRGI**S**QGNDGKL**CT**LT**T**WE**C**GL**C**P**T**H**TC**W**C**

Regarding the 2 structural genes founded by antiSMASH 2.0 (Table 1), the one with the gene locus number KSO_013260 did not show potential to constitute a mature

lanthipeptide, since it can possibly form only one thioether bridge (its core peptide only contains 1 Ser and 1 Cys residues), which is uncommon among this class of compounds. The other structural gene (KSO_013315) codifies for a precursor peptide constituted by a core peptide possessing several Ser, Thr and Cys residues. Thus, this is more likely to be a *lanA* gene. However, the leader peptide removal should occur a few amino acids downstream (GA motif; Table 1) to that proposed by the antiSMASH. This is also supported by the fact that, in class II lanthipeptides, the preferential cleavage site is the "double Gly motif" (GG, GA or GS) (Caetano, 2011; Chatterjee *et al.*, 2005b). Then, the mature peptide (core peptide after modification process) should be composed by 4 MeLan (thioether bond between Dhb and Cys residues) and 1 Lan (thioether bond between Dha and Cys residues). Thus, it was considered that only KSO_013315 encodes a real *lanA* structural gene.

However, 2 *lanM* genes are present in this cluster, making it expectable to find at least other *lanA* gene. Since these clusters usually comprise genes close to each other in *Bacillus* genus, a search for the presence of potential *lanA* ORFs between *lanM1* and *lanT1* was performed using ORF finder. Among the 6 ORFs identified, 3 of them seemed promisor ORFs to constitute lanthipeptide's precursors (Figure 35). The analysis of the encoded sequence demonstrated that 2 of them originate exactly the same peptides containing 58 amino acids (A2 and A3 in Table 2). Moreover, these two peptides showed similarity with an uncharacterized peptide of *B. cereus* and with class II lantibiotics already characterized, such as the β peptide of lichenicidin (Bliβ) and haloduracin (Halβ) (Figure 36). Thus, *A2* and *A3* genes have a high probability to constitute the precursor peptides of lantibiotics, since the majority of class II lanthipeptides known possess antibacterial activity.

The last gene (*A4*) codifies a peptide with 52 amino acids (Table 2) that showed no homology with any lantibiotic. However, its sequence has some characteristics of this class of compounds, such as i) the double Gly-motif, ii) the presence of Cys residues only after the Gly-motif (core peptide) and iii) several Ser and Thr residues close to Cys (Table 2). Therefore, according to this analysis, there are 3 possible lanthipeptides with probability of being produced by IT-45 strain.

Table 2 – Sequence of the potential precursor peptides detected in this study in the *B. amyloliquefaciens* IT-45 genome. ↓ represents the most probable cleavage site and the residues essential for the biosynthesis of lanthipeptides are shown in bold (Ser, Thr and Cys).

Gene	Leader ↓ core peptide sequence
A2	MKKDFQALTPMTEEELKNLAGGS ↓ DA**T**PM**T**V**T**P**T**T**I**T**I**PI**S**LAG**C**P**TT**K**C**A**S**IV**S**P**C**ND
A3	MKKDFQALTP MTEEELKNLAGGS ↓ DA**T**PM**T**VTP**TTI**TIPI**S**LAG**C**P**TT**K**C**A**S**IV**S**P**C**ND
A4	MKENF**S**ALYE**TS**EQELRELIGG ↓ QN**S**V**S**I**TT**IPI**T**NHV**C**P**TI**TVG**C**A**C**PQRQV

Identities: 63%

```
                                  20                                  40
                                   |                                   |
A2 and A3  FQALTPMTEE  ELKNLAGGSD  AT-PMTVTPT  TITIPISLAG  CPTTKCASIV  SPCN 53
      1)   YNGLEEVVEQ  ELMDLAGGND  ESQPQALTPT  TITIPISLWG  CPTTSCASIV  SSCN 54
```

Identities: 57%

```
                                  20                                  40
                                   |                                   |
A2 and A3  KDFQALTPMT  EEELKNLAGG  SDATPMTVTP  TTITIPISLA  GCPTTKCAS  49
      Halβ QGLQFVDEVN  EKELSSLAGS  GDVHAQTTWP  CA-TVGVSVA  LCPTTKCTS  48
```

Identities: 46%

```
                                  20                                  40
                                   |                                   |
A2 and A3  MTEEELKNLA  GGSDATPMTV  TPTT-----I  T--IPISLAG  CPTTKCAS 41
      Bliβ VSEEELKALV  GGNDVNPETT  PATTSSWTCI  TAGVTVSASL  CPTTKCTS 48
```

Figure 36 - BLASTp alignment results between the peptide sequence A2/A3 and: 1) hypothetical protein [*Bacillus cereus*], accession number WP_016132309; Halβ: lantibiotic haloduracin [*Bacillus halodurans* C-125], accession number NP_241319 and Bliβ: lantibiotic lichenicidin [*Bacillus licheniformis* DSM 13 = ATCC 14580] accession number YP_006715399. The matching residues are represented by grey color.

Considering the remaining genes of the cluster, the *lanT* (Figure 35) encodes a classical class II LanT, constituted by an ATP-binding domain and a C-terminal protease domain (through a BLASTp search). The gene KSO_013345 must codify for a LanF protein (*F*, Figure 35), since it possesses an ATP-binding motif (Draper *et al.*, 2008). Moreover, by performing a multiple sequence alignment with LanF from other lantibiotic gene clusters, the conserved motifs characteristic of this nucleotide-binding subunit, including Walker A, Walker B, E-loop, Signature and H-loop (Okuda and Sonomoto, 2011) were identified (Figure 37). It is important to refer that LanF proteins contain the E-loop motif instead of the usual Q-loop of ABC transporters. The E-loop is considered to be involved in the conformational change of the transmembrane subunits E (*lanE*) and G (*lanG*), being a key factor on the lantibiotic-transport mechanisms (Okuda *et al.*, 2010; Okuda and Sonomoto, 2011).

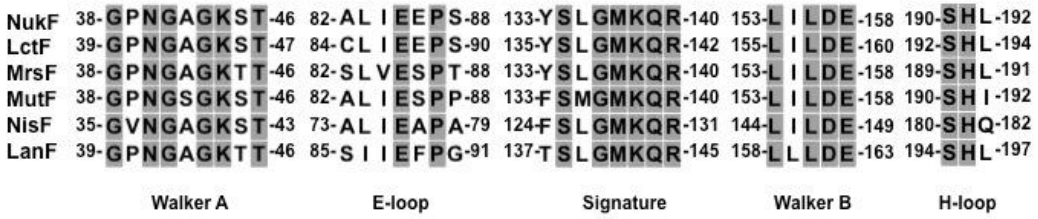

Figure 37 – Multiple sequence alignment of LanF protein identified in IT-45 genome and other LanF proteins involved in the self-protection of already characterized lantibiotics. Residues involved in conserved motifs (Walker A, E-loop, Signature, Walker B and H-loop) are shown. Accession numbers: NukF, NP_940775; LctF, AAC72253; MrsF, CAB60255; NisT, CAA82547; MutF, AAF99691.

The *lanE* and *lanG* genes are usually found next to *lanF*. Then, the gene with the accession number KSO_013340 (*E/G*, Figure 35) must probably codifies for LanE/G protein. The analysis of the correspoding protein using the TMHMM Server v. 2.0 showed that it is composed by 6 transmembrane domains, which are a key characteristic of LanE and LanG proteins (Saris *et al.*, 1996). Thus, it was considered that this gene, together with *F* gene, constitute the self-protection system of the strain to the lantibiotics produced by the biosynthetic cluster. The remaining genes KSO_013320, KSO_013325, KSO_013330 and KSO_013335 (#1, #2, #3 and #4, respectively, in Figure 35) showed homology with LanR and LanK proteins, most probably involved in transcriptional regulation of the remaining genes.

2.6 Screening of mersacidin genetic determinants and production in SL8, Sma1 and MO15

As abovementioned, two different clusters typical of lanthipeptides production were identified in *B. amyloliquefaciens* chromosomes by genome mining analysis: the mersacidin (*mrs*) cluster and an uncharacterized cluster (*bam*). Thus, the presence of some mersacidin genetic determinants in the SL8, Sma1 and MO15 isolates was investigated. In this context, the amplification of a fragment containing the mersacidin structural gene (*mrsA*), the regulator gene (*mrsR1*) and the flavoprotein gene (*msrD*) from SMA1, SL8 and MO15 total DNA was successful (amplicon of 1600 bp). However, the gene codifying the mersacidin modification enzyme MrsM (amplicon with about 3000 bp) was only identified in SL8 and Sma1 isolates. Consequently, the amplification of a *lanM* gene from MO15 strain was attempted using degenerated primers and Sma1 as a positive control. Nevertheless, no amplicons were obtained, indicating the absence of these type of genes in the MO15 isolate. Thus, it will be possible that SL8 and Sma1 isolates

possesses the complete *mrs* gene cluster, whereas MO15 is expected to have the same cluster, but incomplete.

Due to the detection of *mrs* genetic determinants in the SL8, Sma1 and MO15, their ability to produce mersacidin was evaluated. This was performed in order to understand if mersacidin was contributing for their spectrum of activity, detected in this study. To achieve this, the CFSs were obtained from the same conditions as the ones used for stability testing to temperature and proteolytic activity. After extraction and evaporation of the supernatants, the crude extracts were analyzed using MALDI-TOF/MS. The expected mersacidin molecular masses [1826 Da: mersacidin + H; 1848 Da: mersacidin + Na and 1864 Da: mersacidin + K] (Herzner *et al.*, 2011) were not detected in any of the extracts (Figure 38). These results were expected at least for MO15 *B. amyloliquefaciens* subsp. *plantarum* strain, once the amplification of *mrsM* gene was not successful. In this case, the modification process on the mersacidin precursor peptide cannot occur. However, the SL8 and Sma1 isolates possess this gene. The absence of production by these two isolates can have multiple explanations, including the use of inappropriate media for mersacidin production or the absence of other genes in the cluster not evaluated in this study (as *mrsT*). In order to confirm these two possibilities, a PCR screening for the remaining genes should be achieved and these bacteria should be cultivated in media already described for mersacidin production (Bierbaum *et al.*, 1995; Herzner *et al.*, 2011).

Since the lantibiotic mersacidin was not detected in any studied CFSs, the bioactivity of bacterial isolates SL8, Sma1 and MO15 *B. amyloliquefaciens* subsp. *plantarum* was probably conferred by other natural products' classes, which were common to all 13 *B. amyloliquefaciens* genomes analyzed, such as NRPS, PKS and terpenes.

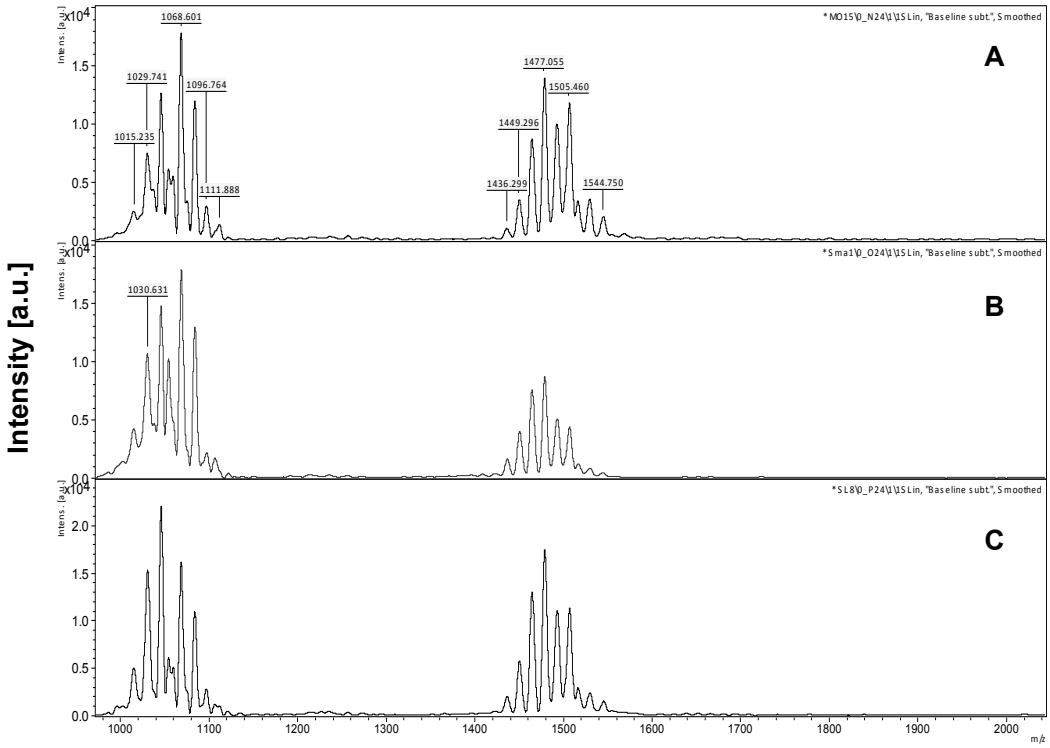

Figure 38 – MALDI-TOF mass spectra of CFSs from MO15 (**A**), Sma1 (**B**) and SL8 (**C**) after butanol extraction. The typical mersacidin-related mass signals [1826 Da: mersacidin + H, 1848 Da: mersacidin + Na and 1864 Da: mersacidin + K] were not detected.

3 Synopsis and Future Perspectives

The sampling sites selected for the present work were source of microorganisms with remarkable antimicrobial activities.

Through colony bioassay we found that a large number of the bacterial isolates collected produced potent antimicrobial compounds capable of inhibiting a wide range of Gram-positive and Gram-negative bacteria, including pathogens such as *Listeria monocytogenes* and *Escherichia coli*. A comparison of the results obtained in solid and in liquid medium led us to conclude that possibly more than one antimicrobial compound was produced by the same isolate, since a different inhibitory spectrum was obtained, depending on the culture conditions used.

Three bacteria, SL8, Sma1 and MO15, identified as *B. amyloliquefaciens*, were selected to test the antimicrobial compounds' stability when the culture supernatant was exposed to different temperatures and proteases. Results showed that these antimicrobials were thermo-sensible, with the exception of those produced by MO15 CFS, that was able to inhibited *B. cereus*. Nevertheless, the antimicrobials produced by SL8 and Sma1, effective against *B. cereus,* retained nearly 50% of their initial activity when placed at 100 °C for 30 min. The effect of proteases suggested that these antibacterials should be mostly of non-peptidic nature. Nevertheless, when these compounds were treated with Pronase E, bioactivity of SL8 and MO15 CFSs against *A. hydrophila* was completely abolished.

A genome mining analysis using the platform antiSMASH allowed the identification of gene clusters encoding secondary metabolites with antibacterial activity in all the sequenced and assembled *B. amyloliquefaciens* genomes. However, it was found that the interpretation of the results retrieved by antiSMASH requires a critical judgment of the user. For instance, herein new structural genes that probably encode lanthipeptides were identified in some of the genomes analyzed that were not detected by the antiSMASH 2.0 algorithm. These genes have not been characterized yet and finding it was an important achievement of the present work. Moreover, the biosynthetic cluster of the lantibiotic mersacidin was found in two of the deposited genomes. But, despite that, this lantibiotic was not detected in any of the supernatants analyzed by MALDI-TOF/MS. Nevertheless, SL8 and Sma1 are *B. amyloliquefaciens* strains that might have potential to produce this lantibiotic because they possess both *mrsA*, *mrsD* and *mrsM* genes.

The results herein obtained showed that the *B. amyloliquefaciens* SL8, Sma1 and MO15 isolates produce natural compounds that inhibit both pathogenic and food spoilage microorganisms, with possible applications in health, food, agriculture and aquaculture

areas. Nevertheless, further studies are still required. Thus, further investigations will be performed and include:

i) Complete the stability tests using CFSs; these will include the evaluation of the impact of pH as well as the impact of other enzymes (such as lipases and amylases) on the antibacterial activity of the compounds produced;
ii) Amplification of the complete cluster involved in the biosynthesis of the lantibiotic mersacidin;
iii) Evaluation of mersacidin production using different culture conditions and other peptide extraction procedures;

Moreover, PCR screening of clusters possibly associated with lantibiotic production, and targeting *lanB*/*lanC* (class I) and *lanM* genes (class II), will be performed on all the remaining isolates possessing antibacterial activity.

4 Experimental procedures

4.1 Bacterial isolates

The bacterial strains used in this study were isolated from soil of an abandoned uranium mine, soil from caves and roots of *Eryngium* sp. collected in an abandoned iron mine (Table 3). The soil samples from caves were collected in Algarve region (Portimão, Vale Telhado and Algar Romão) by Doctor Sofia Reboleira (Department of Biology and CESAM, University of Aveiro). The MO bacterial isolates (Table 3) were provided by Doctor Catarina Marques (Department of Biology and CESAM, University of Aveiro) and Professor Doctor Ruth Pereira (Department of Biology, University of Porto and CESAM, University of Aveiro).

All isolates were grown at 26 °C and maintained in trypic soy agar plates (TSA; Merck) at 4 °C.

Table 3 –Local of isolation and designation of the bacterial isolates used in the present study.

Product	**Sampling area**		**Designation of isolates**
Soil	Abandoned uranium mine, Cunha Baixa, Portugal	Close to the lagoon	SL, LA, NL
		"Lamas"	M, SMA, NM
Soil	Caves, Algarve, Portugal	Algar Romão	AR
		Portimão	POR
		Vale Telhado	VT
Root	Plant *Eryngium* sp. from an abandoned iron mine, Ait Amar, Morocco		MO, α

4.2 Detection of bacteria with antibacterial activity

In order to test the inhibitory bioactivity of the bacterial isolates, three different agar-based tests (deferred-antagonism testing) were performed: colony bioassay, overlay bioassay and "sandwich" bioassay. For each of these assays, several indicator strains were used, including Gram-positive and Gram-negative bacteria (Table 4). The indicator strains were always grown on 20 mL of trypic soy broth (TSB; Merck) using 50 mL falcons, at the appropriate temperature (Table 4) with aeration (180 rpm), overnight.

Table 4 - Indicator strains used in this study and respective incubation temperatures.

Indicator strains	Origin	Growth temperature
Gram positive		
Bacillus cereus Av2	Aveiro Lagoon[1]	26 ºC
Enterococcus faecalis ATCC 29212		37 ºC
Enterococcus faecium 547261	Clinical isolate	
Haemophilus influenzae 121642	Clinical isolate	
Listeria monocytogenes 71	Clinical isolate	
Micrococcus luteus ATCC 9341		
Staphylococcus aureus ATCC 29213		
Gram negative		
Aeromonas hydrophila ATCC 7966		26 ºC
Escherichia coli ATCC 35218		37ºC
Klebsiella pneumoniae 100603	Clinical isolate	
Pseudomonas aeruginosa PAO1		
Salmonella enteretidis ATCC 13076		

[1](Cruz *et al.*, 2007)

4.2.1 **Colony bioassay**

In this assay, 45 mL of melted TSA were inoculated with the respective indicator strain at a final concentration of 0.02 (in terms of OD_{600}) and poured on a petri dish. After solidification, a colony of each bacterial isolate to be tested was streaked on the top of the prepared plate. The plates were incubated at 26 °C for the desired time (24h, 48h, 72h and 7 days), followed by the evaluation of inhibition areas.

4.2.2 **Overlay bioassay**

This assay was performed to assess the antibacterial activity of isolates that are not able to grow at 37 ºC against the indicator strains that grow at this temperature (Table 4). Thus, 45 mL of TSA was used to prepare agar plates and one colony of each strain to be tested for antibacterial activity was streaked on it. After incubation at 26 °C, for the desired time, the plates were overlaid with 35 mL of TSA-soft agar containing the indicator strain at a final OD_{600} of 0.02. The presence of inhibition areas was evaluated after overnight growth at 37 °C.

TSA-soft agar: TSB (Merck) medium containing 0.75% (w/v) of agar.

4.2.3 **Sandwich bioassay**

This assay was performed to assess the antibacterial activity of all the isolates that were not tested by overlay bioassay against the indicator strains with optimal growth temperature of 37 ºC or 26 ºC (Table 4). Thus, the plates containing the colonies to be tested were prepared and incubated for the desired period as abovementioned (section 4.2.2). After incubation, the agar of these plates was cut with a sterile bistoury and transferred to the top of TSA-soft agar plates containing the indicator strain. These plates were prepared as described in section 4.2.2. The inhibition areas were evaluated after incubation at 26 ºC or 37 ºC, according to the indicator strain tested (Table 4).

4.3 Evaluation of antibacterial production in liquid cultures

The production of antibacterial compounds in liquid cultures was also tested for isolates able to inhibit at least one indicator strain. To achieve this, each isolate was inoculated in 3 mL of TSB (tryptic soy broth; Merck) in a 14 mL tube and grown overnight at 26 °C with aeration (180 rpm). Subsequently, 1/100 volume of this culture was added to 20 mL of fresh TSB in a 100 mL erlenmeyer. The culture was incubated at 26 ºC at 180 rpm for 1 week. Along this period, 2 mL of culture were collected in each sampling time point (24 h, 48 h, 72 h and 1 week) in order to assess their antagonistic activity.

Each samples was centrifuged at 16,100 x g for 10 min and the supernatant transferred to a clean microcentrifuge tube. Subsequently, each supernatant was filtered using a 0.45 µm cellulose acetate filter (Whatman^{TM}) to obtain cell-free supernatants (CFS). To assess the antibacterial activity of CFS, TSA plates containing the indicator strains were prepared as described in section 4.2. After solidification, wells with 7 mm diameter were poured off the agar and filled with 50 µL of each CFS to be tested. Finally, the plates were incubated overnight at the appropriate temperature for the indicator strains (Table 4) and the inhibition areas were measured.

4.4 Sensitivity of antibacterial compounds to enzymes and heat

The sensitivity of antibacterial compounds to enzymes and heat was determined for the isolates that showed a broader spectrum of activity (SL8, Sma1 and MO15). To achieve this, CFS of each strain were prepared after 48 h of growth in the conditions described in section 4.3.

4.4.1 **Effect of temperature**

The sensitivity to temperature was evaluated by exposing 100 µL of each CFS during 30 min to the following temperatures: 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C. Subsequently, 50 µL of all the treated and untreated CFS were applied to the bioassay agar plates (section 4.4.3).

4.4.2 **Effect of proteolytic activity**

The sensitivity of the same compounds to four different enzymes was also tested: Proteinase K (Roche Applied Science, REF: 03115879001); Trypsin from bovine pancreas (Sigma-Aldrich, T1426); α-Chymotrypsin (Sigma-Aldrich, C4129) and Protease from *Streptomyces griseus* (also known as Pronase E) (Sigma-Aldrich, P8811). The enzymes α-Chymotrypsin and Trypsin were dissolved in Tris-HCl 50 mM buffer (pH 7.5). The Proteinase K and Pronase E were dissolved in Tris-HCl 100 mM buffer (pH 7.5). All were prepared at a concentration of 10 mg/mL. The assay involved the incubation of the CFS with the corresponding enzyme using a 1:1 proportion in a 100 µL reaction for 2 hours at 37 ºC. Afterwards, 50 µL of treated and untreated CFS were applied to the bioassay agar plates (section 4.4.3).

4.4.3 **Preparation of bioassay agar plates**

The sensitivity of antibacterial compounds to heat and enzymes was tested using *Bacillus cereus* Av2 and *Aeromonas hydrophila* ATCC 7966 as indicator strains. Thus, bioassay TSA plates (75 mL) containing each of these strains were prepared as described in section 4.2.1 and wells with 7 mm diameter were poured off the agar. Afterwards, the wells were filled with treated and untreated CFS as well as their respective controls. The plates were incubated at 26 ºC, overnight and the inhibition areas were analyzed.

4.5 Identification of bacterial isolates

4.5.1 **Analysis of 16S rRNA gene**

The bacterial isolates with the ability to inhibit at least one of the indicator strains tested were identified by the analysis of the 16S rRNA gene. This gene was amplified using the RD1 (5'-AAGGAGGTGATCCAGCC-3') and FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') primers described by Edwards *et al.* (1989). Each reaction was performed at a final volume of 25 µL containing the reagents described on Table 5. The amplification parameters were as follows: one step of denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 56 °C for 30 sec and extension at 72 °C for 2 min. A final extension step at 72 °C for 10 min was included. The reaction product was stored at -20 °C until further use or immediately run in an electrophoresis gel (see appendix 6.1). When necessary, the PCR product was purified from agarose gel (appendix 6.2). The sequence of the amplicon (approximately 1600 bp) was submitted to sequencing reaction (STABVIDA-Portugal). The genus of each isolate was determined using the RDP Classifier tool (Wang *et al.*, 2007).

Table 5 – Components used for the amplification of 16S rRNA.

Component of the reaction	Volume
$MgCl_2$ (25 mM)	3 µL
5x GoTaq polymerase buffer	5 µL
dNTP Mix (10 mM each; NZYTech)	0.5 µL
Forward primer (10 mM)	0.75 µL
Reverse primer (10 mM)	0.75 µL
DMSO	1.25 µL
GoTaq polymerase (5 U/ µL; Promega)	0.125 µL
DNA Template *	~ 1 µL
Sterile, distilled water	Until 25 µL

* The DNA template was either 1 µL of a colony suspension (prepared in 100 µL of distilled water and boiled during 5 min) or 1 µL of genomic DNA (appendix 6.3).

4.5.2 **Analysis of *gyrA* gene**

The analysis of 16S rRNA full sequence did not allow the identification to the level specie of the *Bacillus* sp. isolates SL8, SMA1 and MO15. In this situation, the sequence was extremely highly conserved among different species of the same genera, limiting the power of discrimination (Miranda *et al.* 2013). Thus, the sequence of the *gyrA* gene

(referred to the subunit A of DNA gyrase) was also analyzed for these isolates. Its amplification was performed in a final volume of 25 µL as described in Table 5, using the primers gyrA_Bacilus_Fw (5'-CAGTCAGGAAATGCGTACGTCCTT-3') and gyrA_Bacillus_Rv (5'-CAAGGTAATGCTCCAGGCATTGCT-3') described by Koeppel *et al.* (2008). The amplification parameters were as follows: one step of denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. A final extension step at 72 °C for 10 min was included. The reaction product was stored at -20 °C until further use or immediately run in an electrophoresis gel (see appendix 6.1). The amplicon with expected size (approximately 1025 bp) was then submitted to nucleotide sequencing analysis (STABVIDA-Portugal). The sequences obtained were compared with those accessible in databases using the BLASTn web-based tool. Also, a neighbor joining (NJ) phylogenetic tree based on *gyrA* nucleotide sequences of several *Bacillus* species was constructed with the MEGA 5.1 software (Tamura *et al.*, 2011) using the Neighbor-joining statistical method and 1000 bootstrap replications.

4.6 Molecular genotyping of isolates with the same spectrum of activity

The phylogenetic relationship of bacterial strains was investigated by rep-PCR fingerprinting, using BOX and ERIC-PCR (Rademaker and De Bruijn, 1997). Thus, each amplification reaction was performed in a final volume of 12.5 µL containing the reagents at the same final concentration as described in Table 5. For BOX-PCR, the BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') primer was used as forward and reverse primer. In the ERIC-PCR reactions the primers Eric1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') Eric2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') were used. The amplification parameters were as follows: one step of denaturation at 95 °C for 7 min followed by 35 cycles of denaturation at 94 °C for 30 1 min, primer annealing at 53 °C for 1 min and extension at 65 °C for 8 min. A final extension step at 65 °C for 16 min was included. The products of amplification were submitted to electrophoresis as described in the appendix 6.1. The pattern of amplification was analyzed and isolates differing at least in one band were considered different strains.

4.7 Genome mining analysis

The search for biosynthetic clusters (encoding secondary metabolites) on sequenced genomes was performed through an *in silico* analysis. For this purpose, the antiSMASH 2.0 platform was used (Medema *et al.*, 2011). The accession numbers of all *B. amyloliquefaciens* genomes completely assembled presented on Genome Projects Report at NCBI (Table 6) were introduced to submit the analysis. This procedure was done aiming the potential of SL8, Sma1 and MO15 *B. amyloliquefaciens* isolates as producers of antibacterial secondary products, highlighting the lanthipeptides group.

When necessary, the bioinformatic tool Open Reading Frame Finder (ORF Finder) was used as a complementary methodology, leading to the discovery of omitted genes on antiSMASH results.

Table 6 – List of *B. amyloliquefaciens* genomes completely assembled used to perform the searching for secondary products biosynthetic clusters through an antiSMASH search.

Accession number	**Microorganism affiliation**
NC_009725	*Bacillus amyloliquefaciens* FZB42
NC_017061	*Bacillus amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2
NC_022081	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5113
NC_014551	*Bacillus amyloliquefaciens* DSM 7
NC_020272	*Bacillus amyloliquefaciens* IT-45
NC_017190	*Bacillus amyloliquefaciens* LL3
NC_017188	*Bacillus amyloliquefaciens* TA208
NC_017191	*Bacillus amyloliquefaciens* XH7
NC_017912	*Bacillus amyloliquefaciens* Y2
NC_019842	*Bacillus amyloliquefaciens* subsp. *plantarum* AS43.3
NC_016784	*Bacillus amyloliquefaciens* subsp. *plantarum* CAU B946
HG328253	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5033
NC_020410	*Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5036

The identification of each gene function was conducted by searching homologies with other protein sequences on Protein Basic Local Alignment Search Tool (BLASTp) NCBI's database. The prediction of transmembrane helices domains in certain proteins was accomplished by using the TMHMM Server v. 2.0 (Krogh *et al.*, 2001).

4.8 Detection of genetic determinants involved in the biosynthesis of mersacidin

The genome mining analysis of *B. amyloliquefaciens* genomes revealed the presence of two gene clusters encoding the biosynthesis of class 2 lanthipeptides, including mersacidin. Thus, the presence of some of the mersacidin genetic determinants was investigated in the SL8, SMA1 and MO15 isolates by PCR. Each reaction was performed in a final volume of 25 μL as described in Table 5, using genomic DNA as DNA template. In order to amplify the gene encoding the mersacidin modifying enzyme (*mrsM*), the primers comp_MrsM_fw (5'-ATCAGGATCCATGCATACAAAATTCAAAC-3') and comp_MrsM_rv (5'-TAGATCTCGAGTTACAATTTTAGTTCTAAAG-3') were used. The genes encoding the transcriptional regulator (*mrsR1*), the flavoprotein (*mrsD*) and the mersacidin structural gene (*mrsA*) were amplified with the mrsA_fw (5'-ATATACATATGAGTCAAGAAGCTATC-3') and comp_mrsD_fw (5'-ATCAGGATCCATGAGTATTTCAATATTAAAAG-3') primers. The amplification parameters included one step of denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 53 °C (*mrsM*) or 50 ºC (*mrsR1*, *mrsD* and *mrsA*) for 30 sec and extension at 72 °C for 4 min. A final extension step at 72 °C for 10 min was included. All the samples were submitted to electrophoresis as described in the appendix 6.1 in order to identify the presence of the expected amplicons (3000 bp for mrsM and 1600 bp for *mrsR1*, *mrsD* and *mrsA*).

4.8.1 **Screening of *lanM* genes using PCR**

The genome mining analysis revealed that *B. amyloliquefaciens* strains could encode other class II lanthipeptides in their genome, apart from mersacidin. Thus, the presence of genes encoding the modifying enzymes characteristic of class II lanthipeptides (LanM) was evaluated by PCR using degenerated primers designed in this study.

4.8.2 **Amplification of *lanM* genes**

The amplification of *lanM* genes was performed using the primers LanM_TC_fw (5'-ATHYTIGARYTIAAYGTIGC-3') and LanM_TC_rv (5'-ARISWICKIGGYTTRTAIAC-3'). Each reaction was performed in a final volume of 25 μL as described in Table 5, except that the final concentration of primers used was 3 pmol/μL. The amplification parameters were as follows: one step of denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 46 °C for 30 sec and extension at 72

°C for 45 sec. A final extension step at 72 °C for 10 min was included. The PCR was performed for *B. amyloliquefaciens* SL8, SMA1 and MO15 strains and *Bacillus licheniformis* l89 was included in all the reactions as positive control (Caetano *et al.*, 2011; Mendo *et al.*, 2004). All the amplicons possessing the expected molecular weight (300-400 bp) were purified with the NZYtech PCR Purification kit, according to manufacturer's instructions (see appendix 6.3).

4.8.3 **Cloning of *lanM* genes**

Class II lanthipeptides include the gene clusters of two peptide lantibiotics. Thus, it is possible that the same bacteria possess two distinct *lanM* genes. Therefore, all the amplicons were cloned into a vector, prior to sequencing reaction.

Thus, after purification, the amplification products of each strain were ligated to the pCR2.1 plasmid from the TA Cloning Kit (Invitrogen), independently. Each reaction was performed in a final volume of 20 µL, containing approximately 50 ng of purified PCR product, 50 ng of pCR2.1 plasmid, 1X T4 DNA ligase ligation buffer and 4 U of T4 DNA ligase (Fermentas). The reaction was incubated at 22 °C for 30 min and 5 µL was used to transform chemically competent *E. coli* DH5α cells. Briefly, the ligation:cells mixture was placed on ice for 15 min and transferred to 42 °C for 45 sec. Immediately after, the microcentrifuge tube was placed on ice for 2 min and 950 mL of LB medium (Liofilchem) was added. The cells were grown for 1 hour at 37 °C with aeration (180 rpm). Subsequently, the culture was centrifuged at 2,300 x g for 1 min to collect cells. The majority of the supernatant was discharged and the pellet was resuspended in approximately 50 µL of the remaining supernatant. Finally, cells were spread on LB-agar (Nzytech) plates containing 50 µg/mL of kanamycin and 20 µg/mL of X-Gal. The positive clones were selected after overnight growth at 37 °C based on blue-white selection.

X-Gal stock solution: dissolve 20 mg of X-Gal (Fermentas) in 1 mL of dimethylformamide. Protect the solution from the light covering the tube with aluminium foil and store at -20 °C.

Luria-Bertani broth (LB; Liofilchem): tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 5 g/L, final pH=7.0.

Luria-Bertani Agar (LB-agar; Nzytech): tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar 15 g/L, final pH=7.0.

4.8.4 **Analysis of positive clones**

For the analysis of the *lanM* genes, white colonies were chosen (and also one blue colony, to use as a negative control) to perform colony-PCR in order to confirm the incorporation of the fragment into the pCR2.1 plasmid. The amplification of inserts was performed using the primers M13rv (5'-TCACACAGGAAACAGCTATGAC-3') and M13fw (5'-GTTTTCCCAGTCACGAC-3'). Each reaction was performed in a final volume of 25 μL as described in Table 5. However, a colony was used as DNA template. The amplification parameters were as follows: one step of denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 52 °C for 30 sec and extension at 72 °C for 45 sec. A final extension step at 72 °C for 10 min was included. The PCR products were submitted to an electrophoresis and visualized, as described in the appendix 6.1. The nucleotide sequence of the amplicons with the same (or approximate) size as the positive control (more or less 600 bp) was determined by sequencing reaction (STABVIDA-Portugal).

The nucleotide sequences obtained were compared to sequences available in the database using both BLASTn and BLASTx tools.

4.9 Detection of lantibiotic mersacidin on *B. amyloliquefaciens* cultures' supernatant

The evaluation of mersacidin production was achieved using a mass spectrometry profile generated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/MS). It was known that mersacidin can be isolated from culture filtrate by extraction with solvents which are immiscible with water, such as butanol (Chatterjee *et al.*, 1992). Thus, 0.6 volumes of 1-butanol was added to 30 mL of CFS. The CFS was obtained as described before in section 4.3. The mixture was shacked for 1 hour and the organic phase collected after centrifugation for 1 min at 10,000 rpm. Subsequently, the solvent was evaporated on a rotator evaporator at 50 ºC for 12 h. Finally, the extracts were analyzed for the mersacidin presence as described by Herzner *et al.* (2011).

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6 Appendices

6.1 Agarose gel electrophoresis

Analysis of DNA was performed on agarose gel electrophoresis. The samples were mixed with 6X loading buffer in a proportion of 1:6 (v/v) and loaded in a 1% agarose gel. The gel was prepared with 1X of TAE buffer (Bio-Rad) and EtBr (AppliChem) to a final concentration of 0.5 µg/mL added before transferred the melted agarose in the running tray. All electrophoresis running included 0.5 µg of the DNA marker DNA Ladder Mix (Fermentas). Electrophoresis was generally performed at 120V for the desired time and the DNA was analyzed under UV light and the corresponding image was acquired in the ATTO image acquisition system.

Loading buffer 6X: 2.5 mg/mL of bromophenol blue, 2.5 mg/mL of xylene cyanol FF and 30% (v/v) glycerol; stored at 4°C.

6.2 Purification of DNA from agarose

The purification of DNA from agarose was performed using the kit NZYGelpure (NZYTech), according to manufacturer's instructions. Briefly, the desired DNA fragment was excised from the agarose gel with a clean scalpel and placed in a 1.5 mL microcentrifuge tube. The gel slice was weighted and 300 µL of Binding Buffer were added for each 100 mg of gel. In the cases when the gel slice weights more than 400 mg, two microcentrifuge tubes were used. The tube containing the gel slice was incubated at 50 °C for 10 min and shacked occasionally until agarose was completely dissolved. The mixture was transferred into the NZYTech spin column placed into a Collection tube (2 mL). Each collection tube has a maximum limit of 700 µL of sample. The samples were added and centrifuged at top speed (13,200 rpm) for 1 min. The flow-through was discarded and the column was placed back to the collection tube. This step was repeated until the complete addition of the sample to the NZYTech spin column. For a direct DNA sequencing, 500 µL of Binding Buffer were added to the column, which was centrifuged at top speed for 1 min. The flow-through was discarded. The DNA was washed with 600 µL of Binding Buffer and the centrifugation was performed as in the preceding step. The flow-through was discharged and the column was centrifuged for an additional minute for the complete removal of residual ethanol. The NZYTech spin column was placed in a clean 1.5 mL microcentrifuge tube and the DNA was eluted in 30 to 50 µL of Elution Buffer, concerning the subsequent application. The elution was performed after 2 min of

incubation at room temperature by centrifugation at top speed for 1 min. The sample was stored at -20 °C until further use.

6.3 Genomic DNA extraction

In some bacterial isolates, the extraction of genomic DNA was performed using DNeasy Blood & Tissue Kit (Qiagen). The bacteria were grown overnight on 5 mL of TSB (Merck) in a 50 mL falcon, at 26 °C, 180 rpm. For each bacterium, 2 mL of culture (1 mL in each 1.5 mL microcentrifuge tubes) were used for genomic DNA extraction. The cells were harvested for 10 min at top speed (16,100 x g) and the supernatant were discarded. The pellet were resuspended in 180 µL of enzymatic lysis buffer in one of the 1.5 mL microcentrifuge tubes and the resuspension were completely transferred to the other 1.5 mL microcentrifuge tube and also resuspended. This mixture were incubated at 37 °C for 30 min. After that time, 25 µL of proteinase k were added to the sample and mixed by vortexing. Then, 200 µL of Buffer AL were also added and mixed by vortexing. The samples were incubated at 70 °C for 30 minutes. Next, 200 µL of ethanol (96-100%) were added to the sample and mixed by vortexing. The mixture was then pipetted to the DNeasy Mini Spin column, which is placed in a 2 mL collection tube. The column was centrifuged at top speed (16,100 x g) for 1 min. The flow-through was discarded as also the collection tube. The DNeasy Mini Spin column was then placed on a new and clean collection tube and 500 µL of Buffer AW1 were added. A centrifugation of 1 min at top speed took place. The flow-through and the collection tube were discarded. The column was placed into a new 2 mL collection tube and 500 µL of Buffer AW2 were added. The column was centrifuged at top speed for 3 min. The flow-though was discarded, but this time the column was placed in the same collection tube and centrifuged at top speed during 1 min. This step is to ensure the membrane was dried. Once again, the flow-though and the collection tube were both discarded and the DNeasy Mini Spin column was placed in a clean 1.5 mL microcentrifuge tube and 100 µL of Buffer AE (elution buffer) were pipetted into the column. After this procedure, 5 µL of the genomic DNA solution were run in an electrophoresis gel, in order to confirm its presence on the sample.

Lysis buffer preparation: 20 mM Tris-HCl pH 8; 2mM sodium EDTA; 1,2% Triton X-100. Autoclave this solution. This buffer is also composed by 20 mg/mL of lysozyme, which were added only before use and after autoclaving.

6.4 PCR product purification

The purification of PCR products was performed using NZYGelpure (NZYTech) according to the manufacturer's instructions. Briefly: the volume of the PCR reaction solution was transferred to a 1.5 mL microcentrifuge tube and distilled water was added until a final volume of 100 µL. Then, five volumes of binding Buffer were added and mixed well by vortexing. Thereafter, 100 µL of isopropanol were added and mixed well by vortexing. The mixture was applied to an NZYTech spin column, incubated at room temperature for 2 min and centrifuged at top speed (16,100 x g) for 1 min. The flow-through was discarded and 600 µL of Wash Buffed were added to the spin column. The centrifugation was performed as described previously and the flow-through was discarded. An additional 1 min centrifugation was performed to remove the residual ethanol. Finally, the NZYTech spin column was placed into a clean 1.5 mL microcentrifuge tube and 20 µL of distilled water were added to the center of the column. The DNA-containing column was incubated at room temperature during 2 min and then centrifuged for 1 min at top speed to elute the DNA. The sample was stored at -20 °C until further use.