MARGARETE ALICE FONTES SARAIVA

INHIBITORY SUBSTANCES PRODUCED BY *Lactococcus lactis* STRAINS ISOLATED FROM NATURALLY FERMENTED SAUSAGE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 13 de abril de 2012.

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Hilário Cuquetto Mantovani

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Célia Alencar de Moraes
(Orientadora)

A Deus e à minha família, dedico.

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BIOGRAFIA

MARGARETE ALICE FONTES SARAIVA, filha de Joaquim da Silva Saraiva e Lúcia da Silva Fontes, nasceu em Viçosa, Estado de Minas Gerais, no dia 09 de julho de 1973.

Em agosto de 1997 graduou-se em Economia Doméstica pela Universidade Federal de Viçosa. No período de julho de 1996 a janeiro de 1998, foi bolsista de iniciação científica pela FAPEMIG no Departamento de Microbiologia.

Em março de 1998, iniciou o Programa de Pós-Graduação em Microbiologia Agrícola, no nível de Mestrado com defesa da tese realizada em 17 de dezembro de 2000. No período de 2001 a 2007 dedicou-se a experiência profissional.

Em março de 2008, iniciou o Doutorado em Microbiologia Agrícola. No período de março a outubro de 2011 foi bolsista pela CAPES no Programa de Desenvolvimento de Estágio no Exterior (Bolsa Sanduíche), tendo o estágio sido desenvolvido no Laboratory Microbial of the Gene Technology sob orientação do Dr. Ingolf Figved Nes vinculado ao Department of Chemistry, Biothecnology and Food Science, Norwergian University of Life Sciences, na cidade de Ǻs, Noruega. Em 13 de abril de 2012 foi realizada a defesa da tese de Doutorado.

SUMÁRIO

RESUMO

SARAIVA, Margarete Alice Fontes, D.Sc., Universidade Federal de Viçosa, abril de 2012. **Substâncias inibidoras produzidas por culturas de *Lactococcus lactis* isoladas de salame fermentado naturalmente**. Orientadora: Célia Alencar de Moraes. Coorientadores: Marisa Vieira de Queiroz e Maria Cristina Baracat Pereira.

Neste trabalho, as bacteriocinas produzidas por culturas de *Lactococcus lactis* isoladas de salame fermentado naturalmente no Brasil foram purificadas e identificadas. O sequenciamento dos produtos amplificados por PCR comfirmou que os genes estruturais das bacteriocinas em todas as culturas de *L. lactis* são idênticos ao do lantibiótico nisin Z. Plasmídeos foram detectados em todas as culturas de *L. lactis* e foi observado que culturas curadas foram capazes de produzir bacteriocinas, indicando que os genes relacionados à produção da bacteriocina estão localizados no cromossomo. As bacteriocinas foram purificadas utilizando precipitação das proteínas com sulfato de amônio, cromatografias de troca iônica e de fase reversa, e suas massas moleculares foram confirmadas por MALDI-TOF-TOF. A produção da bacteriocina foi investigada sob diferentes condições de crescimento. Em todas as culturas, a produção de bacteriocina foi paralela ao crescimento celular e alcançou atividade máxima na fase estacionária. Todas as culturas apresentaram produção máxima de bacteriocinas em valores de pH 6.0 e 6.5, em condições de aerobiose e em temperaturas de 25 °C e 30 °C, em meio LAPTg. Triptona e peptona de caseína foram as melhores fontes de nitrogênio para produção das bacteriocinas em todas as culturas. A fonte de carbono foi um dos principais fatores que influenciou a produção de nisin Z. Sacarose foi a fonte de carbono mais eficiente na produção de bacteriocinas pelas culturas de *L. lactis* ID1.5, ID8.5, PR3.1 e PD4.7, enquanto a frutose foi a fonte de carbono mais eficiente para *L. lactis* ID3.1. As culturas apresentaram valores de atividades de bacteriocina diferentes, sendo as máximas atividades observadas nas culturas de *L. lactis* ID1.5 e ID8.5. As sequências dos promotores *nisZ* e *nisF* obtidas de todas as culturas foram idênticas entre si, contudo outros fatores podem estar envolvidos nas diferenças da produção das bacteriocinas entre as culturas. Além disso, dois compostos antimicrobianos, de baixo peso molecular, produzidos por *L. lactis* ID1.5, denominados neste estudo AI e AII, foram purificados e parcialmente caracterizados. O composto AI apresentou um espectro antimicrobiano principalmente contra culturas de *L. lactis*, incluindo a própria cultura produtora. *L. lactis* LMGT 2122, *Bacillus subtilis* DSMZ 347, *Listeria innocua* BL86/26B, *Streptococcus pneumoniae* TIGR 4 e *Pseudomonas aeruginosa* foram os micro-organismos mais sensíveis ao composto AII. A atividade antimicrobiana de ambos compostos foi drasticamente reduzida após tratamento com tween 80, mas a atividade foi mantida após o tratamento térmico e com proteases. Os compostos AI e AII apresentaram propriedades distintas de outras substâncias antimicrobianas produzidas por *L. lactis* e relevante efeito inibitório contra patógenos do sistema respiratório.

ABSTRACT

SARAIVA, Margarete Alice Fontes, D.Sc., Universidade Federal de Viçosa, April, 2012. **Inhibitory substances produced by *Lactococcus lactis* strains isolated from naturally fermented sausage**. Adviser: Célia Alencar de Moraes. Co-advisers: Marisa Vieira de Queiroz and Maria Cristina Baracat Pereira.

In this study, bacteriocins produced by *Lactococcus lactis* strains isolated from naturally fermented sausage in Brazil were purified and identified. PCR product sequencing confirmed that the structural bacteriocin genes of the all strains are identical to lantibiotic nisin Z. Plasmids were detected in all strains of *L. lactis*, and it was observed that cured derivatives from all strains were able to produce bacteriocins, indicating that the genes encoding the bacteriocin are located on the chromosome. Bacteriocins were purified to homogeneity from culture supernatants by ionic exchange and reversed-phase chromatography and their molecular masses were confirmed by MALDI-TOF/TOF. The production of the bacteriocin was investigated under different growth conditions. For all of the strains, bacteriocin production appeared to parallel cell growth and reached its maximal activity at the stationary phase. All strains produced bacteriocin efficiently at initial pH values of 6.0 and 6.5, under aerobic conditions and at 25 °C and 30 °C in LAPTg medium. Tryptone and casein peptone were found to be the optimal organic nitrogen source for the bacteriocin production for all strains. Carbon source appears to be a major control mechanism for nisin Z production. Sucrose was the most efficient carbon source for bacteriocin production of *L. lactis* ID1.5, ID8.5, PR3.1 and PD4.7, while fructose was the most efficient carbon source for strain *L. lactis* ID3.1. The strains presented different activity values of bacteriocins, maximal activity was recorded by *L. lactis* ID1.5 and ID8.5. The *nisZ* and *nisF* promoter sequences obtained from all strains are identical, suggesting that there should be other factors involved in difference of production of bacteriocins among those strains. In addition, two cell-bound antimicrobial compounds, of low molecular weight, produced by *L. lactis* ID1.5 were purified and partially characterized. After purification by cationic exchange, solid phase C_{18} column and three runs of reversed-phase chromatography, antimicrobial activity was recovered with 60% and 100% 2-propanol, suggesting that more than one antimicrobial compound, called in this study AI and AII, were produced by *L. lactis* ID1.5. Compond AI has a spectrum of antimicrobial activity mainly against *L. lactis*, including its producer strain. *L. lactis* LMGT 2122, *Bacillus subtilis* DSMZ 347, *Listeria innocua* BL86/26B, *Streptococcus pneumoniae* TIGR 4 and *Pseudomonas aeruginosa* seem to be the organisms most sensitive to compound AII. The antimicrobial activity of both compounds was drastically reduced by treatment with tween 80, but it was present after heat and proteases treatments. These compounds show distinct properties from other antimicrobial substances produced by *L. lactis*, and have a significant inhibitory effect against clinically important respiratory pathogens.

INTRODUCTION

For many years consumers have requested alternative food grade preservatives without chemical additives. The use of antimicrobial produced by lactic acid bacteria (LAB) instead of chemical additive would enable the food industry to grant this request. The studies of diverse bacteriocins have been focused on their use as food preservatives, because they can inhibit food-born spoilage and pathogenic microorganisms. Furthermore, bacteriocins have demonstrated a remarkable potential as therapeutics for medical or veterinary uses, alone or in combination with other antimicrobial substances. A number of bacteriocins are produced by *Lactococcus lactis* and they have attracted particular attention for commercial applications.

L. lactis is quite desirable for industrial application because it is homofermentative, highly productive and generally recognized as safe. These bacteria are used in food production, and their antimicrobial metabolites may be considered safe agents for preventing growth of undesirable microorganisms. In addition, the incorporation of bacteriocin producing lactococci as starter or adjunct cultures in the manufacture of fermented foods provides an attractive and economic alternative to the addition of purified bacteriocins, indeed, metabolic compounds produced during fermentation are no longer considered as additives. Bacteriocin formation *in situ* may also contribute to the dominance of the producing strains over other bacteria during food fermentation.

Considering the growing interest for commercial application of bacteriocins, many aspects have been studied extensively in order to improve their production rate and productivity. Thereby, the understandings of factors that influence bacteriocin production as well as, the selection of good producer strains are important aspects to enhance peptide yield and to reduce production costs.

Appropriate cultures have been isolated and selected from naturally fermented sausages, because they possess distinctive qualities, partly due to the proprieties of raw material and characteristics of the technology used, but also due to the specific composition of the indigenous microbiota the manufacturing location. In addition, the use of starter cultures has increased with the demand for reducing/eliminating pathogen in fermented sausages.

The aim of the present work was to identify the bacteriocin produced by strains of *L. lactis* isolated during natural fermentation of Italian type sausage in Brazil and investigate production in bach culture. Efforts were also made to characterize two cell-bound antimicrobial compounds produced by *L. lactis* ID1.5, with inhibitory proprieties against Gram positive and Gram negative bacteria. This work was carried out in the Laboratory of Industrial Microbiology, Department of Microbiology at Federal University of Viçosa and in the Laboratory Microbial of the Gene Technology, Department of Chemistry, Biotechnology and Food Science at Norwegian University of Life Sciences, in Aas, Norway.

This thesis consists of the following four chapters: I, *Lactococcus lactis*: a versatile lactic acid bacteria producer of antimicrobial peptides (a review); II, Nisin Z production by wild strains of *Lactococcus lactis* isolated from naturally fermented sausage; III, Detection and activity of two new cell-bound antimicrobial compounds produced by *Lactococcus lactis* ID1.5.; IV, Identification of the natural variant nisin Z produced by *Lactococcus lactis* PD6.9: a potential anti-*Sthaphylococcus aureus* bacteriocin.

CHAPTER 1

Lactococcus lactis: a versatile lactic acid bacteria producer of antimicrobial peptides

1. *Lactococcus lactis*

The genus *Lactococcus* contains five species, *L. lactis*, *L. garviae*, *L. plantarum*, *L. piscium* and *L. raffinolactis* (Basaran *et al.*, 2001). In general, they are gram positive coccoid bacteria, 0.5-1 µm in diameter, which occur in pairs or more or less long chains. They are facultative anaerobes with an optimum growth temperature of 30 ºC. They can grow between 10 ºC and 45 ºC and can survive at 60 ºC for 30 min (Neves *et al.*, 2005; Tauber and Geis, 2006). The *Lactococcus* genus can tolerate up to 6,5% NaCl, and pH up to 9.6 (Tauber and Geis, 2006). *Lactococcus lactis* is perhaps the most extensively studied bacterium, because it is used as starter cultures for the production of fermented dairy products. It grows in a variety of niches and is frequently encountered on vegetable substrates as well as in dairy environment (Bolotin *et al.*, 2001). It has been also isolated from fish, river water, animal and human (Zendo *et al.*, 2003; De Kwaadsteniet *et al.*, 2008; Espeche *et al.*, 2009; Gao *et al.*, 2011). Among the species *L. lactis*, only *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* are used as starter cultures for the production of fermented dairy products (Basaran *et al.*, 2001). In dairy products, *L. lactis* cultures are responsible for production of lactic acid rapidly from lactose and for various enzymatic conversions which affect the organoleptic characteristics of the fermented product (Bachmann *et al.*, 2009). For instance, the caseinolytic activity of lactococcal proteases and peptidases has a major influence on cheese texture (Kunji *et al.*, 1996), in the flavor of cheese especially after cell lysis (Smit *et al.*, 2005) and to the liberation of health-enhacing bioactive peptides from milk (Leroy and De Vuyst, 2004).

The genomes of several *L. lactis* strains have been sequenced (Table 1), but strains IL1403 (Bolotin *et al.*, 2001) and MG1363 (Wegmann *et al.*, 2007) are most commonly used in laboratories. Sequencing of some *L. lactis* plasmids reveled important traits including lactose catabolism, proteolysis, peptide and amino acid uptake, exopolisaccharide, bacteriocin production, bacteriophage resistance and citrate permease (Mills *et al.*, 2006). The success of metabolic engineering approaches in *L. lactis* together with its high transformation efficiency indicates that this lactic acid bacteria (LAB) is a promising candidate for synthetic biology applications (Holo and Nes, 1989). In addition, some of the first synthetic promoters were designed for *L. lactis* (Morello *et al*., 2008).

The relative simplicity of *L. lactis* metabolism that converts sugar by the glycolytic (homofermentative) pathway to pyruvate, generating energy mainly through substrate level phosphorylation and reducing equivalents (NADH) at the level of glyceraldehyde 3-phosphate dehydrogenase, makes it an attractive target for development of effective cell factories (Neves *et al.*, 2005). Regulation of glycolysis in *L. lactis* has been the subject of intensive research. Key glycolytic enzymes, as pyruvate kinase (PK) (Ramos *et al.*, 2004), phosphofructokinase (Fordyce *et al.*, 1982), fructose 1,6-bipfosphate aldolase (Crow and Thomas, 1982), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Even *et al.*, 1999) and lactate dehydrogenase (LDH) (Crow and Pritchard, 1977; Neves *et al.*, 2005) were characterized. Under certain conditions, for example anaerobic, a metabolic shift from homolactic (lactate production) to mixed acid fermentation (ethanol, acetate and formate production) can occur in *L. lactis* (Cocaign-Bousquet *et al.*, 1996). According to the literature, the major transport system mediating the uptake of glucose in *L. lactis* is the mannose-PTS system (PTS^{man}). Two other important sugars, fructose and sucrose, are transported by PTS systems. Lactose can also be translocated either via a lactose-PTS to yield lactose 6-phosphate or by a permease. Lactose uptake by a permease is followed by hydrolysis via β-galactosidase, yielding glucose and galactose. Galactose is metabolized by the tagatose 6-phosphate pathway and by the Leloir pathway (Neves *et al.*, 2005).

L. lactis is also good candidate for the production and delivery of heterologous proteins and peptides that have potential therapeutic activity, because it is acid and bile resistant and well adapted to function as vehicles for oral delivery of vaccine antigens (Teusink and Smid, 2006).

Table 1. Sequenced *Lactococcus lactis* genomes

Strain	Origin	NCBI accession number	Genome size	Plasmids	GC%	References
IL1403	Cheese	AE005176	2.37 Mb	0	35.5	(Bolotin *et al.*, 2001)
SK11	Cheese	NC008527	2.44 Mb	5	35.9	(Makarova *et al.*, 2006)
MG1363	Cheese	NC 09004	2.53 Mb	0	35.8	(Wegmann *et al.*, 2007)
KF147	Mung bean sprouts	CP001834	2.59 Mb	1	34.9	(Siezen *et al.*, 2010)
CV56	Vagina of healthy women	CP002365	2.40 Mb	5	35.0	(Gao *et al.*, 2011)
A76	Cheese	CP003132	2.45 Mb	4	35.9	(Bolotin *et al.*, 2012)

2. Bacteriocins: general aspects

The term bacteriocin was originally coined to describe the *Escherichia coli* colicin type of antimicrobial proteins. However, nowaday, this term is used to define a larger group of ribosomally synthesized antimicrobial peptides (Nes and Johnsborg, 2004; Nes *et al.*, 2007b). These bacteriocins play an important role in the ecology of both Gram negative and Gram positive bacteria, where they offer an advantage for the producer over non-producing cells in the competition for common resources. The spectrum of inhibition for bacteriocins is generally rather narrow, as they mainly kill bacteria closely related to their producers, although some peptides show antimicrobial activity across several bacterial genera (Cotter *et al.*, 2005). Some bacteriocins can inhibit specific food spoilage bacteria and foodborne pathogens such as clostridia, *Listeria monocytogenes* and *Staphylococcus aureus*. They can also function as agents to manipulate microbial populations in food systems (i. e., promote the growth of strains with desirable properties by inhibiting competing strains) (Cotter *et al.*, 2005). Promising results have been reported recently for combined treatments (such as HHP, bacteriocin, or moderate heat) on the inactivation of *Clostridium botulinum* and *Bacillus cereus* spore (Black *et al.*, 2008; Gao and Ju, 2008). Such combined treatments could improve food safety and decrease the impact of the intense heat treatments required for endospore inactivation. In addition, the residual bacteriocin in the finished product affords natural protection against bacterial growth and toxin production during the product shelf life (Galvez *et al.*, 2010).

In the medical field there is a great interest in novel antimicrobial compounds, especially in the light of the ever-increasing antibiotic resistance among pathogenic bacteria. Different bacteriocins are known to target many Gram-positive pathogens *in vitro*, including emerging antibiotic resistant bacteria such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) (Arnusch *et al.*, 2008; Piper *et al.*, 2010). From a health perspective, a study has shown that the bacteriocin Abp118, produced by *Lactobacillus salivarius* UCC118, is effective in reducing *L. monocytogenes* infections in mice, suggesting a role for bacteriocins as anti-infective agents (Corr *et al.*, 2007). This study has led to increased interest in the use of bacteriocin-producing strains as probiotic bacteria.

Bacteriocins constitute a heterogeneous group of peptides with great variations in size, structure and mode of action. Classification is therefore crucial to get an overview of the characteristics and proprieties of the various peptides. However, the classification of the bacteriocins from Gram positive has been continuously revised to accommodate the discovery of new types of antimicrobial peptides in this group. Bacteriocins from Gram positive bacteria are divided in two major groups. Lantibiotics (class I) are small peptides of 19-38 amino acids containing post-translational modifications. They are characterized by thiother-based internal ring structures (known as lanthionine or β-methyllanthionine), which are formed by the dehydration of selected serine and/or threonine residues and the subsequent formation of a thiol-bridge between some of the dehydrated residues and neighboring cysteine residues. The lantibiotics may also contain other unusual amino acids formed by post-translational processes, such as D-alanine (Chatterjee *et al.*, 2005; Rink *et al.*, 2007; Bierbaum and Sahl, 2009). Because of large structural variations, 11 subclasses have been suggested to lantibiotics (Bierbaum and Sahl, 2009). Nisin, Subtilin, Lacticin 481 and Lacticin 3147 are the best known member of the lantibiotics. The other main group is non-lantibiotic bacteriocins, Class II. They are small heat-stable peptides with no modified amino acids (except formation of disulphide bridges and circularization of cyclic peptides) (Cotter *et al.*, 2005; Nes *et al.*, 2007b). Many excellent reviews have described the bacteriocins in detail (Nes and Holo, 2000; Chatterjee *et al.*, 2005; Cotter *et al.*, 2005; Nes *et al.*, 2007b; Maqueda *et al.*, 2008; Asaduzzaman and Sonomoto, 2009; Bierbaum and Sahl, 2009; Papagianni and Anastasiadou, 2009) and the classification scheme for these bacteriocins proposed by (Klaenhammer, 1993; Cotter *et al.*, 2005; Nes *et al.*, 2007b) is shown (Table 2).

The typical mode of action used by bacteriocins to kill target cells is creating pores in the membrane by similar mechanisms as positively charged eukaryotic antimicrobial peptides: bacteriocins bind to anionic lipids and insert unspecifically into the phospholipid bilayer, where in aggregation of peptides leads to the formation of short-lived pore-like structure (Hechard and Sahl, 2002). Pore formation causes leakage of low molecular weight compounds (e.g., ions K^+, PO_4^{2-}, H^+) leading to dissipation of the proton motive force (the transmembrane electric potential, $\Delta\Psi$, and pH gradient, ΔpH) that is deleterious to cells (Christensen and Hutkins, 1992; Hechard and Sahl, 2002). Pore size, stability and selectivity of transferable molecules vary between different bacteriocins. Not all bacteriocins bind to a receptor in the cell membrane. Nisin and some other lantibiotics (class I) specifically bind to the cell wall precursor molecule lipid II, to form lethal pores and/or inhibit cell wall synthesis in sensitive cells (Wiedemann *et al.*, 2001; Asaduzzaman and Sonomoto, 2009). In addition, the membrane proteins belonging to a sugar transporter, the mannose phosphotransferase system (man-PTS) is the target receptor for bacteriocins pediocin-like and lactococcins A and B (Diep *et al.*, 2007; Kjos *et al.*, 2011).

Bacteriocin production is ubiquitous in the bacterial world, and dissemination of this trait has probably been facilitated by the fact that genetic determinants responsible for bacteriocin production often are located on mobile genetic elements, such as conjugative plasmids or transposons (Cotter *et al.*, 2005; Mills *et al.*, 2006).

Table 2. Classification schemes previously proposed for the bacteriocins produced by Gram-positive bacteria

Scheme	Class I	Class II	Class III	Class IV
(Klaenhammer, 1993)	Lantibiotics IA: nisin-like, elongated, screw-shaped, cationic molecules; IB: duramicin-like, globular molecules with low net negative charge;	Non-lantibiotics IIA: pediocin-like antilisterial bacteriocins; IIB: two-peptide bacteriocins; IIC: thiol-activated peptides requiring reduced cysteine residues for activity;	Large heat-labile proteins	Complex protein Composed of protein plus one or more chemical (lipid, carbohydrate) required for activity;
(Cotter *et al.*, 2005)	Lanthionine containing bacteriocins/lantibiotics Includes both single and two-peptides lantibiotics;	Non-lanthionine; Heterogeneous class of small peptides includes pediocin-like (subclass IIa), two-peptide (subclass IIb), cyclic (subclass IIc), non pediocin single linear peptides (subclass IId);	Bacteriolysins, non bacteriocin lytic proteins, large heat-labile proteins;	Bacteriocins (with non proteinaceous moieties) are not include as no member have been demonstrated;
(Nes *et al.*, 2007b)	Lantibiotics AI: modified by a dual-enzyme system generally referred to as LanB and LanC; AII: modified by a single enzyme LanM proteins	Non-modified heat stable bacteriocins IIa: the pediocin-like and strong antilisterial bacteriocins; IIb: the two-peptide bacteriocins; IIc: the bacteriocins that are not included into any other subclasses; IId: the leaderless bacteriocins; IIe: the peptide bacteriocins that are formed by a specific degradation of larger protein	Large heat-labile bacteriocins; Proteins with enzymatic activities that cause cell wall degradation	Cyclic bacteriocins

The genes that are related to the bacteriocin production are usually clustered in operons or regulons. Generally, these clusters include structural genes proper and genes for immunity, processing, transport and of the regulatory systems (Nes and Johnsborg, 2004). Based on the data obtained, there is a tendency that in general the lantibiotics operons are more complex than those encoding no-lantibiotics because they need additional genes encoding enzymes for posttranslational modifications (Kotel'nikova and Gel'fand, 2002; Dimov *et al.*, 2005). An unusual genomic organization is described for the bacteriocin carnobacteriocin BM1 produced by *Carnobacterium piscicola*. While its structural gene is located on the bacterial chromosome, its expression is dependent on the presence of a 61 kb plasmid which carries some of the genes required for the export and the immunity (Quadri *et al.*, 1994).

Bacteriocins are usually synthetized as an inactive pre-peptide that includes an N-terminal leader sequence. The leader sequence presumably maintains the bacteriocin in an inactive form within the producer cell, facilitates interaction with the transporter (Oman and Van Der Donk, 2010). Subsequent cleavage of the pre-peptide at a sequence specific processing site removes the leader sequence from the antimicrobial molecule concomitantly with its export to the outside of the cell (Ennahar *et al.*, 2000). The export of bacteriocins is usually achieved by a dedicated membrane-associated ATP-binding cassete (ABC) transporter that can also contain a proteolytic N-terminal domain belonging to the family of cysteine proteases that is responsible for cleavage of the leader peptide, or a *sec*-independent transporter system (Ennahar *et al.*, 2000; Cotter *et al.*, 2005).

Bacteriocin production has been shown to be regulated by two-component system (Quadri, 2003). In such cases, the inducers are highly specific auto induced bacteriocin-like peptides that are commonly referred to as peptide pheromones (Kotel'nikova and Gel'fand, 2002). The induction of gene expression is achieved by an accumulation of the pheromone peptide through a low constitutive production. When a limiar concentration of the peptide pheromone has been reached, the peptides binds to its receptor (the histidine protein kinase), followed by a phosphorylation cascade leading to phosphorylation of the cognate response regulator, which binds and activates the regulated promoters. A burst in the expression of genes takes place, and mass production of bacteriocin results (Nes *et al.*, 2007a).

Several bacteriocins can be produced in much higher amounts during *in vitro* fermentations under optimal physical and chemical conditions. Due to the complexity of the food matrix and the difficulty of quantifying bacteriocin activities in foods, *in vitro* studies can be performed to stimulate and study the *in situ* functionality of bacteriocinogenic starter (De Vuyst and Leroy, 2007; Trmčić *et al.*, 2010). However, even during fermenter experiments, considerable differences in activity yields are obtained, and an influence of the environmental process conditions on the obtained bacteriocin activity can be seen (De Vuyst and Vandamme, 1992; Simsek *et al.*, 2009). In most cases, bacteriocin production appears to be regulated and is consequently produced only under suitable growth conditions. The cultivation conditions directly affect bacteriocin production. Several factors including carbon and nitrogen sources, and temperature, pH and agitation seem to play a crucial role in bacteriocin production (Cheigh *et al.*, 2002; Todorov and Dicks, 2009). Defining optimal growth conditions therefore represents a major hurdle in the bacteriocin screening system used today.

Bacteriocins can be introduced into food in different ways: bacteriocins can be produced *in situ* in fermented food by bacterial cultures that substitute for all or part of the starter culture and purified or semi-purified bacteriocins directly to food (Cotter *et al.*, 2005). Nisin is the only bacteriocin having a status as a food additive designated as E234 (Eec, 1983). Different strategies for the addition of LAB bacteriocins have been proposed, especially those not accepted as food additives. They may be produced *ex situ* and added as spent fermentation preparations or produced *in situ* in the food by bacteriocinogenic LAB. Food starters known to produce bacteriocins are on the market (Danisco®), resulting in bacteriocin production during food processing. Bacteriocin-producing starter cultures are commercially used both in dairy and meat fermentation industry. Preparations containing nisin (commercialized in the form of Nisaplin™) or pediocin PA-1/AcH (commercialized in the form of ALTA 2341) are commercially available (Cotter *et al.*, 2005).

2.1. Bacteriocins of *Lactococcus lactis*

All lactococcal bacteriocins characterized thus far belong to either class I or class II (Table 3). Members of the class I (lantibiotics) include three lactococcal bacteriocins: nisin, lacticin 481 and lacticin 3147 (Table 3).

Nisin is the most characterized bacteriocin among the antimicrobial peptides produced by lactic acid bacteria. The inhibitory effects of nisin cells in food matrices are well described and have been shown to inhibit the growth of a wide range of gram positive bacteria including food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium perfringens, S. aureus* and *L. monocytogenes* (Hampikyan, 2009; Mitra *et al.*, 2011). Nisin has been commercially exploited on a large scale, due to its low toxicity, stability during processing and storage, efficacy at low concentration, economic viability, and the absence of deleterious effects on the food (Hurst *et al.*, 1981; And and Hoover, 2003). Moreover, this safe and natural food additive has been utilized recently in clinical applications as an antimicrobial agent against the causative bacteria of bovine mastitis, and therefore it has been incorporated into commercial products that are used as an alternative treatment to antibiotics (Sears *et al.*, 1992; Wu *et al.*, 2007).

Four natural nisin variants produced by *L. lactis* have been identified and characterized to date. These are nisin A (Gross and Morell, 1971) was first discovered and one of only bacteriocin used commercially. Nisin Z, was isolated from *L. lactis* NIZO 22186 from a dairy product, differs from nisin A by one amino acid residue in the final active peptide, His27Asn (Fig. 1) (Mulders *et al.*, 1991). Nisin F is produced by *L. lactis* F10 isolated from a freshwater catfish in South Africa, and it differs from Nisin A with respect to two amino acid residues, His27Asn (as Nisin Z) and Ile30Val (Fig. 1) (De Kwaadsteniet *et al.*, 2008). Nisin Q, produced by *L. lactis* strain 61-14 isolated from river in Japan, contains both of the substitution observed in Nisin F as well as two additional variations, Ala15Val and Met21Leu (Fig. 1) (Zendo *et al.*, 2003).

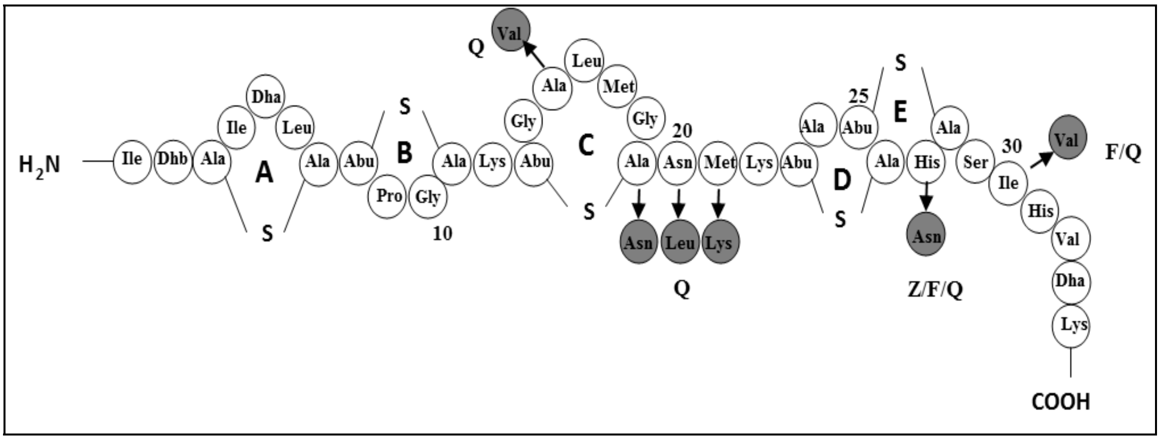

Fig. 1. Structure of nisin A and putative variants nisins Z, Q and F. Gray circles indicate amino acid residues different among the natural nisin variants. Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, 2-aminobutiric acid; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine (adapted from Field *et al*., 2008).

Table 3. Peptide-bacteriocins isolated from *Lactococcus lactis*

Bacteriocin	Class	Producer strain	Mass (Da) (amino acids)	References
Nisin A	I	*L. lactis* ATCC 11454	3353.5 (34)	(Gross and Morell, 1971)
Nisin Z	I	*L. lactis* N8	3330.9 (34)	(Mulders *et al.*, 1991)
Nisin Q	I	*L. lactis* 61-14	3327.5 (34)	(Zendo *et al.*, 2003)
Nisin F	I	*L. lactis* F10	3315.4 (34)	(De Kwaadsteniet *et al.*, 2008)
Lacticin 481	I	*L. lactis* CNRZ 481	2991.8 (27)	(Piard *et al.*, 1992)
Lacticin 3147	I	*L. lactis* DPC3147	A1 3449.3 (30) A2 3005.9 (29)	(Ryan *et al.*, 1996)
Lacticin NK34	ND	*L. lactis*	ND (ND)	(Kim *et al.*, 2010)
Lacticin Z	IId	*L. lactis* QU14	5968.9 (53)	(Iwatani *et al.*, 2007)
Lacticin Q	IId	*L. lactis* QU5	5525.4 (53)	(Fujita *et al.*, 2007)
Lacticin RM	ND	*L. lactis* EZ26	ND (ND)	(Yarmus *et al.*, 2000)
Lactococcin A	IIc	*L. lactis* LMG 2130	5797.31 (54)	(Holo *et al.*, 1991)
Lactococcin B	IIc	*L. lactis* 9B4	5346.8 (54)	(Van Belkum *et al.*, 1992)
Lactococcin DR	ND	*L. lactis* ADRIA85LO30	ND (ND)	(Rince *et al.*, 1994)
Lactococcin G	IIb	*L. lactis* LMG 2081	Gα 4364.5 (39) Gβ 4128.3 (35)	(Nissen-Meyer *et al.*, 1992)
Lactococcin K	ND	*L. lactis* MY23	ND (ND)	(Kim *et al.*, 2006)
Lactococcin MMT24	IIb	*L. lactis* MMT24	Pepα 3765.3 (ND) Pepβ 3255.2 (ND)	(Ghrairi *et al.*, 2005)
Lactococcin MMFII	IIa	*L. lactis*	4144.6 (37)	(Ferchichi *et al.*, 2001)
Lactococcin 972	ND	*L. lactis* IPLA 972	7001.9 (66)	(Martinez *et al.*, 1996)
Lactococcin Q	IIb	*L. lactis* QU4	Qα 4260.4 (39) Qβ 4018.3 (35)	(Zendo *et al.*, 2006)
Lactococcin R	ND	*L. lactis* R	ND (ND)	(Yildirim and Johnson, 1998)
Bacteriocin J46	ND	*L. lactis*	3004.8 (27)	(Huot *et al.*, 1996)
LsbA/LsbB	ND	*L. lactis*	ND	(Gajic *et al.*, 2003)

ND, not determined

Nisin A is ribossomally synthesized as a preptide of 57 amino acid residues. The unmodified precursor of nisin is processed by a specific maturation machinery that is responsible for dehydration reactions and ring formation (NisBC), transport across the cytoplasmic membrane (NisT) and cleavage of the leader peptide (NisP) (Fig.2), which liberates biologically active nisin, consisting of 1 lanthionine, 4 methyllantionines, 1 dehydrobutyrine, 2 dehydroalanines and 21 unmodified amino acids (Fig. 1) (Chatterjee *et al.*, 2005; Rink *et al.*, 2007). Its structure is organized in two-structural domains, located at the N- and C- termini, respectively. The N-terminal domain, containing three posttranslationally incorporated (β-methyl) lanthionine rings (rings A, B and C), is linked to the C-terminal rings (rings D and E) by a flexible region, or hinge, consisting of three amino acids (Asn20-Met21-Lys22) (Fig.1) (Lubelski *et al.*, 2008). Because nisin functionality depends on posttranslational modifications, their operons are some of the most complex among bacteriocins. They are transcriptionally organized in four operons, *nisABTCIPRK*, *nisI*, *nisRK* and *nisFEG* (Fig. 2) (Kuipers *et al.*, 1993; Lubelski *et al.*, 2008). Of these genes, *nisA* encodes the nisin A precursor peptide; *nisB* and *nisC* encode putative enzymes involved in the post-translational modification reactions; *nisT* encodes a transport protein of the ABC translocator family that is probably involved in the extrusion of the modified nisin precursor; *nisP* encodes an extracellular protease involved in precursor processing (Van Den Berg Van Saparoea *et al.*, 2008); *nisI* encodes a lipoprotein involved in the producer self-protection against nisin (Takala and Saris, 2006); and *nisFEG* encodes transporter proteins that have also been involved in immunity (Fig. 2) (Immonen and Saris, 1998). The proteins encoded by nisR, a response regulator, and nisK, a sensor histidine protein kinase, belong to the class of the signal transduction regulatory systems proteins (Fig. 2) (Kleerebezem, 2004). Transcription of nisin genes is facilitated from three major promoter sites of which the promoter proceeding *nisR* is constitutive, whereas the *nisA* and *nisF* promoters are controlled by the two component regulatory system NisRK (Fig. 2) (Ruyter *et al.*, 1996). Quite recently there was an observation, that *nisI* mRNA could be detected without nisin induction, thus suggesting that the *nisA/B/T/C/I/P/R/K* operon might contain another internal and constitutive promoter (Fig. 2) (Li and O'sullivan, 2006). Nisin regulons are located on large transposons (~70 kb); examples of these transposons include Tn5276 (Rauch and De Vos, 1992), Tn5301

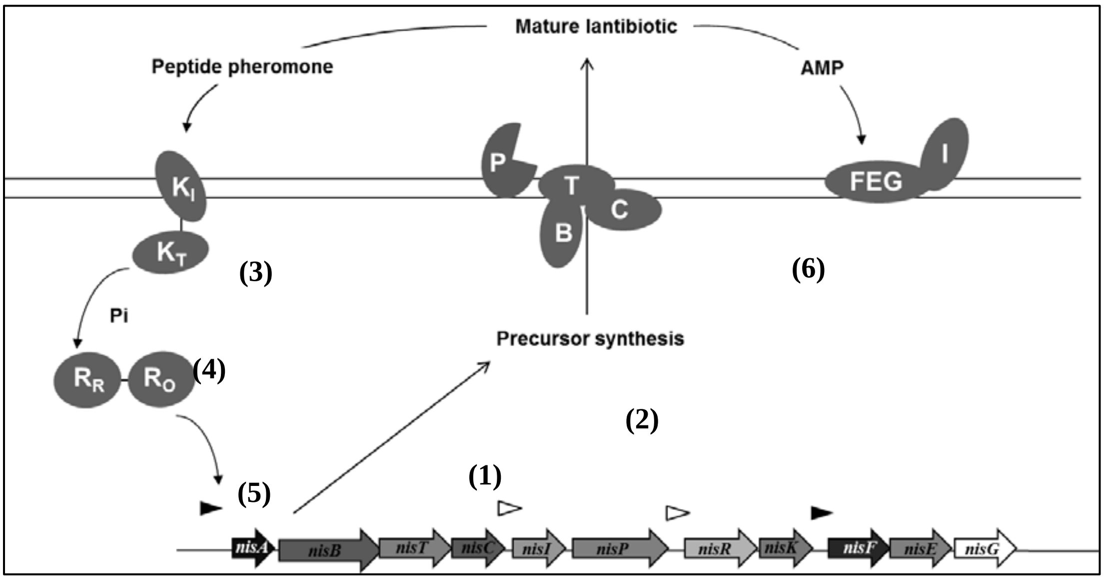

Fig 2. Nisin gene cluster and model of biosynthesis and regulation, i.e. *nisABTCIPRKFEG*. Black and white triangles indicate nisin-inducible and constitutive promoters, respectively. (1) Formation of prepeptide (prebacteriocin); (2) The prepeptide is modified by NisB and NisC, translocated through a ABC-transporter NisT and processed by NisP, resulting in the release of mature bacteriocin; (3) Histidine Protein Kinase (HPK) senses the presence of bacteriocin and autophosphorylates; (4) The phosphoryl group (Pi) is subsequently transferred to the response regulator (RR); RR activates transcription of the regulated genes; and (6) Producer immunity mediated by immunity proteins, NisI, and dedicated ABC-transport proteins, NisFEG (adapted from Kleerebezem, 2004).

(Dodd *et al.*, 1990), Tn5307 (Buchman *et al.*, 1988) and Tn5481 (Immonen and Saris, 1998).

Lacticin 3147 and lacticin 481 are other lantibiotics produced by *L. lactis* (Table 3). Lacticin 3147 is also a broad-spectrum lantibiotic with potential uses in the food industry and in medicine. It is a two-component lantibiotic (two separate peptides act in concert for full activity) produced by *L. lactis* subsp. *lactis* DPC3147 isolated from an Irish kefir grain (Ryan *et al.*, 1996). The genes required for DPC3147 to produce lacticin 3147 are carried on a 60.2 kb conjugative plasmid. Lacticin 481 exhibits a medium spectrum of activity, inhibiting a broad range of other LAB and *Clostridium tyrobutyricum*. This peptide can have a bacteriolytic effect on sensitive organisms (O'sullivan *et al.*, 2002). It has been studied for its potential use in cheese ripening to induce lysis of starter strains and therefore deliver lactococcal enzymes during cheese manufacture to improve both flavor and quality (Guinane *et al.*, 2005).

The class II lactococcal bacteriocins are unmodified, cationic and hydrophobic or partly amphiphilic peptide (Nissen-Meyer *et al.*, 2009). The most well characterized are lactococcins A and B (class IIc), lactococcin G, Q and MMT24 (class IIb – two peptide), lactococcin MMFII (class IIa) and lacticin Q and Z (class IId) (Table 3). Lactococcin A, produced by *L. lactis* LMG 2130 was among the first of the class II bacteriocins to be isolated and is the one that is best characterized (Holo *et al.*, 1991; Van Belkum *et al.*, 1991). It is initially synthesized as a 75 residue pre-bacteriocin that consist of a 21 residue double glycine type leader sequence and the cationic 54 residue mature bacteriocin (Holo *et al.*, 1991). Lactococcin A has a relatively narrow activity spectrum. It increases the permeability of target-cell membranes by bind to the mannose phosphotransferase permease, apparently the part of the permease that is embedded in the target-cell membrane, and thereby dissipates the proton motive force (Van Belkum *et al.*, 1991). Interactions between lactococcin A and the mannose phosphotransferase permease thus apparently alter the conformation of the permease in a manner that results in membrane-leakage, and this leakage is blocked by binding of an immunity protein to the bacteriocin-permease complex (Nissen-Meyer *et al.*, 2009) Lactococcin A and B can have both a bactericidal and bacteriolytic mode of action on target cells. However, such bacteriocins inhibit other lactococci and therefore their associated applications are limited (Guinane *et al.*, 2005). On the other hand, the lytic abilities of these bacteriocins may have an application in the acceleration of cheese ripening to induce starter cell lysis (Morgan *et al.*, 2002). Lactococcin G, lactococcin Q and lactococcin MMT24 produced by *L. lactis* LMG 2081, *L. lactis* QU4 and *L. lactis* MMT24, respectively, are two-peptide bacteriocins (class IIb) with similar characteristics. They are cationic, contain between 30-50 residues long, hydrophobic and/or amphiphilic and both complementary peptides are required to obtain full activity and the individual peptides display little or no antimicrobial activity (Nissen-Meyer *et al.*, 2009).

These bacteriocins have a bactericidal mode of action and a narrow antimicrobial activity against closely related lactic acid bacteria (Nissen-Meyer *et al.*, 1992; Ghrairi *et al.*, 2005; Zendo *et al.*, 2006). Lactococcin MMFII belongs to the class IIa bacteriocins figuring the first example of such a bacteriocin produced by a lactococcal strain described so far. It is one of the most active bacteriocin within these class IIa bacteriocins, rendering this bacteriocin attractive as an anti-*Listeria* compounds to protect food (Ferchichi *et al.*, 2001). Lacticin Q and Z are highly cationic and tryptophan-rich bacteriocin, which have formylated N-terminal methionine residues and are synthesized without leader sequences. They inhibit a nanomolar range of MICs against several of Gram-positive bacteria, and have antimicrobial activity comparable to that of nisin A in terms of both intensity and spectrum. Unlike many of other LAB bacteriocins, the stability of lacticin Z and Q were emphasized under alkaline conditions rather than acidic conditions (Fujita *et al.*, 2007; Iwatani *et al.*, 2007).

Lactococcal bacteriocins have different characteristics and consequently their associated applications differ considerably, it is evident that bacteriocins as well as bacteriocin producer can be used as tools to influence the microbial population in food system. Althoulgh quite diverse, there are a number of potential applications for these lactococcal bacteriocins.

3. Perspectives of new antimicrobial compounds produced by *Lactococcus lactis*

Some LAB also produce other low molecular weight compounds with antimicrobial activity such as reuterin and reutericyclin (Spinler *et al.*, 2008), cyclic peptides (Schnurer and Magnusson, 2005) and biosurfactants (Moldes *et al.*, 2007). Biosurfactants are amphiphilic compounds of microbial origin with a pronounced surface activity that exhibit a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes and fatty acids (Rodrigues *et al.*, 2006). Biosurfactants can be produced by microorganisms in an extracellular position and/or associated with cell walls. Recently, the strain *L. lactis* CET-4434 has shown to be an interesting cell-bound biosurfactant producer, with inhibited the growth of *C. piscicola* used as target microorganisms (Rodríguez *et al.*, 2010). Fractions of crude biosurfactant extract obtained from *L. lactis* 53 have shown antimicrobial activity against *Staphylococcus epidermidis*, *S. aureus* and *Candida albicans* (Rodrigues *et al.*, 2006).

In addition, *L. lactis* CHD-28.3 showed antagonistic activity against several filamentous fungi, that was lost after enzymatic treatment with chymotrypsin, trypsin and pronase E (Roy *et al.*, 1996).

Two possibly different antimicrobial substances produced by *L. lactis* ID1.5 isolated from fermented sausage with inhibitory proprieties against *Pseudomonas aeruginosa* and *Streptococcus pneumonia* TIGR 4 (this thesis, chapter 3) are being investigated and may prove new questions and possibilities regarding the use of lactococcal starter cultures.

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CHAPTER 2

Nisin Z production by wild strains of *Lactococcus lactis* isolated from naturally fermented sausage

Abstract

In this work, we identified a bacteriocin produced by *Lactococcus lactis* strains isolated from naturally fermented sausage in Brazil. PCR product sequencing confirmed that the structural genes of the five strains are identical to lantibiotic nisin Z. By ammonium sulphate precipitation, ionic exchange and reversed-phase chromatographies, nisin Z was purified from culture supernatant of these microorganisms, and their molecular masses were confirmed by MALDI-TOF/TOF. The production of the bacteriocin was investigated under different growth conditions. All strains produced bacteriocin efficiently at initial pH values of 6.0 and pH 6.5, under aerobic conditions and at 25 °C and 30 °C. Tryptone and casein peptone were found to be the optimal organic nitrogen source for the bacteriocin production for all strains. Carbon source appears to be a major control mechanism for nisin Z production. Sucrose was the most efficient carbon source for bacteriocin production of *L. lactis* ID1.5, ID8.5, PR3.1 and PD4.7, while fructose was the most efficient carbon source for strain *L. lactis* ID3.1. The strains produced different amounts of bacteriocins, maximal activity was recorded by *L. lactis* ID1.5 and ID8.5. The *nisZ* and *nisF* promoter sequences obtained from all strains are identical, suggesting that there should be other factors involved in differences of the production among those strains.

1. Introduction

Bacteriocin producing lactic acid bacteria (LAB) are used as starter or protective cultures in the production of foods. In particular, *Lactococcus lactis*, a nisin-producing, has been utilized in fermented foods and it is generally recognized as a safe (GRAS). Hence, *L. lactis*-derived nisin has been extensively studied as a model bacteriocin in several applications. Nisin A, a ribosomally synthesized lantibiotic is the first bacteriocin approved and employed as food preservative commercially (Cotter *et al.*, 2005). The nisin A operon has been studied in detail and consists of eleven genes, i.e., *nisABTCIPRKFEG* (Trmčić *et al.*, 2011). The prepeptide is encoded by the structural gene *nisA* (Kuipers *et al.*, 1993). Transcription of nisin genes is accomplished by three major promoter sites of which the promoter preceding *nisR* is constitutive, whereas the *nisA* and *nisF* promoters are controlled by the two component regulatory system NisRK (De Ruyter *et al.*, 1996). The NisRK-mediated regulatory system, responds to changes in environmental factors (Kleerebezem, 2004). In addition, to the *nisR* promoter, another constitutive promoter has been identified before *nisI. nisI* gene encodes an immunity protein (Takala and Saris, 2006) that together with, *nisF*, *nisE*, and *nisG* encode ATP-binding cassete transporters are responsible for immunity of the producing strain (Immonen and Saris, 1998). In view of the widespread use of this bacteriocin, an important factor to consider for its application is the cost of nisin production. It is well known that nisin production at fermentation systems is influenced by many factors such as type and the level of carbon and nitrogen sources, pH, and temperature (De Vuyst and Vandamme, 1992; Matsusaki *et al.*, 1996; Cheigh *et al.*, 2002), optimization of bacteriocin production and enhancement of its activity may have economic significance. Moreover, nisin production abilities of the producer strains can also differ (Kim *et al.*, 1997).

Some strains nisin producers *L. lactis* have been isolated from cheese, raw milk, grain, fish, fermented vegetable and river (Choi *et al.*, 2000; Zendo *et al.*, 2003; Mitra *et al.*, 2007; De Kwaadsteniet *et al.*, 2008; Hu *et al.*, 2009; Trmčić *et al.*, 2011). However, few nisin producers *L. lactis* have been isolated from sausage fermented (Rodriguez *et al.*, 1995). In fact, among bacteriocinogenic LAB, *Pediococcus* and *Lactobacillus* species are more frequently isolated from meat and fermented meat products (Nieto-Lozano *et al.*, 2002; Albano *et al.*, 2007; Héquet *et al.*, 2007; Müller *et al.*, 2009).

In our previous study on bacteriocin production by lactic acid bacteria isolated from fermented sausage in Brazil (unpublished data), several Gram-positive bacteria were screened for the ability to produce bacteriocin against food-borne pathogens. The purpose of this study was to characterize the bacteriocin secreted by *L. lactis* strains ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7 isolated from Italian type fermented sausage and investigate production in batch culture.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacteria used in this study were *Lactococcus lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7 isolated from Italian type fermented sausage and maintained as a part of our culture collection. All LAB used were grown at 30 °C in LAPTg broth containing (per liter): 10g glucose, 10g tryptone, 10g yeast extract, 15g animal peptone and 1g tween 80 (Juarez Tomas *et al.*, 2004). The susceptible strain *Micrococcus luteus* ATCC 10240 was used as the indicator strain in biological assay for bacteriocin quantification. It was grown at 30 °C in basal media containing (per liter): 10g glucose, 10g animal peptone, 8g meat extract, 4g yeast extract, 5g NaCl and 2,5g Na_2HPO_4.

2.2. Genetic identification of bacteriocin-producing strains

The species was identified by using 16S rRNA gene sequencing and BLAST analysis. Genomic DNA was isolated with Wizard Genomic DNA purification Kit (Promega), applying the protocol for Gram-positive bacteria and using mutanolysin and lysozyme (Sigma-Aldrich). Genomic DNA was used as a template in PCRs, using combinations of primers (Weisburg *et al.*, 1991) listed in Table 1. Amplification of 16s rRNA genes was performed with Taq DNA polymerase (PROMEGA) under the following conditions: 2 min at 95 °C; followed by 30 cycles of 2 min at 95 °C, 30 s at 58 °C, and 2 min at 72 °C; with a final 5 min at 72 °C. Amplicons were purified and ligated to pGEN-T Easy (PROMEGA) and cloned. Plasmids DNA from the clones were isolated by EZ.N.A. TM Plasmid Spin Protocol (Omega, USA), analyzed by PCR and the cloned fragment was sequenced. Sequences were aligned using BLAST software provided online by National Center for Biotechnology Information (USA) to determine the closest known relatives of the partial 16S sequence obtained.

2.3. Sequencing of nisin genes and nisin controlled promoters

Nucleotide sequencing was performed with the PCR products obtained from the amplifications of genomic DNA with primers specific to *nisA* structural gene and *nisA*, *nisF* promoters (designed according to the nisin A regulon, GenBank: HM219853.1) listed in Table 1. The PCR conditions were different for each pair of primers. The PCR thermal cycle program included an initial denaturation at 94 °C for 2 min, followed by 35 cycles, with a denaturation step at 94 °C for 1 min, an annealing step of 30 s, at 40 °C, at 48 °C and at 68 °C for primers sets nqf/naqzr, pnisAf/pnisAr and pnisFf/pnisFr, respectively, followed by extension step during 1 min at 72 °C. Final extension was performed at 72 °C for 7 min. The desired bands after PCR amplification were cut from the gel and purified with Gel Extraction Kit (Nucleospin® Gel and PCR clean up, Machery-Nagel, Germany) and sequenced. The determined sequences were compared with the GenBank database using tools as metioned above.

2.4. Detection and curing of plasmids

The presence of plasmids in *L. lactis* ID1.5, ID3.1, ID8.5, PD4.7 and PR3.1 was detected by EZ.N.A. TM Plasmid Spin Protocol (Omega, USA). Plasmids were cured by using ethidium bromide as a curing agent. Cultures were grown in M17 supplemented with 0.4% of glucose (GM17) and 10 µg mL^{-1} of ethidium bromide, 30°C for 24 hours. After incubation, the same procedure was repeated several times. Cultures, which survived were diluted, plated on GM17 agar and incubated at 30°C for 24 hours. Colonies were further screened for their bacteriocin production by overlaid with soft agar containing *L. lactis* IL1403 as indicator organism, and the presence of plasmids was checked.

2.5. Bacteriocin antimicrobial assay

Quantitative determination of the antimicrobial activity of the bacteriocin was performed by using agar well diffusion assay technique (Ryan *et al.*, 1996). Preparations of the cell-free culture supernatant (boiled and neutralized) as well as purified bacteriocin were serially diluted and tested against indicator strain. One arbitrary unit (AU mL^{-1}) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5 mm in diameter.

2.6. Purification and estimation of the molecular size of bacteriocins

Bacteriocins were purified from the culture supernatant of *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7. Ammonium sulfate (40 g per 100 mL) was added to each supernatant and agitated for 30 min at 4°C. The bacteriocin was precipitated from the supernatant by centrifugation (10000 r.p.m for 30 mim at 4°C) and dissolved in 20 mL sterile distilled water, and the pH was adjusted to 3.5 with 1M HCl. It was them passed through a 5 mL SP Sepharose Fast Flow (GE Healthcare Biosciences, Uppsala) equilibrated with 10 mM acetic acid. The column was eluted with a stepwise gradient consisting of 10 mL of each of 0.1, 0.3 and 1.0 M NaCl at 1 mL min^{-1} flow rate and stored on ice. The fractions showed the highest bacteriocin activities were fractionated on a reversed-phase column (Resource 15 RPC 3 mL; Pharmacia Biotechnology) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water, using Äkta Purifier Fast Protein Liquid Chromatography System. Elution was performed by using a linear gradient from 0 to 100% isopropanol containing 0.1% (v/v) TFA, collected in 1 mL fractions. The fractions with the best bacteriocin activities were collected and were submitted to mass spectrometry analysis. Bacteriocin samples (active fractions) were mixed 1:1 with a solution of 15 mg α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 49.9% ethanol and 0.1% TFA, and deposited on a ground steel Matrix-Assisted Laser Desorption Ionization target. Mass spectra were recovered in a positive reflector mode with an Ultra Flex

TOF/TOF (Bruker Daltonic GmBH, Bremen, Germany) by using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV.

2.7. Effect of carbon and nitrogen sources on bacteriocin production

The carbon sources tested were glucose, lactose, sucrose and fructose at the concentration of 10 g L^{-1}. Bath cultures (1% inoculum, v/v, standardized to OD_{600nm} = 0.6) were grown in LAPTg broth with glucose; and LAPTg broth without glucose, supplemented with lactose, sucrose or fructose. Incubation was at 30 °C, without agitation, for 24 h. Samples were examined each hour for bacterial growth (OD_{600nm}), changes in culture pH and bacteriocin production.

To study the effect of different nitrogen sources on bacteriocin production, the LAPTg medium added of glucose at 10 g L^{-1} was supplemented with each different nitrogen source (at 35 g L^{-1}). The nitrogen sources separately added were tryptone, yeast extract, meat extract, animal peptone, soy peptone, and casein peptone. After 24 hours of incubation at 30 °C, the final optical densities, culture pH value and bacteriocin production (expressed as AU mL^{-1}) were determined.

2.8. Effect of aeration, initial medium pH value and incubation temperatures on bacteriocin production

In a separate experiment, the effect of the temperature, the initial medium pH and aerobic and anaerobic conditions on the production of bacteriocin were determined. To assess the effect of initial pH, the LAPTg medium was adjusted to pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 by addition of 1M HCl or 5M NaOH. Each tube was inoculated with (1% inoculum, v/v, standardized to OD_{600nm} = 0.6) bacteriocinogenic cultures and incubated at 30 °C for 24 h. The effect of temperature on bacteriocin production was determined incubating bacteriocinogenic cultures on LAPTg medium for 24 h at temperatures of 20, 25, 30, 35 and 40 °C. Bacteriocinogenic cultures were also grown in LAPTg medium under anaerobic (with sealed anaerobic tubes) and aerobic conditions (with agitation). After the incubation time, final pH, bacteriocin production, expressed as AU mL^{-1} and bacterial growth (OD at 600 nm) were determined for all conditions described above.

2.9. Statistics

All experiments with regard to bacteriocin production were carried out in triplicate and repeated twice. When error bars are given in the figures, they refer to the standard deviation of the mean.

3. Results

3.1. Identification of bacteriocin-producing strains

In order to study the phylogenetic position of lactic acid bacteria, 1500 nucleotideos of the 16S rDNA of the strains were amplified by PCR, sequenced, and subjected to 16S rDNA sequence analysis. The BLAST analysis showed 99% nucleotide identity of the strains ID1.5. ID3.1, PD4.7 and PR3.1 with the16S rDNA sequence of *L.lactis* CV56 and *L.lactis* SL3 16S ribosomal RNA gene (GenBank: CP002365.1 and AY675242.1, respectively). The 16S rDNA sequence of strain ID8.5 showed 99% nucleotide identity with *L. lactis* KLDS 16S ribosomal RNA gene (GenBank: DQ340068.1). The analysis indicate that all the isolates are strains of *L. lactis* presenting close similarity with the subspecies *lactis*.

3.2. Sequencing of nisin genes and nisin controlled promoters

The PCR products obtained from the amplifications of genomic DNA of *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7, with primers specific to nisin structural gene were subjected to nucleotide sequencing. Results indicated that the nisin gene sequences in all strains were identical to that of nisin Z (GenBank: AB375441). Homology was also observed with nisin A (GenBank: HM219853), except for a C-to-A that resulted in an asparagine (AAT) residue at position 27 of the nisin peptide, instead of histidine (CAT), as the deduced amino acid sequences shown in Fig. 1. This indicated that the bacteriocins produced by all strains are variants of nisin A (Fig. 1).

The nucleotide sequence of the PCR fragment (amplified with primers pnisAf and pnisAr) of genomic DNA of *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7 containing the promoter region of structural gene (*nisA*) have 100% identity to the sequence recorded for the promoter region encoding nisin Z (GenBank: Y13384.1) and the promoter region encoding nisin A (GenBank: Z18947.1). They have a consensus promoter characterized by -35 and -10 sequences that are spaced by an average of 17 nucleotides (Fig 2a). The promoter region upstream of structural *nisZ* gene contains a TCT direct repeat with 8-bp spacer region at the positions -39 to -26, upstream of the transcription start site (Fig. 2a). It also contains a second TCT-N_8-TCT motif present upstream of structural gene *nisZ* at positions -107 to -94 (Fig. 2a). Analysis of the amplified fragment containing the promoter region of *nisF* of all strains have 100% identity to the sequence recorded for the promoter region encoding *nisF* presented in the nisin A regulon (GenBank: HM219853). The sequence/upstream of the *nisF* transcription start site included a TCT-N8-TCT motif present at the same position, -39 to -26, upstream of the transcription start site of *nisZ*. All promoter sequences *nisF* and *nisZ* were identical among *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7 (Fig. 2b).

3.3. Detection and curing of plasmids

Plasmids were found in all strains of *L. lactis* (Fig. 3). Thus, the plasmid curing was conducted to examine whether the genetic determinants for bacteriocin production in *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7 are located on plasmid. It was observed that the strains ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7cured derivatives were able to produce bacteriocin after growth in the presence of ethidium bromide (10 µg mL^{-1}), suggesting that the genes encoding the bacteriocin are located on the chromosome.

3.4. Purification and mass spectrometry analysis of bacteriocin

In order to identify the bacteriocins produced and secreted by *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7, three steps by purification were carried out from the cell free culture supernatant of all strains grown overnight. These supernatant were precipitated with ammonium sulfate and subsequently purified by ion-exchange and reverse-phase chromatography. The highest active fraction of the bacteriocin was eluted between 30-40% isopropanol for all strains (data not shown). These fractions were directly analyzed by mass spectrometry. The molecular masses of the purified fractions from *L. lactis* ID1.5, ID3.1, ID8.5, PD4.7 and PR3.1 were 3330.567, 3330.514, 3329.985, 3329.561 and 3329.591 Da, respectively (Fig. 4), which are similar to the molecular mass of the nisin Z, 3330. 93 Da (Piper *et al.*, 2010). Through the MALDI/MS analysis, the bacteriocins produced by all strains were identified as nisin Z.

3.5. Effect of carbon and nitrogen source on bacteriocin production

The bacteriocin production kinetics of *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 were studied under different conditions. Batch fermentation profiles of microbial growth and bacteriocin production are presented in Fig. 5 and Fig. 6. The strains started producing bacteriocins at early exponential phase, their activity were already detectable after 2 hours of growth. Exponential growth took place during a period of about 1 to 6 hours for all strains. Maximum cell density was reached after 4 and 6 or 7 hours in the medium supplemented with glucose and with other carbon sources, respectively. The activities increase concomitantly with an increase in the growth and reached its maximal activity at the stationary phase when the pH of the medium was decreased to 4.3. However, when the strains were cultivated in medium added with lactose the final culture pH was approximately 5.2 (Fig. 6A). A decrease on bacteriocin production was observed after 8 hours incubation for all strains, except to *L. lactis* ID3.1, when it was grown in medium supplemented with fructose, the bacteriocin activity remained constant (Fig. 5Bb).

The effect of different carbon sources on the bacteriocin production was tested using the LAPT broth supplemented with 10 g L $^{-1}$ of each carbon source (Fig. 5 and Fig. 6). Maximum bacteriocin production was obtained by using sucrose, fructose and lactose (Fig. 5 and Fig. 6). In the presence of sucrose as sole carbon source, about an activity of 226000 AU mL^{-1}, 226000 AU mL^{-1}, 85000 AU mL^{-1}, 136000 AU mL^{-1} was recorded to the strains ID1.5, ID8.5, PR3.1 and PD4.7, respectively. The production of bacteriocin by these strains was stimulated by sucrose presence (Fig. 6B). However, bacteriocin production by strain ID3.1 increased in the presence of fructose (Fig. 5Bb). For all strains, glucose yielded the smallest increase in bacteriocin production compared to the other carbon sources.

In general, the strains presented different values of bacteriocin activities. Maximal bacteriocin activity was recorded by *L. lactis* ID1.5 and ID8.5, their activities were approximately 34000 AU mL^{-1}, 125000 AU mL^{-1}, 136000 AU mL^{-1}, 226000 AU mL^{-1} in medium supplemented with glucose, fructose, lactose and sucrose, respectively, as sole carbon source (Fig. 5 and Fig. 6).

When *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 were cultivated in LAPTg medium that lacked peptone, tryptone and yeast extract and was added of different nitrogen sources (at 35 g L^{-1}), a range of the growth and bacteriocin activities were obtained (Table 2). The strains were capable of growth in medium with each nitrogen source separately, but the highest bacteriocin activities were found in cells grown in medium with tryptone or casein peptone. When meat extract was used as single nitrogen source, all the strains grew well but produced little bacteriocin (Table 2).

3.6. Effect of aeration, initial medium pH and incubation temperatures on bacteriocin production

All strains displayed similar behaviors in terms of cell growth and bacteriocin production at each of the tested temperatures. Cell growth occurred at temperatures ranging from 20 °C to 40 °C (Table 2), with highest levels of bacteriocin production recorded at 25 °C and 30 °C. However, all strains showed a decrease of approximately 28% of bacteriocin production at 40 °C.

The initial pH of the medium was found to significantly influence the growth and bacteriocin production for all strains. In medium adjusted to pH 6.0 or 6.5 higher bacteriocin activity was observed (Table 2). At pH 4.5 and 7.0 bacteriocin activities were approximately 10 and 8 times, respectively, lower than at pH 6.0 or 6.5. Cell growth of all strains was found to be similar under anaerobic and aerobic conditions (Table 2). However, higher levels of bacteriocin production were observed under aerobic conditions, being more pronounced (three times higher) for *L. lactis* ID 3.1, ID8.5, PR3.1 and PD4.7.

4. Discussion

In this study, we report the identification of five strains of *L. lactis* and the production and characterization of their bacteriocins. These strains were isolated from naturally fermented sausage in Brazil, which showed inhibitory effect against foodborne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* (Maciel, 1998; De Paula, 2005).

The structural genes of the bacteriocins produced by these five strains are identical. PCR product sequencing confirmed that the gene structures of the five strains are identical to those nisin Z. The deduced amino acid sequences of all strains showed that they contained an asparagine at position 27 instead of a histidine as in nisin A (Fig. 1). A BLAST search of GenBank sequences indicated that the bacteriocins produced by all strains is the variant nisin Z. Preliminary tests had indicated differences in the inhibitory activity among the isolates. These differences are not accounted for by the gene structure. Nisin Z is a bacteriocin potentially active against pathogenic Gram positive bacteria and purified nisin Z is able to exert activity also against Gam negative bacteria (Kuwano *et al.*, 2005). In addition, De Vos *et al.*, 1993 reported that His27Asn substitution resulted in a higher diffusion rate of nisin Z, which in turn contributed to the larger inhibition zones produced by nisin Z in agar diffusion assays. The higher diffusion rate of nisin Z may be of practical significance, since many products to which nisin A is applied are diffusion limited. The existence of natural variants confirms that the identity of amino acids present at certain locations is flexible and it may thus be possible to generate mutants. The natural variants nisin F and Q possess, as in nisin Z, the His27Asn change. However, nisin F contains one additional variation, Ile30Val and nisin Q contains the additional substitution observed in nisin F as well as two varations, Ala15Val and Met21Leu (Piper *et al.*, 2010).

Development of three steps purification procedure allowed the separation of bacteriocin produced and secreted by *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7. After mass spectrometry analysis of the purified bacteriocins, the apparent molecular masses obtained are similar to the molecular mass of the nisin Z (Fig. 4). Its result confirmed the genetic finding.

The promoter sequences of *nisZ* gene of all strains contain a partially conserved region which could be involved in the transcriptional control function, the TCT-N_8-TCT motif present at position -39 to -26, upstream of the transcription start site of *nisZ* (Fig. 2a). Chandrapati and O'Sullivan, 2002 reported that this motif is supposed to be involved in a co-operative binding of the NisR response regulator of the NisRK two-component regulatory system. They also reported a second TCT-N_8-TCT motif present at position -107 to -94, which was also shown in our results (Fig. 2a). This TCT repeat, together with the first one, is involved in the optimal binding of NisR and also in the induction by some component of the Leloir pathway of galactose metabolism. Thus, it has been reported that galactose and lactose can induce transcription from the *nisA* promoter, but the induction does not occur in *L. lactis* if the Leloir pathway is made redundant by the presence of the preferred PTS-mediated pathways (Chandrapati and O'sullivan, 1999; 2002).

Another promoter in front of the *nisFEG* genes was identified in all strains and it is identical to that observed in the nisin A regulon (GenBank: HM219853) (Fig. 2b). The sequence upstream of the *nisF* transcription start site included a single TCT direct repeat with an 8-bp spacer region similar to the TCT-N_8-TCT motif present at the same position, -39 to -26, upstream of the transcription start site of *nisZ* (Fig. 2a and Fig. 2b). This shows that the *nisF* promoter is possibly regulated in the same way as the *nisZ* promoter. Ruyter *et al*. 1996 reported that the *nisF* promoter is subject to the same *nisRK*-dependent control as *nisA* promoter. The production level of the two transcripts derived from the *nisZ* promoter is probably required to generate a balanced production level of the nisin precursor (nisZ) and of the modification, transport, and processing machinery (nisBCTP) products. The role of the *nisFEG* gene products to immunity of the producing cell nisin is mediated by the regulation of the *nisF* promoter, that providing higher immunity levels when cells meet higher nisin concentrations (Kleerebezem, 2004). Thereby, nisin biosynthesis is directly involved with levels of transcription of these genes. The *nisZ* and *nisF* promoter sequences obtained from all strains are identical (Fig. 2a and Fig. 2b). It suggests that the difference observed in the bacteriocin production among *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 could be due to other events.

Plasmid curing results suggest that the genes encoding nisin Z in *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 are located on the chromosome. Various researchers have found that nisin genes are present on a number of plasmids or also on the chromosome (Gireesh *et al.*, 1992; Rauch and De Vos, 1992; De Kwaadsteniet *et al.*, 2008; Hu *et al.*, 2009). In addition, nisin gene cluster has been showed to be present on a chromosomally located conjugative large transposons (~70 kb) (Rauch and De Vos, 1992), which also contain the genetic determinants of sucrose metabolism. Examples of these transposons include Tn5276 (Rauch and De Vos, 1992), Tn5301 (Dodd *et al.*, 1990), Tn5307 (Buchman *et al.*, 1988) and Tn5481(Immonen and Saris, 1998). De Vos et al. (De Vos *et al.*, 1993) reported that the *nisA* and *nisZ* genes were distributed equally among 23 naturally occurring, sucrose-fermenting *L. lactis* strains. Interestingly, it has been suggested that a genetic regulatory system or a common metabolic control system for sucrose fermentation and nisin production capacity and dynamics may be responsible for the observed linkage between those metabolic traits (De Vuyst and Vandamme, 1992; Rauch and De Vos, 1992).

For all of the strains, bacteriocin production appeared to parallel that of cell growth profile and reached its maximal activity at the stationary phase (Fig. 5 and Fig. 6). In earlier studies, it has been reported that nisin is produced during the growth phase and a sharp decrease occurs in activity at the end of growth, possibly due to protein degradation, adsorption to the cell surface, protein aggregation or complex formation (De Vuyst and Vandamme, 1992; Cheigh *et al.*, 2002; Simsek *et al.*, 2009). The high bacteriocin activity of *L. lactis* ID3.1 cultivated in medium with fructose did not decrease sharply (Fig. 5Bb), indicating that this condition prevented the decrease in bacteriocin activity which occurred with the other strains.

L. lactis have been exploited as a major source of a variety of antimicrobial peptides with potential commercial applications. They are fastidious organisms that often require several organic factors (e.g amino acids, vitamins) for growth and bacteriocin production (Guerra *et al.*, 2001). Bacteriocin production by *L. lactis* is usually enhanced in complex media, such as several commercial media MRS, M17 and CM (De Vuyst and Vandamme, 1992; Cheigh *et al.*, 2002; Yoneyama *et al.*, 2008; Simsek *et al.*, 2009) in formulations which reach high prices. In this study we used LAPTg broth, which can be an alternative medium to bacteriocin production.

In addition, the study and application of bacteriocins are often compromised by limited production of these peptides by the native producer (Cotter *et al.*, 2006). The optimization of fermentation processes offers one option to improve bacteriocin production. Our results demonstrated that bacteriocin production by *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 was affected by temperature, pH, aerobic and anaerobic conditions, as well as carbon and nitrogen sources. Carbon source selection has been reported as a critical control step in nisin production because of its effects on the cell growth and nisin biosynthesis (De Vuyst and Vandamme, 1992). For example, sucrose and lactose were determined to be efficient carbon sources for nisin production in strains *L. lactis* NIZO 22186 (De Vuyst and Vandamme, 1992), *L. lactis* ATCC 11454 (Penna and Moraes, 2002) and *L. lactis* A164 (Cheigh *et al.*, 2002), while fructose was the most efficient carbon source in strain *L. lactis* LL27 (Simsek *et al.*, 2009) and glucose in strain *L. lactis* IO-1 (Matsusaki *et al.*, 1996). Our results indicated that sucrose is the most efficient carbon source in bacteriocin production of *L. lactis* ID1.5, ID8.5, PR3.1 and PD4.7 (Fig. 6B), while fructose was the most efficient carbon source in strain *L. lactis* ID3.1 (Fig. 5Bb). Concluding from these results, bacteriocin produced by *L. lactis* ID3.1 is stimulated by fructose, but not when glucose is present in a disaccharide form as in sucrose, in contrast with other strains. The presence of sugar as a carbon and energy source is essential to bacteriocin biosynthesis. Energy is very important for the translocation of active bacteriocin peptides to the external medium by putative ABC transporter (Ennahar *et al.*, 2000). From a bioenergetics point of view, the sugar transported across cytoplasm membrane by phosphoenolpyruvate phosphotransferase (PTS) system is probably the most efficient, since it is translocated and phosphorylared in a single step at expense of one PEP molecule (equivalent to one ATP). Instead, for carbohydrates that are actively accumulated by non-PTS systems, more than one ATP equivalent must be expended for both transport and subsequent phosphorylation. All the sugar sources used in this study, glucose, fructose and sucrose are transported by PTS system, and lactose can also be translocated either via a lactose-PTS or by a permease in *L. lactis* (Neves *et al.*, 2005). However, lactose uptake by a permease is followed by its hydrolysis via β-galactosidase, yielding glucose and galactose; in this case galactose is further metabolized via Leloir pathway (Neves *et al.*, 2005), whose components as mentioned earlier are involved in the induction of transcription from of *nisA* promoter.

All strains produced bacteriocin efficiently from tryptone and casein peptone as sole nitrogen sources, however, the growth were similar in media supplemented with other nitrogen sources (Table 2). Previous work by Simsek *et al*. 2009 indicated that yeast extract and meat extract were the most efficient nitrogen sources for nisin A production by *L. lactis* LL27. However, our results indicated that yeast and meat extract resulted in the lowest bacteriocin activity among the tested nitrogen sources. Thus, bacteriocin production by *L. lactis* ID1.5, ID8.5, PR3.1 and PD4.7 is stimulated by tryptone and casein peptone, but not by yeast and meat extract. This suggests tryptone and casein peptone contain essential compounds not present in yeast extract and meat extract. It could also be that yeast extract and meat contain proteases that may degrade the peptides. Because cell mass production and bacteriocin activity are not readily related, the effect of nitrogen sources on bacteriocin production by ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 appeared to be involved in multiple physiological responses. Several studies have shown the occurrence of induction by nisin (Kuipers *et al.*, 1995) or by related peptides (Diep *et al.*, 1995), thereby both tryptone and casein peptone could contain peptides that are essential for the synthesis of bacteriocin or could act as inducers of bacteriocin production.

The effect of pH and temperature on the growth of lactic acid bacteria is well known, but these features influence on bacteriocin production should also be considered. *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 were able to grow and produce bacteriocin in LAPTg broth at pH values ranging from 4.5 to 7.0 and temperatures from 30 °C to 40 °C. However, high bacteriocin production were obtained when these strains were cultivated at initial pH 6.0 and 6.5, and incubated at 25 °C and 30 °C (Table 2). High growth rates were reached when all strains were cultivated at pH 7,0 and incubated at 35 °C, but bacteriocin production was lower (Table 2). Our results indicated that pH values below 6.0 and above 6.5 affected negatively bacteriocin production by all *L. lactis* strains. In contrast, Matsusak *et al.* 1996 reported higher nisin production by *L. lactis* IO-1 at pH values ranging from 5.0-5.5. In addition, De Vuyst and Vandamme, 1996 and Mitra *et al*., 2005 showed that *L. lactis* strains produced higher levels of nisin above at pH 6.5. Results similar to ours were reported for other bacteriocins produced by *Lactobacillus plantarum, Leuconostoc mesenteroides* and *Enterococcus mundtii* (Daeschel *et al.*, 1990; Jimenez-Diaz *et al.*, 1993; Drosinos *et al.*, 2006; Todorov and Dicks, 2006; 2009). Our results showed that high temperature (40 °C) did not of influence the growth of the all producer strains but affected negatively bacteriocin production. The difference of optimal cultivation temperature on cell growth and bacteriocin production has been previously reported (Matsusaki *et al.*, 1996; Cheigh *et al.*, 2002; Mitra *et al.*, 2005). Temperature can affect the stability of the peptide by interfering in post-translational modification, adsorption to cells and proteolysis of the bacteriocin (Drosinos *et al.*, 2006). In addition, temperature has an important regulatory effect on its biosynthesis. One example of a temperature-sensitive bacteriocin biosynthesis was published by Diep et al., 2000. They demonstrated that the biosynthesis of sakacin A occurred at 25 °C and 30 °C but not at higher temperatures (33.5 – 35.0 °C). The temperature regulation of sakacin A biosynthesis occurred at the transcription level, and the reduced bacteriocin production at high temperatures was related to a reduced synthesis of the inducer peptide. Additionally it is known that high temperature enhanced the genetic instability of the plasmid carrying the bacteriocin genes (Mortvedt and Nes, 1990; Khouiti and Simon, 2004). However, the loss of bacteriocin production by *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 at 35 °C and 40 °C could not be due to their genetic determinant instability, because plasmid curing results suggest that the genes encoding bacteriocin in all strains are located on the chromosome.

L. lactis is a facultative anaerobe, homofermentative lactic acid bacteria, but under anaerobic conditions, it can shifting from homolactic to mixed acid fermentation (Neves *et al.*, 2005). The oxygen tolerance by lactic acid bacteria is associated to different metabolic pathways that consequently should affect bacteriocin production (Neysens and De Vuyst, 2005). Our results indicated that both aerobic and anaerobic conditions did not affect the growth *L. lactis* ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7. However, bacteriocin production showed high levels at aerobic conditions.

As demonstrated by our study, the structural gene of nisin Z was identified in all strains, which produced nisin at variable concentrations. The production of active bacteriocin was severely affected by environmental factors including carbon and nitrogen sources and fermentation conditions (pH and temperature). Some results indicate that nisin production can be regulated by the carbon source. In addition, increased nisin Z production towards the end of exponential growth phase could be due to delayed formation of the necessary prenisin-modifying enzymes. Whether the regulation is enzymatically mediated or controlled by carbon catabolite repression, inhibition or inactivation is not yet known.

Conditions that favored all strains of *L. lactis* growth were not always related to a higher bacteriocin production or secretion. The strains produced different amount of bacteriocins. We analyzed *nisF* and *nisZ* promoter sequences because these promoters are controlled in the same manner, however the sequences are similar in all strains, demonstrating that there should be other factors involved in increasing nisin concentration.

To our knowledge, this the first report of lantibiotic nisin Z production by *L. lactis* isolated from fermented sausage in Brazil, as well as the first study showing identical bacteriocins produced by different wild strains of *L. lactis* isolated from same environment.

L. lactis ID1.5, ID 3.1, ID8.5, PR3.1 and PD4.7 display good potential for bacteriocin production. They are generally recognized as safe to the health. Besides, the discovering of bacteriocins, it is also important to identify strains producing higher amounts of the antimicrobials (particularly those with broad inhibitory spectrum such as nisin), which would lead to their application as starters or protective cultures in preservation of foods. Autochthonous starters and adjunct cultures composed by bacteriocin-producing strains may further help to reinforce originality of traditional foods.

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Table 1. PCR primers employed in this work

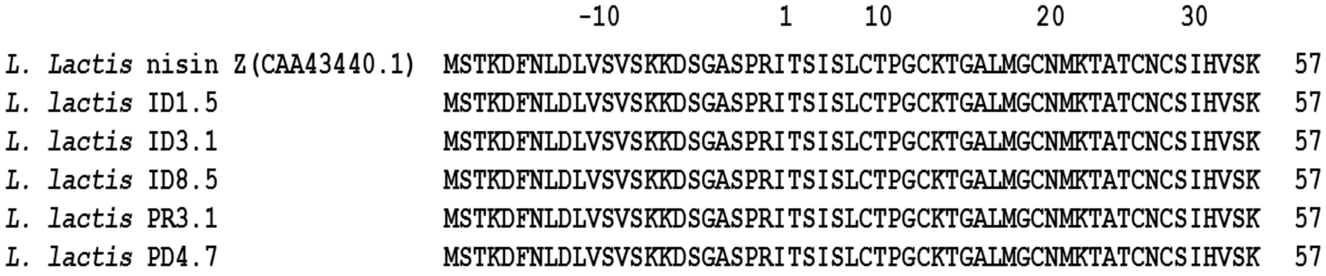
Primer*	Sequence (5’-3’)	Amplicon size
16S rRNAfd	CCGAATTCGACAACAGAGTTTGATCCTGGCTCAG	1500 bp
16S rRNAmd	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC	
nqf	CGTTCGAAGGAACTACAAAATAAATT	515 bp
naqzr	ACAGACCAGCATTATATTTCTGC	
pnisAf	TTGAGTCTTAGACATACTTGAATGACC	378 bp
pnisAr	CAATGACAAGTTGCTGTTTTCA	
pnisFf	TCCTCAAAAAGGTGGGGCAGAAGT	374 bp
pnisFr	GCCTCGATTAAGGCTCCAAGTTGA	

*Primer pairs: 16S rRNAfd/16S rRNAmd (16s rRNA genes); nqf/ naqzr (*nisA* structural gene); pnisAf/ pnisAr (*nisA* promoter); pnisFf/pnisFr (*nisF* promoter).

Table 2. Influence of nitrogen source, initial pH value, temperature, aerobic and anaerobic conditions on the production of bacteriocins by *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7 strains isolated from Italian type fermented sausage

Conditions and components evaluated	*Lactococcus lactis* strains									
	ID1.5		ID3.1		ID8.5		PR3.1		PD4.7	
	Final	AU mL^{-1}	Final	AU mL^{-1}	Final	AU mL^{-1}	Final	AU mL^{-1}	Final	AU mL^{-1}
Tryptone	0.683	**125155**	0.687	**113777**	0.694	**91022**	0.690	**73955**	0.650	**56888**
Animal peptone	0.570	56888	0.577	62577	0.588	51200	0.561	48355	0.573	39822
Soy peptone	0.747	22755	0.670	34133	0.752	14222	0.733	29866	0.630	34133
Casein peptone	0.644	**91022**	0.615	**136533**	0.644	**56888**	0.639	**125155**	0.599	**113777**
Meat extract	0.622	8533	0.638	9244	0.647	4266	0.620	5688	0.582	4266
Yeast extract	0.698	12800	0.700	12800	0.679	9244	0.708	10666	0.675	4266
Initial pH value:										
4.5	0.484	6400	0.491	12800	0.478	9955	0.491	11377	0.469	4266
5.0	0.664	31288	0.658	25600	0.654	19911	0.676	22755	0.591	12800
5.5	0.761	34133	0.746	31288	0.738	45511	0.746	39822	0.748	51200
6.0	0.800	**79644**	0.839	**113777**	0.802	**113777**	0.789	**136533**	0.845	**136533**
6.5	0.958	**91022**	0.959	**91022**	1.029	**113777**	0.955	**113777**	0.775	**79644**
7.0	0.910	12800	0.944	19911	0.977	22755	0.983	15644	0.666	17066
Temperature (°C)										
20	0.717	7111	0.733	5688	0.623	4622	0.698	7111	0.630	3911
25	0.786	**14222**	0.781	**17066**	0.776	**17066**	0.797	**12799**	0.719	**9955**
30	0.750	**14222**	0.754	**17066**	0.718	**18488**	0.758	**14222**	0.688	**9955**
35	0.733	6399	0.725	9955	0.754	7111	0.763	8533	0.693	3199
40	0.650	399	0.700	266	0.662	311	0.619	355	0.573	266
Aerobiose	0.956	**45511**	0.944	**31288**	0.947	**51200**	0.867	**45511**	0.965	**39822**
Anaerobiose	0.917	31288	0.926	17066	0.930	14222	0.874	12800	0.961	11377

O.D., optical density; AU mL^{-1}, arbitrary unit for mL



```
                                    -10         1    10         20       30
L. Lactis nisin Z(CAA43440.1)  MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
L. lactis ID1.5                MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
L. lactis ID3.1                MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
L. lactis ID8.5                MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
L. lactis PR3.1                MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
L. lactis PD4.7                MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
```

Fig. 1. Deduced amino acid sequences of the region encoding nisin Z in *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1, PD4.7 and homologous sequence of *L. lactis* NIZO 22186 obtained in the GenBank.

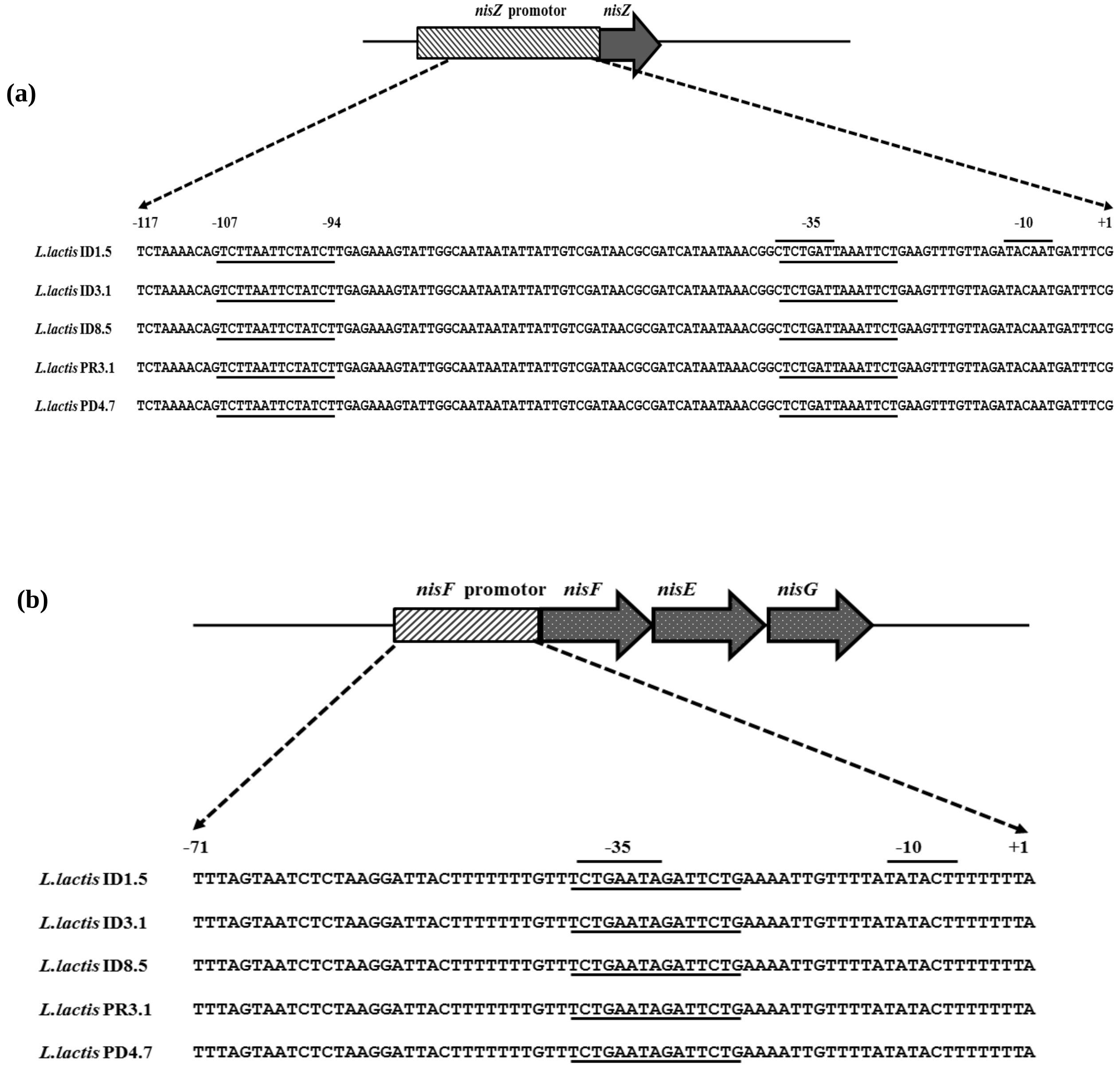


Fig. 2. Alignament of the *nis*Z (a) and *nisF* (b) promoter sequences of *L. lactis* ID1.5, ID3.1, ID8.5, PR3.1 and PD4.7. The -35 and -10 sites and TCT-N_8-TCT are underlined.

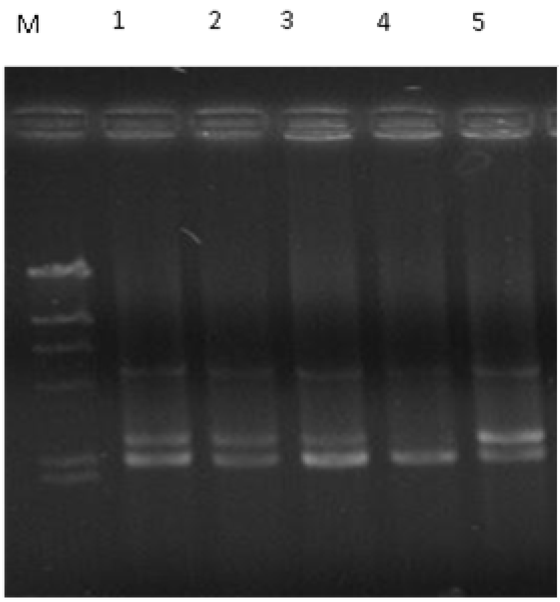


Fig. 3. The plasmids profile of *L. lactis* ID1.5 (lane 1), ID3.1 (lane 2), ID8.5 (lane 3), PR3.1 (lane 4) and PD4.7 (lane 5) and *Hind*III marker (lane M).

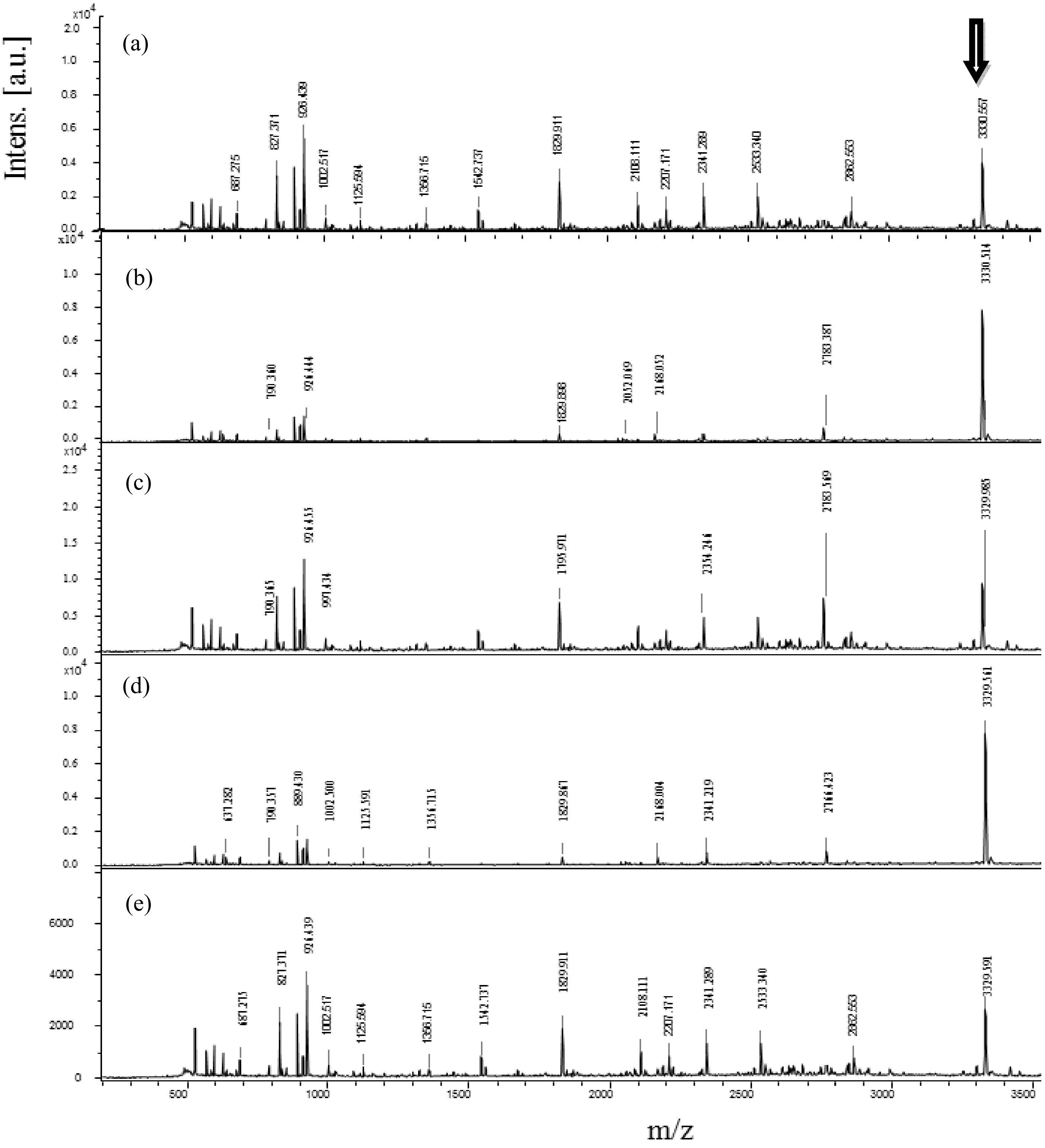


Fig. 4. Mass spectrometry analysis of bacteriocins purified from *L. lactis* ID1.5 (a), ID3.1 (b), ID8.5 (c), PR3.1 (d) and PD4.7 (e). Arrow indicates the molecular mass of bacteriocin.

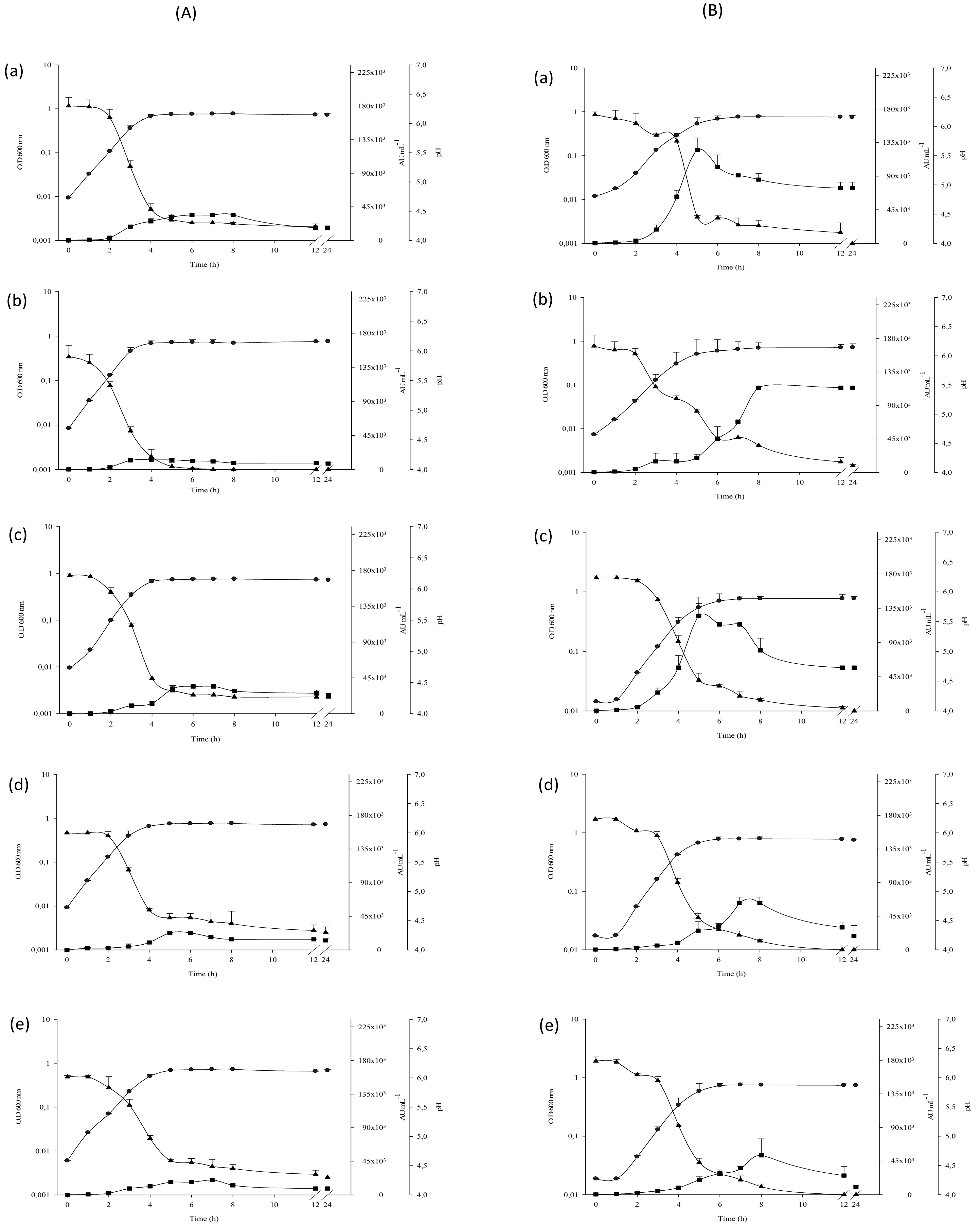


Fig. 5. Production of bacteriocin profile of *L. lactis* subsp. *lactis* ID1.5 (a), ID3.1 (b), ID8.5 (c), PR3.1 (d) and PD4.7 (e) strains. Cells were grown in LAPT broth with 10 g glicose L $^{-1}$ (A) and 10 g fructose L $^{-1}$ (B) as carbono source (see Methods). Antimicrobial activity is presented as AU mL $^{-1}$(■), change in optical density (●) and pH (▲) are indicated.

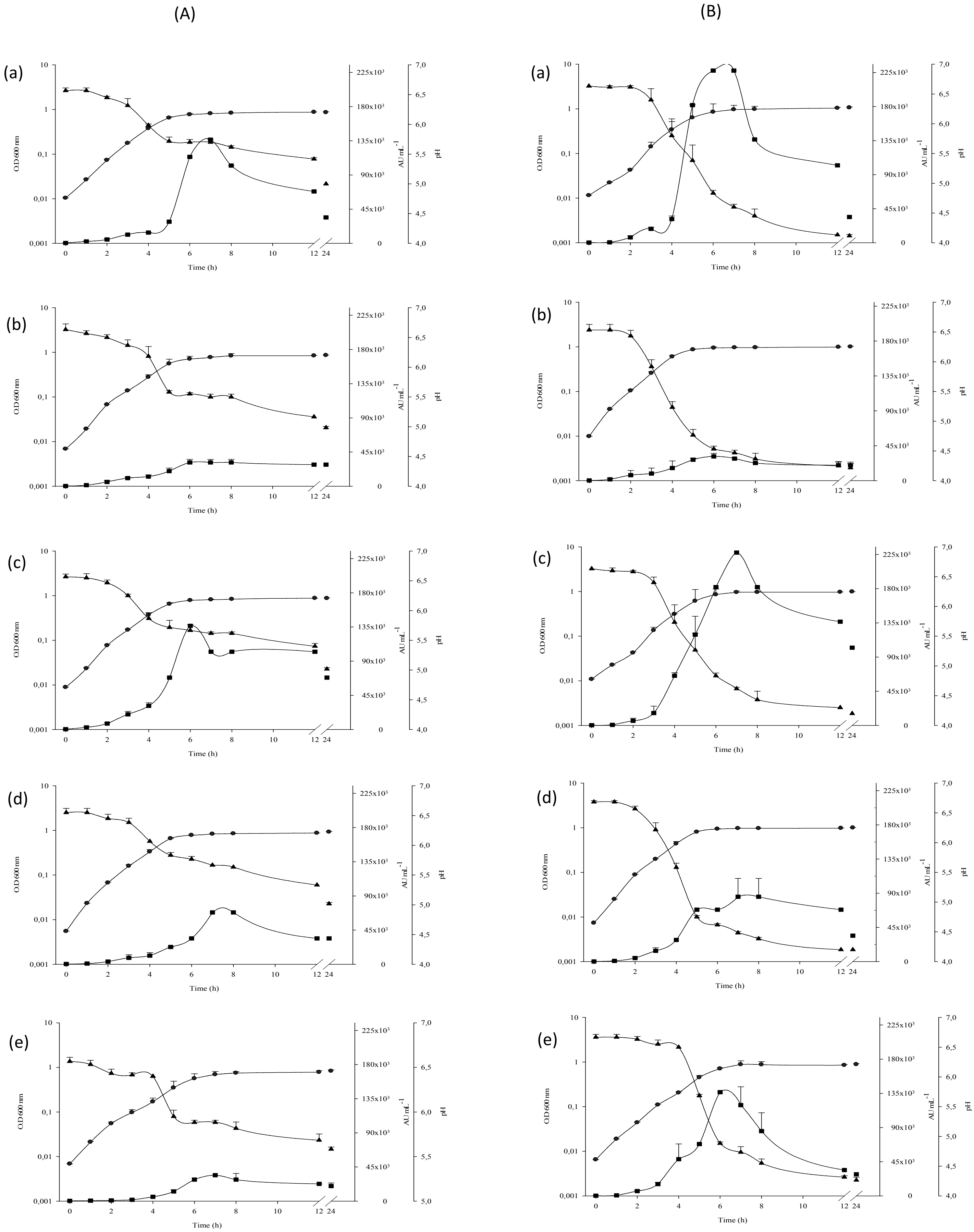


Fig. 6. Production of bacteriocin profile of *L. lactis* subsp. *lactis* ID1.5 (a), ID3.1 (b), ID8.5 (c), PR3.1 (d) and PD4.7 (e) strains. Cells were grown in LAPT broth with 10 g lactose L^{-1} (A) and 10 g sucrose L^{-1} (B) as carbono source (see Methods). Antimicrobial activity is presented as AU mL^{-1}(■), change in optical density (●) and pH (▲) are indicated

CHAPTER 3

Detection and activity of two new cell-bound antimicrobial compounds produced by *Lactococcus lactis* ID1.5

Abstract

In the present study two cell-bound antimicrobial compounds produced by *L. lactis* ID1.5 were purified and partially characterized. After purification by cationic exchange, solid phase C_{18} column and three runs of reversed-phase chromatography, antimicrobial activity was recovered with 60% and 100% 2-propanol, suggesting that more than one antimicrobial compound, called in this study AI and AII, were produced by *L. lactis* ID1.5. Compond AI has a spectrum of antimicrobial activity mainly against *L. lactis*, including its producer strain. *L. lactis* LMGT 2122, *Bacillus subtilis* DSMZ 347, *Listeria innocua* BL86/26B, *Streptococcus pneumoniae* TIGR 4 and *Pseudomonas aeruginosa* seem to be the organisms most sensitive to compound AII. The antimicrobial activity of both compounds was drastically reduced by treatment with tween 80. Nevertheless, both compounds showed high stability to heat and proteases treatments. These compounds show distinct properties from other antimicrobial substances produced by *L. lactis*, and have a significant inhibitory effect against two clinically important respiratory pathogens.

1. Introduction

The antimicrobial activity of bacteria has long been attributed to the production of metabolites such as organic acids, hydrogen peroxide, ethanol and diacetyl. It has gradually become clear, however, that additional metabolites often contribute to the antimicrobial capacity of bacteria. One such category of metabolites are the ribosomally synthesized peptides, called bacteriocins (Nes and Johnsborg, 2004). These Antimicrobial peptides (AMPs) are considered among the most promising candidates for future therapeutics and have resulted in intensive research for discovery of new types and sequences. In addition, it has also become evident that other newly defined antimicrobial compounds, mainly secondary metabolites, such as lipopeptides (LiPs), have been produced by certain bacteria. They are produced non-ribosomally and consist of a short linear or cyclic peptide sequence, with positive or negative charge, to which a fatty acid moiety is covalently attached to the N-terminus (Mangoni and Shai, 2011). Several *Bacillus* strains have been considered to produce LiPs, such as surfactins or lichenysins, iturins and fengycins or plispastatins (Romero *et al.*, 2007). Bacteriocin-like inhibitory substance (BLIS) is a new term applied to antagonistic substances, which are incompletely defined or do not fit the typical criteria defining bacteriocins (Atanassova *et al.*, 2003; Galvez *et al.*, 2010). They tend to have a broader spectrum of activity than currently known bacteriocins. A number of these BLIS were reported to be produced by lactobacilli, inhibiting a wide range of both Gram-positive and Gram-negative bacteria as well as of fungi (Boris and Barbès, 2000; Atanassova *et al.*, 2003; Galvez *et al.*, 2010; Ruíz *et al.*, 2012). Several biosurfactants such as glycolipids, polysaccharides, phospholipids, fatty acids and neutral lipids have shown useful as antibacterial, antifungal and antiviral agents (Rodrigues *et al.*, 2006; Moldes *et al.*, 2007; Rodríguez *et al.*, 2010). Recently, the strain *Lactococcus lactis* CET-4434 has been shown to be an interesting cell-bound biosurfactants producer, which inhibited the growth of *Carnobacterium piscicola* used as target microorganism (Rodríguez *et al.*, 2010). In addition, fractions of crude extract cell obtained from *L. lactis* 53 have shown antimicrobial activity against *Sthaphylococcus epidermidis*, *S. aureus* and *Candida albicans* (Rodrigues *et al.*, 2006).

Among lactic acid bacteria, *L. lactis* is quite desirable for industrial application, because it is homofermentative, highly productive and generally recognized as safe (GRAS) (Guinane *et al.*, 2005; Neves *et al.*, 2005). It is used in food production, and constitutes a significant part of the indigenous flora of mammals, including humans (Espeche *et al.*, 2009; Gao *et al.*, 2011). Thus, their antimicrobial metabolites may be considered safe agents for preventing growth of undesirable microorganisms.

We describe the purification and partial characterization of two rather different antimicrobial substances produced by *L. lactis* ID1.5 isolated from fermented sausage, with inhibitory proprieties against Gram positive and negative bacteria.

2. Material and methods

2.1. Bacterial strains and culture conditions

Lactococcus lactis ID1.5 was grown in LAPTg both (Juarez Tomas *et al.*, 2004) at 30 °C for 16h. Indicator strains for determination of antimicrobial spectra were propagated at appropriate temperatures (30 °C or 37 °C), recommended by culture collections, for 18 hours. *L. lactis* IL1403 (nisin sensitive), LMGT 2115 (nisin producer) and LMGT 2122 (nisin producer) were grown in M17 medium (Oxoid) supplemented with 0,4% (w/v) glucose (GM17). *L. sake* NCDO 2714 was grown in MRS broth (Oxoid). *Bacillus subtilis* DSMZ 347, *Enterococcus faecalis* v583, *Listeria innocua* BL86/26B, *Micrococcus luteus* ATCC 10240, ATCC 4698, *Staphylococcus aureus* 2002-05-ME 8245-3, 2002-60-8452, *Pseudomonas aeruginosa*, *Pseudomonas* ssp., *Salmonella Thyphimurium* SL 1344 and *Shigella soneii* ATCC 11060 were grown in BHI medium (Oxoid). *Streptococcus pneumoniae* TIGR4 was grown in Todd-Hewitt broth (BD) supplemented with 0,8% (w/v) glucose and *Escherichia coli* ATCC 14763 was grown in LB medium (Bertani, 2004). Routinely, fresh bacterial cultures were obtained from frozen stocks before each experiment.

2.2. Antimicrobial activity assay

Antimicrobial activity was detected by an agar well diffusion assay, as described by Ryan *et al.* (Ryan *et al.*, 1996). GM17 agar at 30 °C was seeded with the indicator strains *L. lactis* LMGT 2115, LMGT 2122 and IL1403 (20 µl of an overnight culture per 20 mL agar), dispensed into sterile plates, and allowed to solidify. Wells of approximately 4-6 mm in diameter were made. 30µl aliquots of cell-bound antimicrobial compounds extract (CE) obtained with 70% 2-propanol/0,1% trifluoroacetic acid prepared according to (Field *et al.*, 2008) and cell-free culture supernatant (CFS) of *L. lactis* ID1.5 were dispensed into the wells. After incubation overnight at 30 °C, antimicrobial activity was observed.

Antimicrobial activity was quantified by using a microtiter assay method (Holo *et al.*, 1991). A twofold serial dilution (in medium) of 100 µL antimicrobial compounds and cell extract samples were prepared in a microtiter plate well containing 50 µL of the culture medium to which 150 µL of a diluted overnight culture of the indicator strain was added. The microtiter plate cultures were incubated for 12 hours, after which growth inhibition was measured spectrophotometrically at 620 nm with a microtiter plate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). One Arbitrary Unit (AU) was defined as the amount of antimicrobial compounds which inhibited growth of the indicator strains by 50%.

2.3. Extraction and purification of the cell-bound active compounds

The extraction of the cell-bound antimicrobial compounds was performed by a bacteriocin extraction protocol (Field *et al.*, 2008) with some minor modifications. *L. lactis* ID1.5 was grown in two litres of LAPTg broth at 30°C, overnight. The cells were collected by centrifugation at 7500 r.p.m. for 20 min. The cells pellet were re-suspended in 300 mL of 70% isopropanol (Arcus, Norway) containing 0.1% trifluoroacetic acid (Merck) and stirred at room temperature for 3 hours. The cell debris was removed by centrifugation at 7500 r.p.m. for 20 min and the supernatant retained. For purification, the sample was applied to 5 mL SP Sepharose Fast Flow (GE Healthcare Biosciences, Uppsala) equilibrated with 10 mM acetic acid. The active compounds were collected in the unbound fraction and applied in solid phase C_{18} column. The sample was diluted and pH adjusted to 4 before applying to a 10g solid phase C_{18} column (Resprep, Resteck, Norway) pre-equilibrated with methanol and water. The inhibitory activity compounds were eluted in 140 mL of 70% isopropanol/0.1% trifluoroacetic acid (TFA). The purification was followed by reversed - phase chromatography using Äkta Purifier Fast Protein Liquid Chromatography System. The reversed-phase column (Resource 15 RPC 3 mL); Pharmacia Biotechnology, Norway) was equilibrated with 0.1% TFA in water. Elution was performed with a gradient of isopropanol (Merck) containing 0.1% TFA at a flow rate of 1 mL/min as follows: 0-5 min, 0%-30% (v/v); 5-15 min, 30% (v/v); 15-20 min, 30 to 60%, 20-30 min, 60%; 30-35 min, 60-100%; 35-45 min, 100% of isopropanol, and collected in 1 mL fractions. From RPC I, active fractions eluted in 60% (AI) and 100% isopropanol (AII) were diluted in sterile water, and applied separately to reversed-phase column (RPC II). This step was repeated once (RPC III) to obtain one peak of each compound, AI and AII.

2.4. Effect of heat, proteolytic enzymes and tween 80 on stability of antimicrobial compounds

Samples of fractions purified were dispensed in micro tubes and treated separately in a water bath at 100 °C for 15 min and 100 °C for 30 min. After, samples were cooled and the residual activity was determined by the microtitre plate assay.

The protein nature of the antimicrobial compounds was verified by treatment with the enzymes trypsin (Sigma-Aldrich) and proteinase K (Finnzymes) at 10 mg mL^{-1} concentration, in 0,01 M phosphate buffer at pH 7,0, were added to 0,1 mL of fractions samples to give a 1mg mL^{-1} enzyme final concentration. The samples were filtered through 0.22 μm pore-size filters (Millipore) and incubated for 5 hours at 37°C. The reactions were stopped by boiling the mixture for 3 min. The residual activity was tested against *L. lactis* LMG 2122 by the microtitre plate assay.

To investigate the effect of tween 80 (Sigma-Aldrich), samples of fractions were added separately with different final concentrations (0.01%, 0.02%, 0.05%, 0.1%, 0.5% and 1.0%) of detergent. The samples were filtered and tested against *L. lactis* LMG 2122 by the microtitre plate assay.

In all steps untreated samples were used as controls.

3. Results

We have earlier shown that the culture supernatant of strain *L. lactis* ID1.5 possesses a good bacteriocinogenic potential. In order to confirm the presence of novel cell bound active compounds produced by *L. lactis* ID1.5, the activity of cell extract was compared to the cell-free culture supernatant using *L. lactis* nisin producer and nisin sensitive as indicators (Table 1). Only one lactococcal strain, *L. lactis* IL1403, was sensitive to cell-free culture supernatant, whereas all other strains were insensitive to cell-free culture supernatant and sensitive to the cell extract. This indicates that there is other inhibitory substance present in cell extract different than nisin.

3.1. Extraction and Purification of the cell-bound active compounds

In order to establish the presence of other compounds and to study their proprieties, the CE was subjected to sequential purification steps. The consequent increase in the specific activity and the fold purification obtained at each step is summarized (Table 2). In cation exchange chromatography, the antimicrobial activity was mainly retained in the unbound fraction. The volume of the unbound fraction was them applied subsequently to a solid phase C_{18} column, in which two fold increases in specific activity was observed (Table 2). Upon chromatography on RPC I, two distinct fractions with antimicrobial activity were obtained. These antimicrobial substances, termed compounds AI and AII, were eluted at 60% isopropanol and 100% isopropanol, respectively, which indicate high hydrophobicity of bioactive agents. These fractions were separated and purified in two further steps RPC (II and III). The third step of reverse phase chromatography (RPC III) yielded pure fractions of each compound with antimicrobial activity, suggesting the presence of two different antimicrobial compounds (Fig.1a and 1b). The most active fractions revealed 43 and 110 fold increasing in specific activity to AI and AII compounds, respectively (Table 2). Agar well diffusion assay of fractions obtained by RPC III of each compound is shown in Fig. 1c. The clear halos around the wells charged with the fractions revealed also the antimicrobial activity of both compounds in the plates inoculated with the indicator strain. However, compared AI resulted in the apparent strongest antimicrobial activity demonstrated by the largest halo (Fig. 1c).

3.2. Effect of heat, proteolytic enzymes and tween 80 on stability of antimicrobial compounds

The inhibitory action of compounds AI and AII was not reduced when they were treated with trypsin and proteinase K. Furthermore, the activity of both compounds AI and AII was maintained after heat treatment at 100°C for 15 and 30 minutes. These results demonstrated that the bioactive substances produced by *L. lactis* ID1.5 are heat stable. On the other hand, when a purified preparation of both compounds was treated with different concentrations of tween 80, the inhibitory action was significantly reduced. When the purified preparations were treated with 0.01% of tween 80 the activities of compound AI and AII were reduced 87% and 75%, respectively. After treatment with 0.05% of tween 80 the activity of compound AI was completely lost. The activity of compound AII was lost after treatment with 1.0% of tween 80.

3.3. Determination of inhibitory spectrum

The inhibitory spectrum of AI and AII compounds produced by *L. lactis* ID1.5 is presented in Table 3. They showed inhibitory activities against several species Gram-positive (*L. lactis*, *B. subtilis*, *S. pneumoniae* and *L. innocua*) and Gram-negative (*P. aeruginosa, Pseudomonas* ssp.*)*. The sensitivity varied considerably among the different target strains, *Pseudomonas* ssp appears to be less sensitive for both compounds. *L. lactis* LMGT 2122, *B. subtilis* DSMZ 347, *L. innocua* BL86/26B, *S. pneumoniae* TIGR 4 and *P. aeruginosa* seem to be the organisms most sensitive to compound AII. Interestingly, the compound AI showed relatively high activity against all strains of *L. lactis*. However, it also showed considerable activity against *L. lactis* ID1.5, its own producer strain. Among all *L. lactis* tested, the strain LMGT 2122 was the only which showed sensitivity to compound AII, thus it was used as indicator during all steps of purification and in the stability tests.

4. Discussion

L. lactis ID1.5 isolated from fermented sausage produces a natural extracellular nisin Z variant. In this study, we report the detection, purification and partial characterization of new inhibitor compounds, which are different from other antimicrobial peptides or bioactive compounds produced by *L. lactis*. The antimicrobial compounds appears to be cell associated, as cell-free supernatant from *L. lactis* ID1.5 cultures lacked antimicrobial activity against *L. lactis* LMGT 2122 used as indicator when assessed by agar well diffusion method (Table 1).

To characterize and identify these compounds, several purification approaches were applied to the cell extract of the producer strain. Even though, this method is designed for the isolation of cationic and hydrophobic peptides, it led us to purify the inhibitory substances. However, the antimicrobial substances did not bind to the cationic exchange resin, indicating the anionic nature of the compounds. This characteristic contrasts with the bacteriocins produced by lactic acid bacteria, which are cationic peptides (Lubelski *et al.*, 2008; Nissen-Meyer *et al.*, 2009). Compound AI was detected as the peak at 60% isopropanol and compound AII was eluted in 100% isopropanol, indicating that AII is more hydrophobic than AI (Fig. 1). Furthermore, the antimicrobial substances were extracted with isopropanol, suggesting the presence of a hydrophobic moiety in the substances. Rodrigues et al. (Rodrigues *et al.*, 2006) reported the presence of glycoprotein-like in the composition of cell extract obtained of *L. lactis* 53, which showed antimicrobial activity against several bacterial strains.

For an accurate characterization of the different purified compounds, mass spectra were recorded by electrospray ionization-mass spectrometry (ESI-MS), however the results showed variations among ions. The mass spectra of compound AI showed a series of mass number of m/z = 367 to 1071 and of compound AII, m/z = 437 to 956 (data not shown).

The two compounds proved to be resistant to heat and stable to proteolytic enzymes, and these properties could be advantageous for commercial applications. Sensitivity to proteolytic enzymes is an important characteristic which distinguishing bacteriocins from other antimicrobial compounds. A few bacteriocins produced by lactic acid bacteria proved to be resistant to proteolytic degradation, such as gassericin A and reutericin 6 (Kawai *et al.*, 2001; Maqueda *et al.*, 2008). The activity of both compounds purified was susceptible to treatment with tween 80 (Table 3). This result suggests that tween 80 may reduce the level of action of these compounds to the cells, or to increase the resistance of indicator strain, causing barrier protection of the site of action on the cell. Tween 80 is a nonionic detergent, hydrophilic, mostly composed of oleic acid and has been used for some time in bacterial cultures to assist in growth. It has also been found to promote the entrance of compounds and their exit from the cell (Keren *et al.*, 2004). Kimoto *et al.* (Kimoto *et al.*, 2002) reported that tween 80 produced a variation in the fatty acid composition in *L. lactis* strain. They concluded that these fatty acids contributed to enhancing the lipid membrane stability. In contrast, it has been shown previously that tween 80 increased the sensitivity of indicator strain to bacteriocin lacticin RM or increased its activity (Keren *et al.*, 2004). This suggests that the mode of action of these compounds might be different than that of bacteriocins, and should be clarified in detail.

The inhibitory spectrum of AI and AII compounds purified from cell extract of *L. lactis* ID1.5 effectively inhibited several Gram positive and Gram negative bacteria including spoilage and pathogenic bacteria, such as *B. subtilis*, *S. pneumoniae* TIGR 4 and *P. aeruginosa*. Pulusani et al. (Pulusani *et al.*, 1979) reported strong inhibitory activity of a methanol-acetone extract from *S. thermophilus* grown in milk against *Bacillus* sp., *P. aeruginosa*, *P. fluorencens* and various strains of *L. lactis*. Interestingly, in our work the compound AI showed antimicrobial activity against all *L. lactis* strains tested. Moreover, the compound AI was able to inhibit its producer strain, suggesting that an effective immune system for specific protection against this compound is not found its producer strain.

Previous work indicated that *L.lactis* ID1.5 produces a bacteriocin, nisin Z, and in this study we report the production also of two inhibitor compounds. Antimicrobial compound production is a bacterial defense mechanism which gives the producer strain a competitive advantage towards non-producer and sensitive strains in the same niche (And and Hoover, 2003). Thereby, microorganisms that simultaneously produce more than one inhibitory substance show additional advantages in terms of capacity for colonization and thus ability to be a health protective industrial strain.

The results of our study indicate that *L. lactis* ID1.5 produces cell-bound antimicrobial substances not previously reported by other *L. lactis*. Even though, they are produced by lactic acid bacteria are heat stable and are active against Gram positive bacteria. Several characteristics make these substances different from the bacteriocins. They are hydrophobic, anionic and resistant to proteases. Additionally, *L. lactis* ID1.5 does not seem to produce these bioactive substances extracellularly. The low molecular weight and antimicrobial activity against gram-negative bacteria, suggest that the antimicrobial substances may be a short-chain fatty acid or other amphiphilic substance. We were unable to obtain the identification of these compounds, perhaps, due to low concentrations obtained after purification.

However, due to spread and increase of resistance to classical antibiotics there is a need for new antimicrobial compounds. As mentioned earlier, these compounds showed antimicrobial activity against *P. aeruginosa* and *S. pneumoniae* TIGR 4, two clinically important respiratory pathogens (Lomovskaya *et al.*, 2001). These pathogens can cause difficulty to treat respiratory infection because of their intrinsic resistance to antibiotic therapy with many available antibiotics (Zhanel *et al.*, 2004). Thus, the new compounds obtained this study may allow efficacy against these pathogens and may be exploited for other applications.

Further studies are underway and will be crucial to investigate the chemical proprieties and structural analysis in order to clarify the exact structure of bioactive compounds extracted from cell surface of *L. lactis* ID1.5, as well as to know their distinct mechanism of action. Their precise identification may impact the efficacy of the extraction protocols and improve production levels for further studies.

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Table 1. Antimicrobial activity of cell extract and cell-free culture supernatant from *L. lactis* ID1.5

Indicator	Description	CFS	CE
L. lactis ID1.5	This study	-	+
L. lactis IL1403	nisin sensitive	+	+
L. lactis LMGT 2115	nisin producer	-	+
L. lactis LMGT 2122	nisin producer	-	+

Abbreviations: CFS, cell free supernatant; CE, cell extract; +, activity; -, absence of any activity

Table 2. Purification of cell-bound antimicrobial compounds produced by *L. lactis* ID1.5

Purification step[a]	Volume (mL)	Protein concentration (mg mL^{-1})[b]	Antimicrobial activity ($\times 10^3$) (AU mL^{-1})	Specific Activity (AU mg^{-1})	Purification Factor
CE	300	3.88	1.28	330	1.00
IEX	300	2.90	1.28	441	1.33
Resprep C_{18}	140	1.58	1.28	810	2.45
Compound AI					
RPC I	3	0.61	1.28	2.09×10^3	6.33
RPC II	2	0.32	1.28	4.00×10^3	12.12
RPC III	2	0.18	2.56	1.42×10^4	43.03
Compound AII					
RPC I	3	0.90	2.56	2.84×10^3	8.6
RPC II	2	0.30	5.12	1.70×10^4	51.51
RPC III	1	0.14	5.12	3.65×10^4	110.60

[a] CE, cell extract; IEX, ion-exchange chromatography, RPC, reversed-phase chromatography. [b] The protein concentration was determined either by determining the optical density at 280 nm.

Table 3. Effect of heat, enzyme and Tween 80 treatments on the antimicrobial activity of the compounds AI and AII produced by *L. lactis* ID1.5

Application	Antimicrobial activity (AU mL^{-1})[a]	
	AI	AII
Untreated	2560	5120
100°C, 15 min	2560	5120
100°C, 30 min	2560	5120
Trypsin	2560	5120
Proteinase K	2560	5120
Tween 80		
0.01%	640	640
0.02%	40	320
0.05%	NI	40
0.1%	NI	40
0.5%	NI	40
1.0%	NI	NI

[a] *L. lactis* LMGT2122 was used as indicator strain; AU mL^{-1}; Arbitrary Unit for mL.

Table 4. Inhibition spectrum of purified compounds AI and AII produced by *L. lactis* ID1.5

Indicator	Strain	AU mL^{-1}	
		AI	AII
Lactococcus lactis	ID1.5	640	NI
Lactococcus lactis	IL1403	1280	NI
Lactococcus lactis	LMGT 2115	1280	NI
Lactococcus lactis	LMGT 2122	2560	5120
Bacillus subtilis	DSMZ347	320	1280
Enterococcus faecalis	v583	NI	NI
Lactobacillus sake	NCDO 2714	NI	NI
Listeria innocua	BL86/26B	NI	1280
Micrococcus luteus	ATCC 4698	NI	NI
Micrococcus luteus	ATCC 10240	NI	NI
Staphylococcus aureus	2002-05-ME8245-3	NI	NI
Staphylococcus aureus	2002-60-8452	NI	NI
Streptococcus pneumoniae	TIGR4	640	1280
Escherichia coli	ATCC 14763	NI	NI
Pseudomonas aeruginosa	-	320	1280
Pseudomonas ssp.	-	160	640
Salmonella Thyphimurium	SL1344	NI	NI
Shigella soneii	ATCC 11060	NI	NI

Abbreviations: ATCC, American Type Culture Collection; LMGT, Laboratory of Microbial Gene Technology (UMB); NI, no inhibition; AU mL^{-1}; Arbitrary Unit for mL.

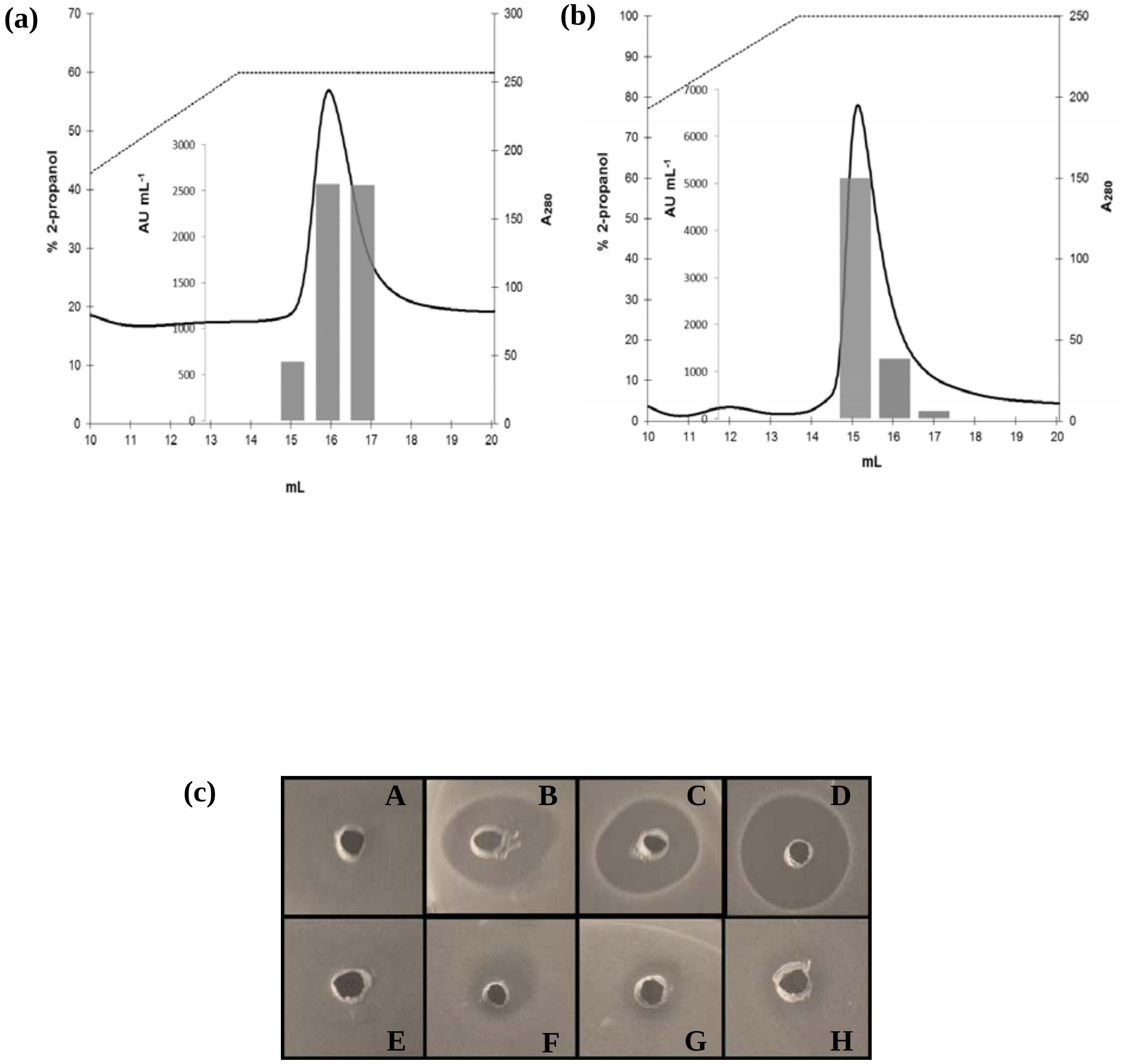

Fig 1. The chromatographic elution profile from RPC III shows (a) compound AI and (b) compound AII. Solid lines show the absorbance at 280 nm and elution gradient of isopropanol (%). The antimicrobial activity quantified by microtiter assay system is shown as grey columns. (c) Formation of clearing halos around the wells charged with the fractions 15 (B), 16 (C) and 17 (D) obtained by RPCIII of compound AI, and with the fractions 15 (F), 16 (G) and 17 (H) obtained by RPCIII of compound AII in the plates inoculated with *L. lactis* LMGT 2122 as indicator strain. (A) 60% isopropanol/0.1% TFA (E) 100% isopropanol/0.1% TFA.

CHAPTER 4

Identification of the Natural Variant Nisin Z Produced by *Lactococcus lactis* PD6.9: a Potencial Anti-*Staphylococcus aureus* Bacteriocin

Abstract

Nisin is a bacteriocin belonging to the lantibiotic group that shows important therapeutic potential. In this study, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* PD6.9, was purified and identified. The presence of the nisin gene was determined by PCR and DNA sequencing. Bacteriocin was purified to homogeneity from culture supernatant by ionic exchange and reversed-phase chromatography, and molecular weight was determined by mass spectrometry. Sequencing of the *L. lactis* PD6.9 nisin gene showed that it was the natural nisin variant, nisin Z, as indicated by substitution of asparagine residue instead of histidine at position 27. The nisin determinant in strain PD6.9 was found to be located in the chromosome. Purification of nisin and mass spectrometry analysis confirmed the genetic finding. The ability of the nisin Z produced by *L. lactis* PD6.9 to inhibit significant pathogens including *S. aureus* strains isolated from bovine mastitis may be useful for future applications.

1. Introduction

Lantibiotics are antimicrobial peptides that have attracted widespread scientific attention not only as promising safe and natural food additives but also as potential therapeutic agents to combat medically significant bacteria and their multi-drug resitant forms (Field *et al.*, 2008). These ribosomally synthesized peptides are distinguished by the presence of post-translationally modified amino acids such as dehydroalanine (Dha), dehydrobutyrine (Dhb) and eponymous lanthionine (Lan) and *β*-methyllanthionine (MeLan) formed by thioether linkages between dehydrated amino acid residues and neighboring cysteines (Rink *et al.*, 2007). Nisin A is one of the most intensively studied lantibiotic, it has a long record of safe use, is US Food and Drug Administration (FDA) approved (Delves-Broughton, 1990) and is one of a few bacteriocins to has been applied commercially (Bierbaum and Sahl, 2009). It is a 34 residue-long peptide bacteriocin produced by strains of *Lactococcus lactis*, which is active against many Gram-positive bacteria including food-borne pathogens such as staphylococci, bacilli, clostridia and mycobacteria (Field *et al.*, 2010), but it can be very potent and is able to kill bacteria at nanomolar concentrations (Cotter *et al.*, 2005b). Nisin exerts its antimicrobial activity both by pore formation and by inhibition of cell wall synthesis through specific binding to lipid II, an essencial precursor of the bacterial cell wall (Breukink *et al.*, 1999; Nes *et al.*, 2007). Natural variants of nisin have been described. These are Nisin Z (Mulders *et al.*, 1991), F (De Kwaadsteniet *et al.*, 2008), Q (Zendo *et al.*, 2003) produced by strains of *L. lactis* and nisin U (Wirawan *et al.*, 2006) and U2 (Piper *et al.*, 2010) are produced by *Streptococcus uberis* and *Streptococcus agalactiae*, respectively.

Potentially, the most significant application of lantibiotics may be in the treatment of antibiotic resistant pathogens. Nisin A has been shown to be active against a number of multidrug-resitant Gram-positive pathogen (Goldstein *et al.*, 1998; Severina *et al.*, 1998) incluing a wide range of mastitis-causing pathogens (Cotter *et al.*, 2005a).

We have earlier described a natural isolate of *L. lactis* subsp. *lactis* strain PD6.9 which produces bacteriocin possessing some importants features (De Carvalho *et al.*, 2006). Here, we report the purification and identification of this bacteriocin designated as nisin Z. Nisin Z purified form was tested against pathogen bacteria including some strains responsible for bovine mastitis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. lactis subsp. *lactis* PD6.9 was cultured in M17 broth (Oxoid) supplemented with 0.4% (w v^{-1}) glucose at 30 °C, as was the indicator strain *Lactococcus lactis* IL1403. All target strains were grown in brain heart infusion (BHI) broth (Oxoid) at 37 °C for 12 hours.

2.2. Assay of bacteriocin activity

Quantitative determination of the antimicrobial activity of the bacteriocin was performed by using a microtiter assay method (Holo *et al.*, 1991). A twofold serial dilution (in medium) of 100 µL bacteriocin samples were prepared in a microtiter plate well containing 50 µL of the culture medium to which 150 µL of a diluted overnight culture of the indicator strain was added. The plate was incubated for 12 hours, after which growth inhibition was measured turbidometrically at 620 nm with a microtiter plate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). One bacteriocin Unit (BU) was defined as the amount of bacteriocin that inhibited the growth of the indicator strain by 50%.

To study the antimicrobial spectrum of nisin produced by *L. lactis* subsp. *lactis* PD6.9, a wide range of indicator organisms (Table 1) were used in microtiter assay system.

2.3. Sequencing of nisin genes

Nucleotide sequencing was performed with the PCR products obtained from the amplifications of genomic DNA of *L. lactis* subsp. *lactis* PD 6.9 using the following primers specific to nisin structural gene, nqf (5’-GTTCGAAGGAACTACAAAATAAATT-3’) and naqzr (5’-ACAGACCAGCATTATATTTCTGC-3’), and *nisA* promoter region, pnisAf (5’-TTGAGTCTTAGACATACTTGAATGACC-3’) and pnisAr (5’-CAATGACAAGTTGCTGTTTTCA-3’). The PCR conditions were different for each pair of primers. The PCR thermal cycle program included an initial denaturation at 94 °C for 2 min, followed by 35 cycles, with a denaturation step at 94 °C for 1 min, an annealing step of 30 s, at 40 °C and 48 °C for primers sets nqf/naqzr and pnisAf/pnisAr, respectively, followed by an extension step during 1 min at 72 °C. Final extension was performed at 72 °C for 7 min. The desired bands after PCR amplification were cut from the gel and purified with Gel Extraction Kit (Nucleospin® Gel and PCR clean up, Machery-Nagel, Germany) and sequenced using the a BigDye Terminator v3.1 cycle Sequencing Kit and ABI Prism 377 DNA sequencing system (Applied Biosystems, United States) as described by the manufaturer. The determined sequences were compared with the GenBank databases using basic local alignment search tools (BLAST) software provided online by National Center for Biotechnology Information (United States).

2.4. Plasmid curing

To obtain plasmid curing, the *L. lactis* subsp. *lactis* PD6.9 was grown in GM17 with 5 μg mL^{-1} of ethidium bromide at 30°C for 24 hours. After incubation, cells were collected by centrifugation and resuspended in the same volume of fresh GM17 containing 5 μg mL^{-1} of ethidium bromide and incubated again for 24 hours. The same procedure was repeated several times, but in the same volume of fresh GM17 containing 10 μg mL^{-1} of ethidium bromide. Cultures, which survived were diluted, plated on GM17 agar and incubated at 30°C for 24 hours. After replica plating, the colonies were overlaid with soft agar containing *L. lactis* IL1403 as indicator organism and incubated once again at 30°C for 24 hours and checked for zones of growth inhibition. The colonies were purified and propagated in GM17 broth. The presence of plasmid was checked by EZ.N.A. ^{TM} Plasmid Miniprep Kit II Spin Protocol (OMEGA Bio-Tek, United States).

2.5. Purification of bacteriocin

The supernatant from a 200 mL overnight culture of *L. lactis* subsp. *lactis* PD6.9 was collected. Ammonium sulfate (40 g per 100 mL) was added to the supernatant and agitated for 30 min at 4°C. The bacteriocin was precipitated from the supernatant by centrifugation (10000 rpm for 30 mim at 4°C) and dissolved in 20 mL sterile distilled water, and the pH was adjusted to 3,5 with 1M HCl. It was them passed through a 5 mL SP Sepharose Fast Flow (GE Healthcare Biosciences, Uppsala) equilibrated with 10 mM acetic acid. The column was eluted with a stepwise gradient consisting of 10 mL of each of 0.1, 0.3 and 1.0 M NaCl at 1 mL per min flow rate and stored on ice. The fractions displaying the highest bacteriocin activities were used for further purification. The purification was followed by reversed - phase chromatography using Äkta Purifier fast protein liquid chromatography system. The most active fractions from the ion-exchange were applied to a reverse-phase column (Resource 15 RPC 3 mL; Pharmacia Biotechnology) equilibrated with 0.1% trifluoroacetic acid (TFA) in water. Elution was performed with a water-isopropanol gradient from 0 to 100%, containing 0.1% TFA (v v^{-1}). The most active fractions were stored at 4°C for further analysis.

2.6. Mass spectrometry

The molecular weight of the purified bacteriocin was determined by mass spectrometry. Bacteriocin samples (active fractions) were mixed 1:1 with a solution of 15 mg α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 49.9% ethanol and 0.1% TFA and deposited on a ground steel matrix-assisted laser desorption ionization target. Mass spectra were recorded in the positive reflector mode with an Ultra Flex TOF/TOF (Bruker Daltonic GmBH, Bremen, Germany), using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV.

2.7. Results

The bacteriocin was purified from the cell free culture supernatant of *L. lactis* subsp. *lactis* PD6.9, grown overnight, by ammonium precipitation, ion-exchange and reverse-phase chromatography (Table 2). The cell free culture supernatant contained 1280 bacteriocin units mL^{-1} as determined with the indicator strain *L. lactis* IL1403. The bacteriocin specific activity was concentrated 10-fold from the cell free culture supernatant by ammonium sulphate precipitation. This concentration step resulted a recovery of 20% of activity. Upon subsequent prepurification step by ion-exchange chromatography, the specific activity was about 300-fold higher than that of the cell free culture supernatant, and the recovery was about 20%. The specific activity of the final purified bacteriocin eluted from reversed-phase chromatography was about 2500-fold higher than that of the cell free culture supernatant, with a recovery of about 8%. The molecular mass of the purified fractions 14 and 15 (Fig. 1A) was determined by mass spectrometry to be 3329.571 Da (M+1, 3330. 6) (Fig. 1B), which is close to the molecular mass of the nisin Z, whose monoisotopic molecular mass is 3330. 93 Da (Piper *et al.*, 2010).

The inhibitory spectrum of nisin produced by *L. lactis* subsp. *lactis* PD6.9 is presented in Table 1. It inhibited a broad range of several species Gram-positive (*Lactococcus*, *Bacillus*, *Enterococcus*, *Listeria*, *Micrococcus*, *Streptococcus*, *Staphylococcus*) but not Gram-negative bacteria (*Escherichia coli* ATCC 14763 and *Pseudomonas aeruginosa*). The bacteriocin unit concentration varied considerably among the different target strains, *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* 4119 and *Staphylococcus aureus* 3975 seem to be the organisms most sensitive to nisin Z, whereas *Enterococcus faecalis* v583 appears to be less sensitive. All the *S. aureus* strains isolated from bovine diagnosed with mastitis were sensitive to nisin Z.

The PCR products obtained from the amplifications of genomic DNA of *L. lactis* subsp. *lactis* PD6.9 with primers specific to nisin structural gene were subjected to nucleotide sequencing (Fig. 2A). Results indicated that the sequence of PD6.9 nisin gene was identical to that of nisin Z (GenBank accession number AB375441.1). Homology was also recorded with nisin A (GenBank accession number HM219853.1), except for a C-to-A transversion at position 148 (Fig. 2A). This resulted in an asparagine (AAT) residue at position 27 of the nisin peptide, instead of histidine (CAT), as the deduced amino acid sequence encoded by it showed significant similarity (100% identity) to nisin Z (GenBank accession number P29559.1). This indicates that the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9 is a natural variant nisin Z, as shown in Fig. 2B.

The nucleotide sequence of the PCR fragment (amplified with primers pnisAf and pnisAr) containing the *nisA* promoter region has 99% identity to the sequence recorded for the promoter region encoding nisin Z (Gen Bank accession number Y13384.1). It has a consensus promoter characterized by -35 and -10 sequences that are spaced by an average of 17 nucleotides. The promoter region upstream of structural gene *nis* contains a TCT direct repeat with 8-bp spacer region at the positions (-39 to -26) upstream of the transcription start site. It also contains a second TCT-N_8-TCT motif present upstream of structural gene *nisZ* at positions -107 to -94 (Fig. 2A).

The plasmid curing was conducted to examine whether the genetic determinant for nisin production in *L. lactis* PD6.9 is located on plasmid. However, strains PD6.9 cured derivatives were able to produce bacteriocin after growth in the presence of ethidium bromide (10 µg mL^{-1}), suggesting that the genes encoding the bacteriocin are located on the chromosome.

3. Discussion

In the last few years, a variety of bacteria such as lactic acid bacteria have attracted attention for their production of compounds with potential uses in many fields. In this investigation, we have carried out the identification and purification of the antimicrobial compound produced by an naturally fermented sausage isolate of lactic acid bacteria, *L. lactis* subsp. *lactis* PD6.9 (Maciel, 1998).

Development of three steps purification procedure allowed the separation of bacteriocin and the reliability of each step were demonstrated by significant increase in the specific activity of bacteriocin. After mass spectrometry analysis of the purified bacteriocin, the apparent molecular mass was confirmed to be 3329.571 Da (M+1, 3330.6), corresponding the native form of nisin Z.

The deduced amino acid sequence of the PD6.9 nisin showed that it contained an asparagine at position 27 instead of a histidine as in nisin A. A BLAST search of GenBank sequences indicated that the PD6.9 nisin is a variant nisin Z. De Vos *et al.* (De Vos *et al.*, 1993) reported that the His27Asn substitution resulted in a higher diffusion rate of nisin Z. Natural variants of a number of lantibiotics have been described (Cotter *et al.*, 2005a). The existence of natural variants suggests that the identity of amino acids present at certain locations is flexible and it may thus be possible to generate mutants. These natural variants may highlight regions of lantibiotics that demonstrate a greater propensity and permissiveness to change while the comparison of more distantly related peptides permits the identification of conserved regions that are likely to be essential for activity (Cotter *et al.*, 2005a). In addition, nisin variants may have potential as novel antibiotics, because, generally it is not recommended to use the same compound for both food conservation and for antibiotic treatment (Lubelski *et al.*, 2008).

The promoter sequences of *nisZ* of the PD6.9 strain contain a partially conserved region which could be involved in the transcriptional control function, the TCT-N_8-TCT motif present upstream of structural gene *nisZ* (Fig. 2A). Chandrapati and O'Sullivan (Chandrapati and O'sullivan, 2002) reported that this motif is supposed to be involved in a co-operative binding of the NisR response regulator of the NisRK two-component regulatory system. They also reported a second TCT-N8-TCT motif present upstream of *nisA* at positions -107 to -94, which was also shown in our results. This TCT repeat, together with the first one, is involved in the optimal binding of NisR (Trmčić *et al.*, 2011).

Plasmid curing results suggest that the genes encoding nisin Z in *L. lactis* PD6.9 are located on the genome. Various researchers have found that nisin genes are present on a number of plasmids or also on the chromosome (Gireesh *et al.*, 1992; Rauch and De Vos, 1992; De Kwaadsteniet *et al.*, 2008; Hu *et al.*, 2009). In addition, nisin gene cluster has been showed to be present on a chromosomally located conjugative transposon (Rauch and De Vos, 1992).

The spectrum of target bacteria against which the nisin Z produced by PD6.9 strain is effective is also interesting. Among the bacteria tested, the pathogens *L. monocytogenes*, *S. pneumoniae* and *S. aureus* were sensitive. Studies with nisin variants against a series of clinically significant pathogens establish differences in specific activities against selected targets (Piper *et al.*, 2010). Piper *et al.* (Piper *et al.*, 2010) stated that Nisin Z had an inhibitory effect against methicillin-resistent *S. aureus* (MRSA) and (heterogeneous) vancomicin intermediate *S. aureus* [(h)VISA]. Here, in addition, most of the *S. aureus* strains more sensitive to nisin Z were isolated from bovine mastitis. It is significant due to the severity of the clinical symptoms and mortality associated to the infection (Espeche *et al.*, 2009). Mastitis is the main disease affecting dairy cattle herds in Brazil and worldwide (Pinto, 2008). More recently this area has received renewed attention, undoubtedly as a consequence of the ability of nisin to inhibit a wide range of mastitis-causing pathogens (Cao *et al.*, 2007). The development of non-antibiotic formulations has the potential to reduce the dependence on antibiotics for prophylactic therapies in the future.

L. lactis subsp. *lactis* PD6.9 displays a good potential for bacteriocin production. It is generally recognized as safe to the health. It is characterized in the present work. *L. lactis* PD6.9 is potentially active against pathogenic Gram positive bacteria of food safety. The use of bacteriocin and bacteriocin-producing strains as starters or protective cultures in preservation of foods contributes to the production of safe and wholesome foods. *L. lactis* PD6.9 is a new strain, originated from fermented meat products, presenting good production and desirable characteristics.

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Table 1. Inhibition spectrum of Nisin Z produced by *L. lactis* subsp. *lactis* PD6.9

Indicator	Strain	Source or reference	BU mL^{-1}
Bacillus subtilis	DSMZ347	LMGT	640
Enterococcus faecalis	v583	LMGT	160
Listeria innocua	BL86/26B	LMGT	1280
Listeria innocua		CERELA	1280
Listeria sw		CERELA	640
Listeria monocytogenes		CERELA	1280
Listeria monocytogenes	Scott A	CERELA	2560
Micrococcus luteus	4698	ATCC	640
Micrococcus luteus	10240	ATCC	5120
Streptococcus pneumoniae	TIGR4	LMGT	1280
Staphylococcus aureus	ME8245-3	LMGT	2560
Staphylococcus aureus	8452	LMGT	1280
Staphylococcus aureus	4759	[27]	2560
Staphylococcus aureus	4052	[27]	2560
Staphylococcus aureus	4784	[27]	1280
Staphylococcus aureus	3870	[27]	640
Staphylococcus aureus	4119	[27]	5120
Staphylococcus aureus	3212	[27]	640
Staphylococcus aureus	3702	[27]	1280
Staphylococcus aureus	4716	[27]	2560
Staphylococcus aureus	3975	[27]	10240
Staphylococcus aureus	3129	[27]	1280
Escherichia coli	14763	ATCC	NI
Pseudomonas aeruginosa		LMGT	NI

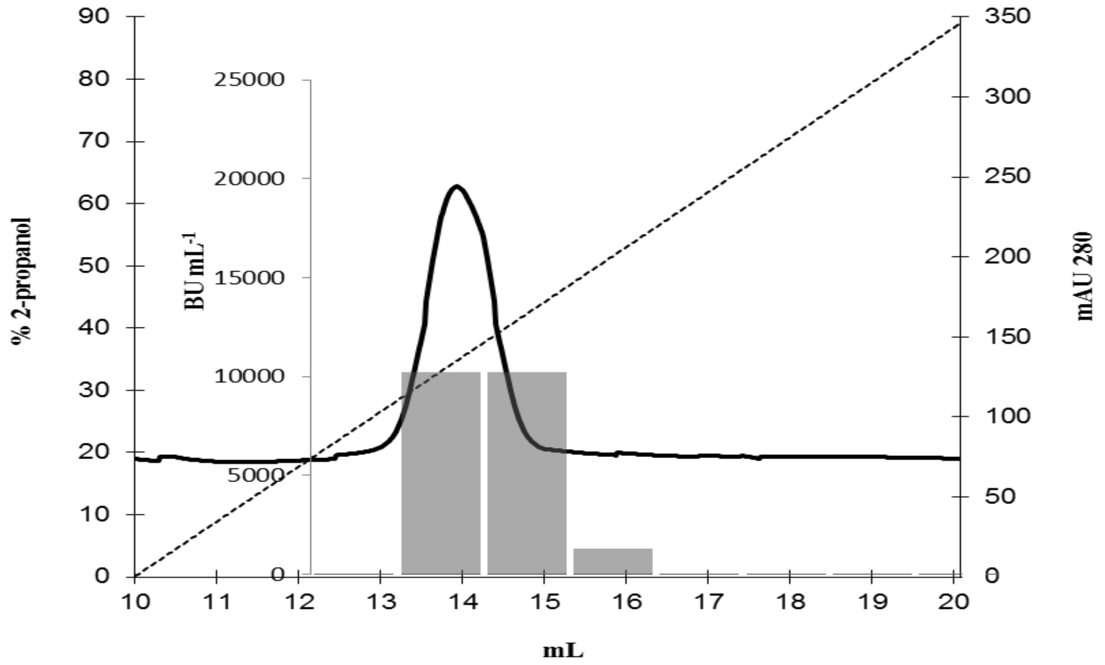
Abbreviations: ATCC, American Type Culture Collection; CERELA, Centro de Referencia para Lactobacilos; LMGT, Laboratory of Microbial Gene Technology (UMB); NI, no inhibition

Table 2. Purification of the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9

Purification step	Volume (mL)	Recovery (%)	Protein concentration (mg mL^{-1}) [a]	Antimicrobial activity (BU mL^{-1})	Specific Activity (BU mg^{-1})	Increase in specific activity (fold)
Cell free culture supernatant	200	100	24,87	1.28×10^3	51,46	1,0
Ammonium sulphate precipitate	20	20	4,44	2.56×10^3	576,58	11
Ion-exchange chromatography	10	20	0,32	5.12×10^3	1.6×10^4	311
Reversed- phase chromatography	2	8	0,08	1.02×10^4	1.28×10^5	2490

[a] The protein concentration was determined either by determining the optical density at 280 nm.

(A)



(B)

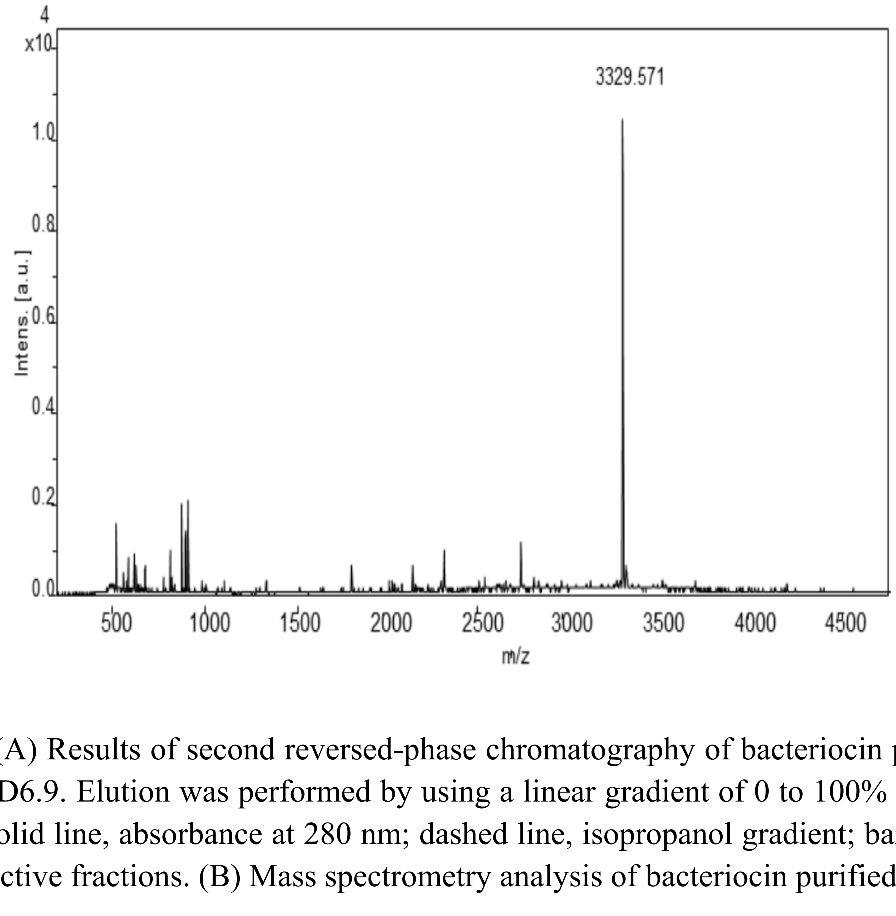
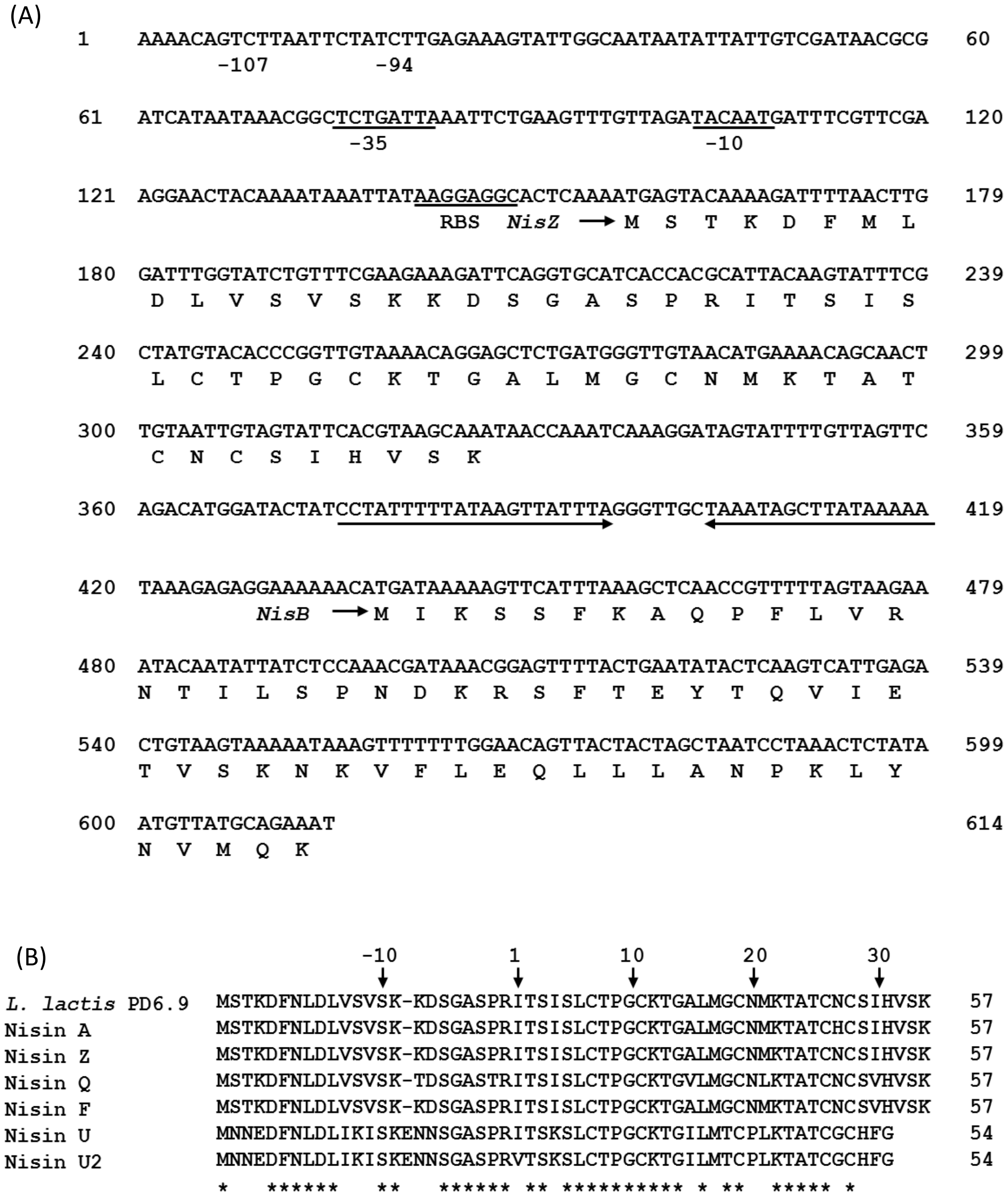


Fig. 1. (A) Results of second reversed-phase chromatography of bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9. Elution was performed by using a linear gradient of 0 to 100% 2-propanol containing 0,1% TFA. Solid line, absorbance at 280 nm; dashed line, isopropanol gradient; bars, bacteriocin units (BU) in eluted active fractions. (B) Mass spectrometry analysis of bacteriocin purified.



```
(A)
1     AAAACAGTCTTAATTCTATCTTGAGAAAGTATTGGCAATAATATTATTGTCGATAACGCG   60
             -107         -94

61    ATCATAATAAACGGCTCTGATTAAATTCTGAAGTTTGTTAGATACAATGATTTCGTTCGA   120
                         -35                              -10

121   AGGAACTACAAAATAAATTATAAGGAGGCACTCAAAATGAGTACAAAAGATTTTAACTTG   179
                                 RBS  NisZ  → M  S  T  K  D  F  M  L

180   GATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACGCATTACAAGTATTTCG   239
       D  L  V  S  V  S  K  K  D  S  G  A  S  P  R  I  T  S  I  S

240   CTATGTACACCCGGTTGTAAAACAGGAGCTCTGATGGGTTGTAACATGAAAACAGCAACT   299
       L  C  T  P  G  C  K  T  G  A  L  M  G  C  N  M  K  T  A  T

300   TGTAATTGTAGTATTCACGTAAGCAAATAACCAAATCAAAGGATAGTATTTTGTTAGTTC   359
       C  N  C  S  I  H  V  S  K

360   AGACATGGATACTATCCTATTTTTATAAGTTATTTAGGGTTGCTAAATAGCTTATAAAAA   419
                     ──────────────────────→      ←──────────────────

420   TAAAGAGAGGAAAAAAACATGATAAAAAGTTCATTTAAAGCTCAACCGTTTTTAGTAAGAA  479
                   NisB  → M  I  K  S  S  F  K  A  Q  P  F  L  V  R

480   ATACAATATTATCTCCAAACGATAAACGGAGTTTTACTGAATATACTCAAGTCATTGAGA   539
       N  T  I  L  S  P  N  D  K  R  S  F  T  E  Y  T  Q  V  I  E

540   CTGTAAGTAAAAATAAAGTTTTTTTGGAACAGTTACTACTAGCTAATCCTAAACTCTATA   599
       T  V  S  K  N  K  V  F  L  E  Q  L  L  L  A  N  P  K  L  Y

600   ATGTTATGCAGAAAT                                                614
       N  V  M  Q  K

(B)
                           -10       1        10        20        30
                             ↓       ↓         ↓         ↓         ↓
L. lactis PD6.9   MSTKDFNLDLVSVSK-KDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
Nisin A           MSTKDFNLDLVSVSK-KDSGASPRITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK   57
Nisin Z           MSTKDFNLDLVSVSK-KDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK   57
Nisin Q           MSTKDFNLDLVSVSK-TDSGASTRITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK   57
Nisin F           MSTKDFNLDLVSVSK-KDSGASPRITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK   57
Nisin U           MNNEDFNLDLIKISKENNSGASPRITSKSLCTPGCKTGILMTCPLKTATCGCHFG      54
Nisin U2          MNNEDFNLDLIKISKENNSGASPRVTSKSLCTPGCKTGILMTCPLKTATCGCHFG      54
                  *   ******   **   ****** ** ********** * **  ***** *
```

Fig. 2. (A) Nucleotide Sequence of the Region Encoding Nisin Z in *L. lactis* subsp. *lactis* PD6.9 and deduced Amino Acid Sequence. The putative -35 and -10 promoter regions and a putative ribosome binding site (RBS) are underlined. (B) Alignament of Nisin Z in *L. lactis* subsp. *lactis* PD6.9 and its homologs sequence of Nisin A, Z, Q, F, U and U2 obtained in the GenBank data. Identical amino acid residues are indicated with an asterisk.

CONCLUSION

In this thesis, bacteriocins produced by different wild strains of *L. lactis* isolated from a good quality meat processing plant in Brazil were identified as the lantibiotic nisin Z, a natural variant of nisin A. This bacteriocin showed inhibitory effect against foodborne pathogens, including *S. aureus* strains isolated from bovine mastitis.

The structural gene of nisin Z was identified in all strains of *L. lactis*, which produced nisin at variable concentrations. Conditions that favored all strains of *L. lactis* growth were not always related to a higher bacteriocin production. The production of active bacteriocin was affected by environmental factors including carbon and nitrogen sources and fermentation conditions (pH and temperature). Several results indicated that nisin production can be regulated by the carbon source.

In addition, *L. lactis* ID1.5 strain produces two inhibitor compounds, which are different from other antimicrobial compounds produced by *L. lactis*. The antimicrobial compounds of low molecular weight appear to be cell associated. These compounds showed antimicrobial activity against *P. aeruginosa* and *S. pneumoniae* TIGR 4, two clinically important respiratory pathogens.

To our knowledge, this the first report of lantibiotic nisin Z production by *L. lactis* isolated from fermented sausage in Brazil, as well as the first study showing two antimicrobial compounds extracted from cell surface of *L. lactis*.

The origin of the strains and the diversity in inhibition profiles against several relevant pathogens indicate that these strains may be suitable for commercial starter cultures for fermented meat products. The low molecular compounds are being investigated, may prove to be novel inhibitory substances.