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IDENTIFICATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA AND *IN VITRO* EVALUATION OF THEIR POTENTIAL ROLE IN MASTITIS CONTROL

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Mamma e papà

L'inferno dei viventi non è qualcosa che sarà; se ce n'è uno, è quello che è già qui, l'inferno che abitiamo tutti i giorni, che formiamo stando insieme. Due modi ci sono per non soffrirne. Il primo riesce facile a molti: accettare l'inferno e diventarne parte fino al punto di non vederlo più. Il secondo è rischioso ed esige attenzione e apprendimento continui: cercare e saper riconoscere chi e cosa, in mezzo all'inferno, non è inferno, e farlo durare, e dargli spazio.

Italo Calvino, LE CITTA' INVISIBILI

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ABSTRACT

Bovine mastitis is one of the most significant causes of economic losses for the dairy industry. On the other hand, public health authorities advise prudent use of antibiotics because they could promote bacterial resistance and leave residues in food chain. The dairy industry could benefit from the development of safe antimicrobial agents and bacteriocins could be attractive alternatives to antibiotics. Due to the safety of Lactic acid bacteria (LAB), their bacteriocins have the potential to be used as antimicrobials in veterinary clinical application. We analyzed the efficacy of antibacterial substances produced by bacteriocinogenic *Lactococcus lactis* subsp. *lactis* strains against contagious and environmental mastitis pathogens. Thereafter, we investigated how lactococcal strains or their bacteriocins could influence mammary gland innate immune response *in vitro*. Out of 65 LAB strains tested, 3 were active against mastitis pathogens: 2 strains produced Nisin, one Lacticin 481 and in addition a novel molecule with likely antibacterial activity. To analyze the immune response of mammary epithelial cells when stimulated with lactococcal strains or bacteriocins, a stabilized epithelial cell line, BME-UV1, was used. Both lactococcal live cultures and their antibacterial products were shown to modulate the non-specific immune response of BME-UV1 cells: Lysozyme and N-acetil-β-D-glucosaminidase excretion were overall enhanced by bacteriocins and live-culture treatments, while intracellular amounts were unaffected by treatments. Proinflammatory cytokine expression of treated BME-UV1 was similar to that observed in control cells, except for *Lactococcus lactis* subsp. *lactis* SL153. Such strain induced a significant reduction of TNFα transcriptional level.

The stimulation of enzyme secretion due to the administration of lactococci or of their antibacterial products, with potential enhancement of pathogens cleaning, can be of interest for the prevention of intra mammary infections. In addition, *Lactococcus lactis* subsp. *lactis* SL153 strain could be advantageous for its potential anti-inflammatory properties and could be of interest for the development of intra-mammary probiotic treatments.

Key words: mastitis, bacteriocins, *Lactococcus lactis* subsp. *lactis*, antibacterial treatments, non-specific immunity.

STATE OF THE ART

1. LACTIC ACID BACTERIA (LAB): NOVEL APPLICATIONS IN MASTITIS FIELD

1.1. LAB bacteriocins

The finding of bacteriocin production in several bacterial species has increased researchers' interest to study these new antibacterial molecules. Due to their efficacy they are investigated as alternatives to traditional antibiotic treatments both in human and veterinary clinical application.

Bacteriocins are termostable, ribosomally synthesized peptides secreted by many varieties of Gram-positive and Gram-negative bacteria, to kill other bacteria. They can be active either in the same species (narrow spectrum) or across the genera (broad spectrum). Producer organisms are resistant to their own bacteriocin activity, producing specific proteins which provide to sequestration or competition for bacterial receptors or to pump bacteriocins from the producer membrane (Cotter et al., 2005). It has been proposed that 99% of all bacteria may produce at least one bacteriocin; the only reason we haven't isolated more is that few researchers have looked for them (Riley and Wertz, 2002). In addition to ribosomally synthesized antimicrobial peptides, bacteria also produce antibiotic molecules through the activity of some enzymes called nonribosomal-peptide synthetases wich provide to modify peptides through ring formation, glycosylation or acylation. Among this type of antimicrobial peptides polymyxin B and gramicidin S have found an application in topical treatment of infections, while vancomycin and daptomycin have become important for their efficacy against multiple resistant strains of Gram-positive bacteria (Hancock et Sahl, 2006).

Bacteriocin production provides bacteria a competitive advantage in the environment, eliminating competitors, thus gaining resources (Lohans and Vederas, 2012).

Among producer strains, lactic acid bacteria (LAB) are the mostly investigated because they are approved as Generally Recognized As Safe (GRAS) organisms by Food and Drug Administration and they are permitted as additives in food. LAB are often found in foods and used to manufacture fermented foods, they are known not only to contribute improvement of taste, but also to prevent contamination by spoilage bacteria. Especially

bacteriocins-producing LAB, which have antibacterial activity, are considered suitable in food preservation. On the basis of the safety of LAB, their bacteriocins have the potential to be used as antimicrobial in all fields in which safety and absence of residues are required. Although pollution of antibiotics in the environment is an emerging problem, bacteriocins have proteinaceous nature and they are degraded easily in our body and in the environment (Zendo *et* al., 2010).

1.1.1. Production and classification

Bacteriocins are produced as inactive pre-peptides with an N-terminal sequence guide (Macwana and Muriana, 2012). These precursors are transported through specific carriers at the cell surface during the exponential growth fase and enzymatically converted into their active form. Bacteriocin production is managed by a three-component regulation system: an inducing peptide (or pheromone-activating factor), the transmembrane histidine kinase (pheromone receptor) and a response regulator protein. The inducing peptide is the pre-peptide synthesized in the ribosome in small amount. When the inducing peptide concentration reaches a threshold level, it activates the trasmembrane histidine kinase, which leads to autophosphorylation of the histidine residue, thus transferring phosphate to the response regulator protein. The phosphorylated regulator activates the transcription of the bacteriocin in addition to the elements that form the regulatory system, initiating a positive feedback. In some cases, such as in Nisin, the bacteriocins itself exert the induction function, leading an exponential increasing in bacteriocin synthesis (Balciunas et al., 2013)

The classification scheme of LAB bacteriocins has been subjected to ongoing revision, Klaenhammer (1993) proposed four classification groups, but recently a new scheme of classification was proposed. Class I bacteriocins are the lantibiotics, which are highly post-translationally modified peptides thermostable and with very low molecular weight (<5 kDa). Class II consists of small thermostable peptides (<10 kDa) which are free of modified residues. A third class (class III bacteriocins) includes non-bacteriocin lytic proteins, termed bacteriolysins which are large (>30 kDa) and heat-labile proteins with a distinct mechanism of action (Cotter et al., 2005).

Class I bacteriocins

The lantibiotics (lanthionine-containing antibiotics) are small peptides (19–38 amino acids in length) containing lanthionine or β-methyllanthionine residues. These unusual residues form covalent bridges between amino acids, resulting in internal 'rings' and giving lantibiotics their characteristic structural features. The lantibiotics carry out their antibacterial activity with two different modes of action: either forming pores in the membrane of sensitive cells and/or binding lipide II and preventing the formation of peptidoglycan (Jung G, 1991). Generally they are active against Gram-positive bacteria, but their activity can be extended to Gram-negatives if the integrity of outer membrane have been affected (Cotter et al., 2005).

Among the LAB bacteriocins identified to date, Nisin has been the most investigated. It is approved for use as a food preservative since 1969 (Balciunas *et* al., 2013) and remains the only bacteriocin approved for use by the European Union and Food and Drug Administration. Several variants of Nisin, which is a 34 amino acids, are produced by *Lactococcus lactis* subsp. *lactis*. Nisin has a broad-spectrum of activity against strains of Gram+ bacteria and exert its activity forming pores in cell membranes even if a secondary antimicrobial mechanism has been observed consisting in the inhibition of peptidoglycan biosynthesis after Nisin-lipid II binding (Wiedemann et al., 2001). Nisin and Lacticin 3147 need a docking molecule as initial receptor: the N-terminal binds to a peptidoglycan precursor, lipid II, while the C-terminal penetrates cytoplasmic membrane resulting in pore formation with the consequent leak of ions and ATP from the cells. Although Nisin is primarily active against gram-positive bacteria, some strains can become nisin resistant (Mantovani et Russell, 2001). It is thought that there are at least four processes involved in nisin resistance: preventing the bacteriocin from reaching the cytoplasmatic membrane, reducing the acidity of the extracellular medium thereby stimulating binding of bacteriocin to the cell wall, preventing the insertion of the bacteriocin into the cell membrane and transporting the peptide out of the membrane (Kramer et al., 2006). It should be noted that bacteria differ greatly in their sensitivity to bacteriocins activity, due to the differences in cell envelope composition. Alterations within the envelope are repeatedly seen in bacteria with altered resistance to Nisin. Kaur et al. (2011) observed after repeated exposure to Nisin, changes in cell membrane or cell wall and a nisin-degrading protease. The cell-wall thickening appears beneficial to nisin-resistance: this

thickening seems to protect membrane and lipid II against the Nisin effect (Kramer et al., 2006). The development of resistance can interestingly bring to negative consequence; for example a *L. monocytogenes* nisin-resistant variant results in an acid -sensitive phenotype (McEntire et al., 2004). Naghmouchi et al. (2007) noted that the development of bacteriocin resistance can be associated with a cross-resistance among different class of bacteriocins. Nevertheless, to several bacteriocins, such as Lacticin 3147, bacteria do not readily develop resistance and, when it occurs, it happens at low frequency (Guinane et al., 2006).

Table 1. Classification of LAB bacteriocins produced by strains belonging to *Lactococcus* spp. (Zendo et al., 2010)

Class	Remark	Bacteriocin	Molecular weight (amino acids)
I	Lanthionine-containing bacteriocins, lantibiotics. Includes both single- and two-peptide lantibiotics	Nisin A	3,354 (34)
		Nisin Z	3,331 (34)
		Nisin Q	3,327 (34)
		Nisin F	3,317 (34)
		Lacticin 481	2,901 (27)
		Lacticin 3147	A1, 3,306 (30) A2, 2,847 (29)
II	Non-lanthionine-containing bacteriocins. Heterogeneous class of small peptides		
IIa	Listeria-active bacteriocins with a consensus sequence in the N-terminal of YGNGVXC	Lactococcin MMFII	4,143 (37)
IIb	Two-peptide bacteriocins	Lactococcin G	α, 4,346 (39) β, 4,110 (35)
		Lactococcin MN	LcnM, 4,325 (48) LcnN, 4,377 (47)

		Lactococcin Q	α, 4,260 (39) β, 4,018 (35)
IIc	Cyclic bacteriocins	Lactocyclicin Q	6,063 (61)
IId	Other class II bacteriocins	Lactococcin A	5,778 (54)
		Lactococcin B	5,327 (47)
		Lactococcin 972	7,381 (66)
		Lacticin Q	5,926 (53)
		Lacticin Z	5,971 (53)

Class II bacteriocins

The class II are small, heat stable, non-lanthionine-containing peptides, not subjected to extensive posttranslational modification. Many class II bacteriocins are active in the nanomolar range and pore formation is the most common mode of action. Due to their amphiphilic helical structure, these bacteriocins can exert an antibacterial activity through the insertion in the cytoplasmic membrane of the target cell, thereby promoting membrane depolarization and cell death. Cotter et al. (2005) suggested to divide class II bacteriocins into three subclasses: subclass IIa (pediocin-like bacteriocins), subclass IIb (two-peptide bacteriocins), subclass IIc (circular bacteriocins) and subclass IId, non-pediocin single peptide linear bacteriocins. Even though up to 8% of wild type *L. monocytogenes* strains appear to be naturally resistant (Collins et al., 2010), the subclass IIa show high specificity against *L. monocytogenes*: Pediocin PA-1 is the only bacteriocin produced not only by different species, but also by different genera of LAB (*Pediococcus* spp. and *Lactobacillus* spp.). Enterocin (a bacteriocin from *Enterococcus* spp.) is another bacteriocin that firstly belonged to class IIa, but several bacteriocins from *E. faecalis* subsequently found and characterized, were classified in more than one class of bacteriocin (Balla et al., 2000). Into the class IIb are gathered those bacteriocins whose the foundamental characteristic is to be formed by two peptides, such as Lactococcin G and Enterocin 1071 (Balla et al., 2000). Since the activity of both units is crucial to exert their antimicrobial effect, the encoding genes are located on the same operon and expressed simultaneously. Indeed it is common for one or both peptides to lack activity when they are assessed separately, while their combination demonstrate an important synergic action and a potent activity (Balciunas et al., 2013). The remaining groups of class II bacteriocins are subclass IIc, which is characterized by a covalent binding among the N and C-terminii resulting in a circular structure, such as Lactocyclicin Q (Sawa et al.,

2009) and Lactococcin B (Venema et al., 1993). In the subclass IId are grouped lactococcal bacteriocin, such as Lacticin Q, which forms particular, very large pores into the membranes, inducing high membrane permeabilization without the need of docking molecules (Zendo et al., 2010). Resistance to class II bacteriocins has been frequently investigated in relation to the mannose-phosphotransferase systems (man-PTS) that is the target receptor for subclass IIa. The lack of a component of man-TPS was sufficient to determine the resistance for both Leucocin A and Pediocin PA-1, two pediocin-like bacteriocins (Collins et al., 2010).

1.1.2. Application fields

The research on the antibacterial activity of bacteriocins has been addressing two practical applications: a) food production and preservation and b) use in medical and veterinary medicine. In food production, only Nisin and Pediocin PA-1 have found widespread use (Cotter et al., 2005). They have shown a potential in preservation of meat, dairy products, eggs products, beverage, bakery products and fermented vegetables. They can be produced *in situ* from producers cells added instead of traditional starter culture, or added in purified or semi-purified form. The presence of bacteriocins in milk seems to have no negative impact on human health, because they are degraded by the proteolytic enzymes of the stomach, so they don't have any detrimental effect on the intestinal bacterial flora (EFSA, 2005). Moreover, these molecules do not affect dairy transformations, rather they could promote technological improvement, for example by controlling adventitious non-starter flora (Cotter et al., 2005).

Even though investigations about LAB bacteriocins have been mainly driven by food industry, the non-toxicity conjugated with the activity of these molecules against Gram-positive human and animal pathogens has led to investigate potential clinical applications. With the widespread development of antibiotic resistant strains, the importance of alternative antimicrobials is becoming increasingly urgent (Dicks et al., 2011) and bacteriocin-producing organisms could be considered as important source of antimicrobial agents in the medical and veterinary fields (Pieterse and Todorov, 2010).

1.1.3. Bacteriocins as alternatives in mastitis prevention and therapy

Bovine mastitis is one of the most significant causes of economic losses for the dairy industry, due to reduction of milk quality and production, discarded milk, early culling of

cows, drug costs and enhance of labor for farmers (Coelho et al., 2007). The widespread of post-dipping adoption and the antibiotic treatment at drying-off has led to a significant progress in mastitis control (Gruet et al., 2001). Antibiotics still represent the election treatment for mastitis. Nevertheless, the biggest challenge in the modern dairy industry is the reduction of the antibiotics use in food production animals (Bradley, 2002): public health authorities advise prudent use of antibiotics because they could promote bacterial resistance and leave residues in food chain (Coelho et al., 2007). Since starter cultures for cheese and yoghurt manufacture are inhibited by these substances (Miles et al., 1992), the dairy industry could benefit from the development of safe antimicrobial agents and bacteriocins could be an attractive alternatives to antibiotics (Pieterse and Todorov, 2010). Furthermore, the dramatic increase in organic production still requires an effective alternative against mastitis: the dry period remains difficult to manage in the absence of antibiotic dry cow therapy, with significantly more new affected quarters (Berry and Hillerton, 2000). In addition to improved environmental management and optimal nutrition, Bradley (2002) recognizes the pivotal role of the development of new therapeutic agents coming from 'mammary probiotics' as an area of possible future research that may yield novel approaches to the control of bovine mastitis.

The lantibiotic Nisin was recently assayed in experimental trials and tested as treatment of clinical and subclinical mastitis. The papers highlighted the possibility to achieve a cure rate similar to antibiotic treatments avoiding milk withdrawal. The use of antibiotics is often inadvisable in subclinical mastitis because of the low cure-rate and the withdrawal period for milk (Gruet et al., 2001).

Two nisin-based products namely Mast Out® (intrammary treatment) and Wipe-Out® (dairy wipes) were developed by ImmuCell Corporation (Maine, USA) (Cotter et al., 2005). Although Mast Out® showed a significant cure rate in an initial field trial and was licensed by Pfizer Animal Healt, the product was not made available on the market and no trial was further reported. In two recent studies, the efficacy of Nisin in the treatment of clinical and subclinical mastitis was compared with that obtained with antibiotic administration. Clinical mastitis caused by *Staphylococcus aureus* or *Streptococcus agalactiae* (Cao et al., 2007) and subclinical mastitis by *Staphylococcus aureus*, Coagulase-Negative Staphylococci, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* or *Streptococcus uberis* were considered (Wu et al, 2007). Both studies concluded that

administration of Nisin was effective in the treatment of mastitis caused by several mastitis pathogens in lactating dairy cows.

Nisin was also tested for its efficacy in a post-dipping formulation: Ambicin® N showed a significant reduction of several pathogens in experimentally challenged teat surfaces after 1 minute exposure to the germicidal formulation. Furthermore the formulation containing Nisin showed a lower potential for skin irritation after repeated exposure in contrast to 1% iodophore or 5% chlorexidine digluconate (Sears et al., 1992). Using bacteriocin-based products would be advantageous because the complete removal at milking time is not required, contrarily to chemical products.

Also Lacticin 3147 produced by *Lactococcus lactis* subsp *lactis* was investigated for use as antimicrobial agent in a teat sealant formulation. The results demonstrated Lacticin efficacy against mastitis pathogens and stability in teat environment (Ryan et al., 1998). Moreover, Pieterse et al. (2010) showed the *in vitro* susceptibility of mastitis pathogens to the new bacteriocin macedocin ST91KM added to a sealing formulation. Traditional teat seal formulations are recommended during dry period as a prophylactic measure to reduce the number of new infections (Berry and Hillerton, 2002). The inert property of the seals formulation has no antimicrobial effect and treatment efficacy relies on good udder hygiene practices, therefore the addiction of bacteriocins could improve the efficacy of teat sealants.

Antibiotics administration is recommended at drying-off to improve mastitis control programs. However prolonged exposure to low levels of antibiotics could stimulate antibiotic resistance development, so bacteriocins could replace antibiotics in these formulations (Twomey et al., 2000). The protection given by a teat sealant plus Lacticin 3147 was tested against experimental challenge with *Streptococcus agalactiae* and the results indicated higher percentage of quarters free from infections when treated with the bacteriocin-added teat sealant (Ryan et al., 1998). Similar results were observed by Twomey et al. (2000) against *Staphylococcus aureus*, with the conclusion that Lacticin concentration was determinant for the teat sealant to be effective against the pathogen.

In contrast to antibiotics, bacteriocins are considered as safe because they have been always present in various foods since ancient times. Furthermore their proteic nature ensures the inactivation by enzymes (such as trypsin and pepsin) during the passage through the digestive tract, avoiding the destruction of the gastrointestinal tract

microbiota. This means that bacteriocins do not remain in the environment, thus they do not promote the development of resistance among bacterial strains into the environment. In addition, while antibiotics generally need micromolar level for their activity, bacteriocins exert their antibacterial effect at nanomolar level. They act specifically on target cell membrane through quick pores formation at extremely low concentration. This rapid action is one of the reason for which the development of resistant strains was not evidenced as problematic in the practical application of these molecules (Zendo *et* al., 2010, Pieterse and Todorov, 2010).). Although resistance could become an issue in the future, especially if bacteriocins will be applied in clinical field, it should be considered that Nisin has a 50 years of safe usage in food industry and in more than 50 countries without the emergence of acquired resistance (Zendo et al., 2010). On the other hand, in the last years the interest on the study of the basis for bacteriocin resistance has increased. Also, even if no development of resistance has been reported in the practical usage, further insights into resistance development allow us to build up strategies to minimize the possibility of such happening. The lowest minimum inhibitory concentration (MIC) of the bacteriocin should be established, to reduce the amount of bacteriocin in treatment products. Due to the risk of resistance development following antibiotic treatments, milk with antibiotic residues cannot be sold. To the contrary, bacteriocin residues in milk are accepted and no withholding period would be required if bacteriocin therapy were used instead of antibiotics, avoiding economic losses due to wastage (Pieterse and Todorov, 2010).

Furthermore the presence of antibiotic residues in milk can affect cheese and yoghurt manufacture because LAB used as bacterial starter cultures are inhibited, thus the quality of products compromising (Pieterse, 2009). For these reasons, the implementation of new antibacterial molecules, which are safe for human consumption and do not affect milk transformations, can be recommended in mastitis prevention and therapy (Pieterse and Todorov, 2010).

1.2. Lactic acid bacteria and mammary immune response against pathogens

As previously described, an effective, non-antibiotic treatments for mastitis could reduce the costs of the depletion of milk during therapy and relieve some of the pressure of agricultural and veterinary sectors to limit the use of antibiotics. Several alternatives to

antibiotics have been explored and proposed, including milking quarters many times at day, hydrotherapy, ultrasonic therapy, topically applied liniment, intramammary infusions of glucose solution, lysotaphin and Nisin (Klostermann et al., 2008). The use of probiotic bacteria has been proposed as a novel approach to prevent and cure several infections in humans and animals, especially in vaginal and in the gastrointestinal tracts (Frola et al., 2012). In particular, LAB with a GRAS status and an inhibitory activity against pathogens are still under investigation. Probiotic LAB may exert their beneficial actions through different mechanisms such as adhesion to epithelial cells, colonization, biofilm formation, production of antagonistic metabolites, competition for nutrients and immune system modulation (Beecher et al., 2009). In recent studies, the efficacy of treatments using different genera of LAB (*Lactobacillus* spp and *Lactococcus* spp.) and the response of mammary gland were evaluated. A first study (Klostermann et al., 2008) was performed using the best characterized strain of the lactococcal species, *Lactococcus lactis* DPC 3147 producer of Lacticin 3147. A live culture of the microorganism was tested for its efficacy in the treatment of clinical and subclinical mastitis, demonstrating similar results when compared to antibiotic therapy, with the advantage of no cost for milk withdrawal. Furthermore, in the first two days after infusion an increase in SCC was noted, due to the influx of PMN into the mammary gland, followed by a rapid decrease and the achievement of normal levels to a pre-treatment level. These two observations indicate the enhancement of local defenses induced by the infusion of *Lactococcus lactis* and the safety of the treatment, since this microorganism is unable to colonized the treated quarters. Such alternative treatments, would also be of interest in organic dairy farms where the use of antibiotics is restricted or even prohibited. The application of the same *Lactococcus* strain was subsequently used to study the stimulation of mammary immune response through the evaluation of cytokine gene expression. Beecher at al. (2009) concluded that infusion with live culture of *Lactococcus lactis* caused a rapid and considerable response with the highest expression levels observed for IL-1β and IL-8 corresponding to peaks in SCC values. A different LAB, belonging to the genus *Lactobacillus*, was under investigation for its characteristics of mastitis pathogen inhibition and co-aggregation, and for its ability to adhere to bovine teat canal epithelial cells: the inoculation with *Lactobacillus perolens* CRL 1724 in non-lactating bovine udders was performed providing important information for further studies in the development of

probiotic products for bovine mastitis prevention (Frola et al., 2012). This histological study highlighted the adherence of LAB on the epithelial cells of cistern accompanied with a mild inflammation and the absence of morphological modifications, lesions or necrosis indicating that no damage occurred after the inoculation (Frola et al., 2013). This novel control strategy was proposed against the major pathogen *Staphylococcus aureus* by Bouchard et al. (2013), demonstrating the protective effect of *Lactobacillus* strains in the preventing adhesion and internalization of the pathogen into bovine mammary epithelial cells.

2. MAMMARY INNATE IMMUNE DEFENSES

The mammary gland is open to the environment and therefore is subjected to the invasion of pathogen microorganisms. For this reason, an effective immune defense play a pivotal role in preventing infection establishment (Ganz, 2004). The overall impact of mastitis disease on the quality and quantity of milk produced for human consumption has provided the impetus to study the mechanisms of mammary immune response and how to prevent disease through immune modulation. Innate immunity is a non-specific function, quickly activated at the site of infection by numerous stimuli, but not increased by repeated exposure to the same agent (Sordillo and Streicher, 2002). This immune reaction is mediated by physical barrier of the teat end, macrophages, neutrophils, natural killer cells and certain soluble factors. If non-specific defense mechanisms work adequately, most of the pathogens are eliminated within a short period of time and before the activation of the specific immune response. To the contrary, the acquired immune system is activated when pathogens evaded the innate defense or is not completely eliminated: the recognition of specific pathogen antigens results in an immunological memory which provides a faster and a stronger response in clearing the pathogen. The innate immune response is mediated by macrophages, lymphoid populations and immunoglobulins. In the mammary gland both innate and acquired immunity are required in order to provide an optimal defense from mastitis, but here we consider only innate immunity, since it is the predominant protection of the mammary gland during the first stage of infection (Bannerman, 2009).

2.1. Anatomical defenses

The teat canal is the first line of defence against mastitis, since this is the route by which invading pathogens can gain entrance to the mammary gland. In the distal part of the teat sphincter muscle maintains tight closure between milkings and hinders bacterial penetration. Increased opening of the sphincter is directly correlated to increased incidence of mastitis (Rainard et al*.,* 2005).

This canal is locked between milkings and in the dry period by a keratin plug derived from the stratified epithelial lining. The role of the plug is creating a physical barrier preventing the penetration of bacteria in the gland cistern. Indeed, keratin is able to bind and immobilized most strains of non-encapsulated mastitis-causing bacteria (Rainard *et al*. 2005). Some components of keratin have antimicrobial activity: esterified and not esterified fatty acids, such as myristic acid, palmitoleic acid and linoleic acid, are bacteriostatic. Furthermore, keratin cationic proteins can bind electrostatically to the mastitis-causing bacteria, altering their cell wall and affecting their resistance to osmotic pressure (Hogan et al., 1987). The bactericidal efficiency of keratin may be limited by different factors, above all inadequate milking procedures. Close to the parturition, a high accumulation of fluid occurs within the udder, , causing increased intramammary pressure. Thus, the leakage of mammary secretions and dilatation of the teat canal increase susceptibility to mammary infections (Sordillo and Streicher, 2002).

2.2. Soluble defenses

Cytokines

This group of small soluble proteins (less than 50kDa) has a crucial role in cell signalling, in the mammary gland they provide the balance between humoral and cell-based immune responses. Although cytokines play an essential role in the host response to infection, they can have deleterious effects. Thus, there is a fine balance between the positive and negative effects of cytokines on the host that is dictated by the duration, amount, and location of their expression (Bannerman, 2009). Some cytokines clearly promote inflammation, while others suppress the production of inflammatory mediators. They

comprise interleukins (IL), chemokines, interferons (IFN), tumor necrosis factors (TNF) and colony stimulating factors (CSF) (table 1).

Pro-inflammatory cytokines

Tumor necrosis factor-α

Tumor necrosis factor-α (TNFα) is produced very early in inflammation stimulating the acute phase reaction. TNFα is an essential mediator of inflammation because in combination with IL-1 it triggers changes in the vascular endothelial cells that line small blood vessels. A local increase in TNFα causes the classic signs of inflammation, including heat, swelling, pain, and redness The massive inflammatory response observed during coliform mastitis is accompanied by elevation of TNFα levels causing such severe symptoms. In coliform mastitis, TNFα induces plasma haptoglobin, promotes recruitment and activation of neutrophils and raises intra-mammary and systemic nitrite and nitrate (Tizard, 2013). In contrast to the continuous and sustained release of TNFα in coliform mastitis, in *S. aureus* infected glands, TNFα transcriptional activity has only a short episodic elevation at 24 h post infection (Alluwaimi, 2004), sustaining the immunosuppressive nature of *S. aureus* infections (Allowaimi et al., 2003). To the contrary, in vitro studies using bMEC, showed a significant increment of TNFα one hour after *S. aureus* experimental challenge, demonstrating the increase of cytokine production in the early phase of infection (Griesbeck-Zilch et al., 2008). Despite this early increment, *S. aureus* challenge results overall in lower production of TNFα if compared with *E. coli.* These results can explain the undetectable level of TNFα in mastitic milk during the *in-vivo S. aureus* challenge (Lahouassa et al., 2007). Despite its pro-inflammatory activity, TNFα has been detected not only in infected bovine mammary gland but also in normal quarters during periparturient period, lactation and involution, demonstrating its essential role in regulating and maintaining immunological function during physiological changes of mammary gland (Alluwaimi, 2004).

Interleukin-1

Interleukin-1 (IL-1) includes the secreted IL-1β and the cytoplasmatic IL-1α; 10- to 50-fold more IL-1β is produced than IL-1α. They are produced by macrophages and epithelial cells and are mediator of local and systemic immune response: it can regulate cells proliferation and apoptosis, and the expression of other cytokines, acute phase proteins and enzymes for the eicosanoid synthesis (Schukken et al. 2011). During severe infections, IL-1β circulates in the bloodstream where, in association with TNF-α, it is responsible for sickness behaviour (Tizard, 2013) and signs of acute septic shock (Ohtsuka et al., 2001). Due to the influx of neutrophils in association with the increase in IL-1 level observed in *E. coli* mastitis, it has been postulated that IL-1 is indirectly involved in chemo-attraction of neutrophils (Schuster et al. 1997). To the contrary, in *S. aureus* mastitis the role of IL-1 is negligible or transient, indicating the minor role in S. aureus mastitis (Riollet et al., 2001). Despite its pro-inflammatory nature, the immunotherapeutic properties of IL-1 have been investigated: IL-1 has been proposed as adjuvant in the *S. aureus* mastitis, demonstrating its efficacy in neutrophils influx enhancement, in the oxygen radical upregulation and in the increase of intracellular potency of some antibiotics (Alluwaimi, 2004).

Interleukin-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine with additional anti-inflammatory properties. It promotes some aspects of inflammation, especially in response to tissue damage and severe infections, since it is a major mediator of acute-phase reaction and of septic shock (Tizard, 2013). Macrophages, T lymphocytes and epithelial cells produce IL-6 (Okada et al., 1997). In the mammary gland IL-6 regulates the acute phase protein synthesis and promotes a shift from neutrophils to monocytes influx, essential to prevent the detrimental effect of neutrophils allowing a suitable immune response (Allowaimi, 2004). It is expressed both in infected mammary glands and in healthy ones. Hagiwara et al. (2001), in an *in vivo* study of naturally infected cows, observed the highest concentration of IL-6 during *E. coli* mastitis, when compared with other pathogens. In contrast to its active presence in coliform mastitis IL-6 activity was confirmed to be negligible in *S. aureus* infection (Alluwaimi et al., 2003).

Chemokines

Chemokines are a family of at least 50 small (8 to 10-kDa) chemotactic cytokines. They coordinate the migration of cells and hence dictate the course of many inflammatory and immune responses. Chemokines act predominantly on macrophages and dendritic cells (Tizard, 2013).

Interleukin-2

Interleukin-2 (IL-2) is produced mainly by T-lymphocyte, it is involved in the growth and activation of B-lymphocytes, activation of NK cells and induction of cytotoxic T-cells activation. Bovine IL-2 has been detected in epithelial cells of both normal and mastitic mammary gland (Alluwaimi, 2004). It has been demonstrated that decreased IL-2 endogenous production contributes to reduce immune capabilities, with the consequent development of disease. In some studies, a correlation between low amount of IL-2 in colostrums and increased susceptibility to mastitis was observed and the possibility to enhance mammary gland defences with IL-2 has received considerable attention (Sordillo and Streicher, 2002). IL-2 was investigated for its therapeutic use in the treatment of *S. aureus* mastitis. The infusion of IL-2 in infected quarters caused a significant immunopotentiation by overwhelming recruitment of lymphocytes, neutrophils, macrophages and eosinophils (Alluwaimi, 2003). Nevertheless, apparent enhanced immunomodulation in the IL-2 infused normal and/or mastitic mammary glands did not result in prevention or cure of the infection (Alluwaimi, 2004).

Interleukin-8

Interleukin-8 (IL-8) is produced by macrophages, monocytes, mast cells, T-lymphocytes, as well as epithelial and endothelial cells. It has the function to attract and activate neutrophils, stimulating their respiratory burst and the release of their granule contents (Tizard, 2013). In the mammary gland IL-8 plays a crucial role in neutrophil recruitment (Sordillo and Streicher, 2002). Barber and Yang (1998), demonstrated the biological role of IL-8 in attracting neutrophils into infected bovine udder, showing lower neutrophil chemotactic activity in the presence of anti-IL-8 antibodies in mastitic mammary secretion. The main source of IL-8 in udder has been further investigated by measuring its level in milk and in

lymph from afferent and efferent lymphatic vessels of the supra-mammary lymph node. The level of IL-8 was higher in milk than in lymph, indicating that mammary epithelium rather than sub-epithelial tissue is a major source of the cytokyne (Waller et al., 2003). Mammary epithelial cell lines stimulated with LPS, IL-1β, *S. aureus* and/or *E. coli* showed a copious production of IL-8 (Alluwaimi, 2004). IL-8 is actively produced in *E. coli* mastitis, whereas it is present in lower concentration in mastitis caused by *S. aureus* both in *in vitro* (Griesbeck-Zilch et al., 2008) and in *in vivo* studies (Bannerman et al., 2004). The increase of IL-8 concentration is associated with higher somatic cell count, highlighting the crucial role of IL-8 in the recruitment of leukocytes into the mammary gland, which is essential for the elimination of invading pathogens. Furthermore, in contrast to the more transient effects of other chemoattractants, IL-8 is able to exert a longer lasting effect, presumably because of its resistance to proteolytic degradation and slower clearance from tissues (Bannerman, 2009).

Anti-inflammatory cytokines

Interleukin 10

Interleukin-10 (IL-10) is one of the best characterized anti-inflammatory interleukins in udder immune response. IL-10 has two main functions, the inhibition of cytokine synthesis and the reduction of factors of the MHC-II complex (Schukken et al. 2011). A significant increase in the amount of IL-10 in milk after infection with *E. coli* was observed, while *S. uberis* infection showed a delayed response (Bannerman, 2009). Natural *S. aureus* infected quarters showed a limited IL-10 response (Bannerman et al., 2004), but IL-10 had been previously detected in milk somatic cells from chronic infected quarters, supporting the lower inflammatory status that characterized *S. aureus* chronic mastitis (Riollet et al., 2001). Interestingly, IL-10 secretion is subsequent to TNFα increasing, but differently to what observed for TNFα, the response seems not to be related to the bacterial cell wall type. For instance, *S. uberis* has been shown to evoke milk IL-10 concentrations similar to *E. coli*. However increases in IL-10 production are

detected earlier in response to gram-negative bacteria than to gram-positive or wall-less bacteria, similarly to TNFα (Bannerman, 2009).

Interleukin 12

Interleukin-12 (IL-12) is a key cytokine that determines Th1/Th2 polarization. IL-12 is produced by macrophages, dendritic cells, B cells, and neutrophils (Tizard, 2013). Similarly to IFN-γ, IL-12 acts to link the innate and adaptive arms of the immune system, and plays an essential role in modulating the host immune response to bacterial and parasitic intracellular pathogens (Bannerman, 2009). In mammary cells isolated from cows experimentally infected with *E. coli* or *S. aureus* an increase in IL-12 mRNA was detected (Alluwaimi et al., 2003), as well as in naturally occurred *S. aureus* mastitis (Riollet et al., 2001).

Interferon-γ

Interferon-γ(IFN-γ) plays a key role in the immunity against intracellular pathogens and is the most extensively studied interferon in udder immunity (Schukken et al. 2011). Increasing abundance of IFN-γ mRNAs was detected in milk cells as well as increasing protein amounts in milk isolated in the course of mastitis (Bannerman, 2009; Riollet et al., 2001). Cellular sources of IFN-γ include lymphocytes, natural killer cells, and cells of monocyte lineage. The influence of IFN-γ on the innate immune system is most evident from its effects on macrophages and neutrophils. IFN-γ enhances the microbial activity of these cells by increasing receptor-mediated phagocytosis, inducing respiratory burst activity, and priming nitric oxide production (Bannerman, 2009). Furthermore, IFN-γ links innate with acquired immunity inducing the production of IL-12 (Alluwaimi, 2004).

Complement system

The complement system is is a set of proteins that help the lysis of bacteria through pore-formation, the opsonization and attraction of phagocytes, after a cascade of events, in which each step leads to the next. The components of complement complex are synthetized mainly by epathocytes but also by monocytes and tissue macrophages (Sordillo and Streicher, 2002). In milk, the highest concentrations of complement complex proteins (C) were observed for colostrum, mastitic milk and in the mammary secretions

during involution, while they were lower in healthy glands during lactation (Sordillo and Streicher, 2002). Although the overall function of complement system in mammary gland defence has yet to be fully determined, available knowledge supports its predominant pro-inflammatory role in intramammary infections (Riollet et al., 2000; Bannerman, 2009). It has been postulated that the classical pathway of complement activation is impossible in milk from healthy quarters due to the lack of the component C1q (Reinard et al., 2003). Nevertheless the alternative pathway can operate with a double outcome: deposition of opsonic C3b and iC3b on bacteria, and generation of the pro-inflammatory fragment C5a. The C5a fragment is among the most potent chemoattractants for neutrophils and for cells of monocytic lineage inducing the migration of these cell types through the mammary epithelium. The fragment C5a is also a potent stimulator of the phagocytic function and respiratory burst activity of neutrophils (Alluwaimi, 2004).

N-acetil-β-D-glucosaminidase and Lysozyme

During mastitis, while the enzymes related with the synthesis of milk decrease, the enzymes associated to inflammation increase, such as N-acetil-β-D-glucosaminidase (NAGase; Pyörälä, 2003) and Lysozyme (LZ; Osman et al., 2010). They are lysosomal enzymes, considered as reliable indicators of inflammation (Pyörälä, 2003).

NAGase is mainly released from the polymorphonuclear leucocytes during phagocytosis and cell lysis (Åkerstedt et al., 2011) and to some degree from damaged epithelial cells. It plays a role in mammary gland immune response (Torben and Karen, 2012) and its amount in the milk is related to pathogen strain (Kitchen *et* al., 1981; Ebling *et* al., 2001). The enzyme activity in milk was shown to increase in subclinical mastitis (Åkerstedt et al., 2011). NAGase activity was also found in clinically healthy cows (Åkerstedt et al., 2011) with the highest levels in the first 20 days post-parturition, followed by a decline and a stabilization after 160 days of lactation (Piccinini et al., 2007). Despite the increase of this enzyme in mastitic milk, in the absence of inflammation the inconsistency between somatic cell count mean values and NAGase activity suggests that tissue factors may contribute to the release of some immune components (Piccinini et al., 2007). Recently, mammary epithelial cells of a continuous cell line, BME-UV1, were shown to produce NAGase when infected with *S. aureus* strains, as well as in the absence of stimuli (Mazzilli and Zecconi, 2010). NAGase distribution in a variety of tissue and in extracellular

compartments has led some studies to investigate the suitability of this enzyme as a marker of tissue damage. Recent studies demonstrated a correlation between high level of extracellular NAGase and tubular damage: Forman *et al.* (1996) published the first paper regarding NAGase in the urinary excretion of hens, concluding that this enzyme could be successfully used as a marker of kidney damage in hens. Later, Sato *et al.* (2002) showed the clinical usefulness of measuring NAGase in urinary tract diseases of cats.

While NAGase is considered a marker of inflammation and tissue damage, LZ, with its antibacterial properties, is an indicator of mammary gland immune response.

LZ is one of the most extensively studied antibacterial milk proteins. It has a well-recognized bactericidal effect against both Gram-positive and Gram-negative bacteria (Lopez et al., 2006) and represents one of the components of mammary gland innate immune defenses, fundamental in preventing pathogen invasion. Indeed, together with lactoferrin, LZ is one of the most abundant proteins contained in neutrophils-specific granules (Ganz, 2004). It plays an antibacterial activity killing ingested bacteria in the phagolysosomes and preventing colonization through exocytosis of polymorphonuclear leucocytes secondary granules (Zecconi and Smith, 2003). The mode of action of LZ is not yet completely understood: while it is well known for its catalytic function related to the damage of bacterial cell walls (it cleaves the bond between N-acetyl muraminic acid and N-acetyl glucosamine and destroys cell wall peptidoglycans in Gram-positive bacteria; Tizard, 2013), a non-enzymatic mechanism exhibiting antibacterial activity against Gram-negative microorganisms has been proposed (Ibrahim et al., 1996). As a matter of fact, LZ is highly active against many Gram-positive species but it can synergize with antibodies, complement or lactoferrin, allowing the disruption of the outer membrane of Gram-negative bacteria and the access to peptidoglycan (Ganz, 2004). Due to a small concentration of IgA in ruminant milk and to a low amount of LZ, it is unknown if this mechanism is active in the bovine mammary gland (Sordillo and Streicher, 2002). Furthermore, a recent study provides a different mode of action of LZ, demonstrating the antimicrobial activity of LZ-deriving peptides. These peptides, isolated from proteolytic digests of hen egg white LZ, were internalized in both *E. coli* and *S. aureus* after the interaction with membrane phospholipids. These internalized peptides did not only affect the membrane permeability, but affected intracellular functions (Hunter et al., 2005). As for NAGase, LZ was secreted in the milk also in the absence of inflammatory reaction.

Furthermore the higher levels of enzyme in latent infections in comparison with subclinical and healthy mammary quarters, showed a role of this molecule in the control of invading pathogens (Piccinini et al., 2007).

Lactoferrin

Lactoferrin (Lf) is an iron-binding protein produced by leukocytes and epithelial cells. It is first known for its iron-chelating functions, which are the basis of two activities: a non-specific bacteriostatic activity and protection against oxygen radicals. These properties depend on Lf capability to bind free ferric ion in milk, avoiding the growth of iron-depending bacteria and blocking the reactions of free radicals, which are catalysed by free iron. On the other hand, bovine mammary epithelial cells secrete citrate in milk and this secretion creates an ideal buffer that chelates iron and makes it available to bacteria, abolishing the bacteriostatic activity of lactoferrin (Sordillo and Streicher, 2002). Bovine milk contains small amount of Lf (20-200 µg/mL) in comparison to bovine colostrum (2-5 mg/mL) and to the secretions of non-lactating mammary gland, which can contain very high concentration of Lf (20-100 mg/mL). Thus, the secretion of Lf is inversely related to alveolar development and Lf bacteriostatic and bactericidal activities are likely to be more important when the mammary gland is fully involuted (Molenaar et al. 1996).

Transferrin

Transferrin (Tf) is similar to lactoferrin for its iron-binding functions, and is found in milk. This protein comes from blood, by transcytosis in the normal gland and through exudation of plasma during mastitis. Bovine milk contains low amounts of transferrin (20-40 µg /mL); higher amounts are present in colostrum (1mg/mL), but the highest Tf amounts are in the blood (4-5mg/mL). Transferrin may allow a first iron-chelating bacteriostatic effect, before the increase of Lf concentration (Alluwaimi, 2004).

Xanthine oxidase

This enzyme is located in the membrane of milk fat globules. It catalyses the formation of nitric oxide from inorganic nitrite, which under aerobic condition leads to generation of peroxynitrite, a powerful bactericidal agent (Alluwaimi, 2004).

Antibodies

"Natural" antibodies circulate in normal healthy individuals without the need of antigenic stimulation: they are mostly the IgM antibody isotype (Boes, 2000). Two types are distinguished, the one directed against exogenous antigens and the other against self-antigens (Baumgarth et al., 2005). IgM are polyreactive and mostly directed against bacterial antigens, such as lipopolysaccharide, lipoteichoic acid and peptidoglycan and opsonize efficiently *S. aureus* and *E. coli* in rather low concentrations (less than 1%; Wijga et al., 2013). They are components of innate immunity and can cooperate with complement system in the first line of defence. Van Knegsel et al. (2007) suggested that concentration of IgM in both plasma and milk of dairy cows in early lactation was negatively influenced by compromised metabolic health in the periparturient period with consequent reduce immune functions.

The primary isotype found in healthy mammary secretion are IgG, produced by antigen-activated B lymphocytes and components of specific immune response. Togheter with IgG, IgM act opsonising bacteria and thus enhancing the phagocitic activity of neutrophils and macrophages (Sordillo and Streicher, 2002).

2.3. Cellular defenses

Leucocytes are the most representative cells of innate immunity of the mammary gland, including neutrophils, macrophages and lymphocytes. Other cell types such as dendritic cells and mammary epithelial cells should be added as components of milk somatic cells (SC). Mammary epiyhelial cells (MEC) are the interface between udder and invading microorganisms, thus they have a crucial role for an efficient immune response. Even though the amount of MEC depend on the lactation phase, milk from healthy bovine mammary gland contains a small number of epithelial cells and leukocytes. Recently a count of somatic cells (SCC) of 100.000 cells/mL was suggested in absence of inflammation (Hillerton, 1999). Lactation stage affects the SCC so that immediately after parturition SCC is high, but , but in healthy quarters decrease within 3 days after calving (Barkema et al., 1999). Towards the end of the lactation period, SCC increases slightly until drying-off, when udder tissues undergoes several physiological changes.

Nevertheless, according to more recent studies, the physiological effects affect very little the SCC of truly healthy cows (Leavens et al., 1997).

Neutrophils are non specific leukocytes that are recruited actively in the infection site. While they are in low number in the healthy mammary gland, they increase to 90% of total mammary cell population in mastitic quarter milk (Sordillo and Streicher, 2002). During infection, the neutrophils are recruited by chemotactic stimuli from blood into the milk and they play an efficient phagocytosis and killing of pathogens (Schukken et al., 2011), exploiting their crucial role in the elimination of invading bacteria.

Macrophages represent the major cell type in the secretion of involuted udder and in the tissues of a healthy mammary gland, but not in the milk (Pilla *et al.,* 2013). The nonspecific function of these cells is to phagocytise bacteria and destroy them with proteases and reactive oxygen species. The phagocyte activity of these cells is lower if compared with neutrophils, but it can increase in the presence of opsonic antibodies against particular pathogens. Furthermore, they can secrete substances that promote both migration and bactericidal activity of neutrophils. Indeed, activated macrophages are triggered to release prostaglandins, leukotrienes, and cytokines that can augment local inflammatory processes. Therefore, the ability of macrophages to secrete substances that mediate the migration and bactericidal activity of neutrophils is considered of higher importance to the non-specific mammary gland defense, than their phagocytic activity. Macrophages also play a role in the development of specific immune response, through antigen processing and presentation (Sordillo and Streicher, 2002).

Lymphocytes are able to recognize antigens through specific membrane receptors, which give the immunological characteristics of specificity, diversity, memory, and self/non-self recognition. In healthy mammary gland T lymphocytes represent the major lymphocyte subset, including CD4+ (T helper) and CD8+ (T cytotoxic or T suppressor) lymphocytes. Even though the specific leukocyte populations mediating the immune response during IMI are not well defined (Schukken et al., 2011), it is known that lymphocyte subset which prevail after staphylococcal infections is CD4+ T cells, while with streptococcal mastitis there is a parallel increase in both CD4+ and CD8+ T-cells (Sordillo, 2005). Mehrzad et al. (2008) studied the T-cell dynamics after

an experimental *E. coli* challenge infection. They observed there was a significant decline in the CD4+/CD8+ ratio at 6–24 h after challenge due to greater CD8+ cell concentrations in milk. CD8+ T lymphocytes has a scavenger activity, eliminating old or damaged secretory cells (Schukken at al., 2011). Furthermore, they are thought to modulate immune response during bacterial infections through their suppressor function. This phenomenon is dependent on stage of lactation and is observed in particular in the early stage of lactation (Sordillo and Streicher, 2002). The primary role of B lymphocytes is to produce antibodies against pathogens. They use specific surface receptor molecules to recognise specific antigens and similarly to macrophages and dendritic cells they function also as antigen-presenting cells. Natural killer (NK) cells are a lymphocyte subpopulation with an antibody-dependent cytotoxic activity, which is independent from MHC. NK cells are capable to eliminate tumoral and virus-infected cells, but NK cells has also potent bactericidal activity against both Gram+ and Gram- bacteria (Sordillo and Streicher, 2002).

Bovine mammary epithelial cells (bMEC) have shown to be highly immune competent (Strandberg et al., 2005; Lahouassa et al., 2007; Gunther et al., 2010). bMEC are able to release a variety of inflammatory mediators such as cytokines, antimicrobial peptides, and arachidonic acid metabolites. The role of these messenger molecules is not completely understood, but they may be involved in the recruitment of neutrophils and lymphocytes into milk (Günther et al., 2010). The epithelial cells have both a sentinel and an effector role and they are well suited to exert these functions due to their abundance and exposed position to invading microorganisms (Günther et al., 2010). A burst of cytokines synthesis follows the contact between bMEC and pathogens: the reaction is stronger after *E. coli* challenge in comparison with *S. aureus* experimental infection (Günther et al., 2010). These cells are the first to come in contact with invading pathogens, thus activation of immune response of bMEC is involved in the evolution of infection. *In vitro* studies using a continuous cell line (MAC-T) showed that the cells secreted IL-8 under stimulation with LPS, in a time and dose-dependent manner (Boudjellab et al., 1998). Analogously, primary cultures of bMEC showed enhanced expression of chemokines, when stimulated by LPS (Strandberg et al., 2005). The secretion of pro-inflammatory cytokines by bMEC has been investigated after *S. aureus* challenge, with different results, however TNFα was

secreted after stimulation in all of the studies (Wellnitz et al., 2004; Strandberg et al., 2005, Lahouassa et al., 2007). In the latter study, IL-1β and IL-8 increased, but less than in *E. coli* bMEC infection. To the contrary, the two major anti-inflammatory cytokines IL-10 and TGF-β were not affected by the presence of both pathogens, indicating that bMEC are not the source of the high IL-10 levels observed during cronic *S.aureus* mastitis that is characterised by moderate inflammatory response (Lahouassa et al., 2007).

3. *IN VITRO* STUDY OF BOVINE MAMMARY GLAND

In vitro models provide a useful system for the analysis of bovine mammary gland response. They take advantage from the growth of udder secretory cells in a controlled environment, in relatively short time and at low costs.

In recent years, considerable progress has been made in understanding the molecular mechanisms driving the different functions of the ruminant mammary gland and in-vitro experimental models based on mammary epithelial cells culture have been applied for this proposal (Matitashvili et al., 1997). These models are currently used to investigate the mechanisms involved in milk production, cellular proliferation and differentiation, or to study mammary gland metabolisms or again the interactions between mammary tissue and pathogens and/or their products (Matitashvili et al., 1997). Nowadays two immortalized cell lines derived from bovine mammary epithelium are available: MAC-T cells and BME-UV1 cells (Zhao et al., 2010).

BME-UV1 are established from primary bovine mammary epithelial cells by stable transfection with a plasmid encoding the SV40 large T-antigen, a viral oncogene that prevent the senescence upon continuous passage and not affects metabolic activity. BME-UV1 cells are able to synthesize α-lactoalbumin andαs1-casein (Zavizion et al., 1994). Moreover they are the only known bovine mammary epithelial cell line showing enhanced proliferation in the presence of epidermal growth factor (EGF) (Zavizion et al., 1996). Based on these characteristics this continuous cell line has been used as a valid model for the investigations on bovine mammary epithelium. In comparison with primary cell cultures, the established ones show a reduced possibility of alteration of their original

characteristics caused by genetic drift, which is the major limitation of primary cultures (Matitashvili et al., 1997). Furthermore the researcher can avoid both the biological variability associated with the natural differences between individual organisms and some of the complexity inherent for freshly isolated cells, which may contain cells at different stages of differentiation. Additionally, immortalized cells are available at different passages and the use of the same cell lines in different laboratories makes the results more comparable (Matitashvili et al., 1997). BME-UV1 cells express immunological and inflammatory molecules (Mazzilli and Zecconi, 2010) and therefore can be successfully applied to test the interactions of mammary gland epithelial cells with bacteria and antimicrobials (Didier and Kessel, 2004; Fitzgerald et al. 2007).

AIM OF THE STUDY

The use of antibiotics for prophylactic treatment is being subjected to considerable debate all over the world for its connection with the emergence of antimicrobial resistance in pathogens (Frola et al., 2013). Moreover, the use of antibiotic treatment during lactation causes the presence of antibiotic residues in milk. For these reasons a pressure for the reduction of the use of antibiotics in veterinary field is ongoing, and the researchers are interested in finding novel molecules and in the development of new alternative approaches. The current use of lactic acid bacteria (LAB) as probiotic cultures and their ability to produce antimicrobial molecules encourage investigations of the employment of their bacteriocins or viable cell cultures into the mammary gland with the subsequent *in situ* production of antimicrobial molecules. In recent studies, the efficacy of treatments using different genera of LAB or their antibacterial products was evaluated (Frola et al., 2012; Wu et al., 2007; Cao et al., 2007). Furthermore, there is the evidence that some probiotics can stimulate a protective immune response enhancing resistance to invading pathogens (Klostermann et al., 2008; Beecher et al., 2009). However, the information regarding this subject is still scarce. Either, the antimicrobial proteins (bacteriocins) or viable bacteriocin-producing *Lactococcus* spp. cultures, applied into the mammary gland come in contact with both invading pathogens and epithelial cells, which are involved in the activation of immune response and thus in the evolution of infections. The stabilized line of bovine mammary epithelial cells (BME-UV1) produces proinflammatory cytokines upon stimulation with antimicrobial peptides (Tomasinsig et al., 2010). Therefore, it is a suitable *in vitro* model to assess the response of bovine mammary epithelial tissue to bacteriocins and to evaluate the interaction between epithelial cells and viable *Lactococcus* spp. cultures.

The aim of the present study was to evaluate the potential use of LAB bacteriocins against mastitis pathogens and the role of both LAB live cultures and their bacteriocins in modulating the response of mammary epithelial cells.

Most specifically, the tasks were:

1. To set up the optimal culture conditions for improvement of the growth of LAB and for optimization of bacteriocin production and extraction.
2. To establish the best antibacterial assays to screen large numbers of producer strains.
3. To evaluate the bacteriocin efficacy against mastitis pathogens.
4. To characterize the antibacterial proteins in the extracts of selected LAB producers.
5. To assess the immune response of mammary epithelial cells to the challenge with both antibacterial extracts and live producer bacterial strains.

MATERIALS AND METHODS

1. SCREENING OF LAB STRAINS

1.1. Preparation of cell-free culture supernatants

Overall 65 strains of LAB come from Centro Sperimentale del Latte (CSL, Zelo Buon Persico, Lodi) collection were tested: *Lactococcus lactis* subsp. *lactis* (25 strains), *Lactococcus lactis* subsp. *cremoris* (16 strains), *Lactococcus lactis* subsp. l*actis* bv. *diacetylactis* (11 strains), *Lactobacillus plantarum* (one strain), *Lactobacillus rhamnosus* (2 strains) *Lactobacillus paracasei* (one strain), *Lactobacillus acidophilus* (2 strains), *Lactobacillus fermentum* (one strain), *Lactobacillus helveticus* (4 strains), *Lactobacillus rheuteri* (one strain) and *Bifidobacterium* spp. (one strain).

Six different media were used to grow *Lactococcus lactis* subsp. *lactis* (LL11), in order to investigate which medium could allow the highest antibacterial activity: TGE1 (1% Tripticase, 1% Glucose, 1% Yeast extract), TGE2 (TGE broth additioned with 0,5% sodium citrate, 0,1% sodium acetate and 0,05% dipotassium phosphate, Yang et al., 1992), GYB1 (1% Glucose, 0,5% Yeats extract), GYB2 (2% Glucose, 1% Yeast extract), SG (3% milk whey, 1% glucose), MRS (DeMan, Rogosa, Sharpe broth; Oxoid, UK). One colony of a 24h-culture was picked from MRS plate and inoculated in 15 mL of each medium. After 18h incubation at 30°C, the culture broth was plated on MRS agar (Difco^{TM}) and counted, while the cell-free supernatants (CFS) were tested with *spot-on-lawn assay* and MIC test against *S. agalactiae* MB386 (see below).

Cell-free supernatants (CFS) of all screened strains were obtained directly from the cultures in deMan, Rogosa and Sharpe broth (MRS), by acid extraction method following a previously described protocol (Yang et al., 1992). Briefly, after incubation for 18-24 h at 30°C for *Lactococcus* spp. or at 37°C for *Lactobacillus* spp., culture broths were acidified at pH 2. After the release of bacteriocins, the broths were centrifuged at 29.000 x g for 15 min and adjusted to pH 7. Finally CFSs were sterilized by filtration through 0,22 µm filter and stored at -20°C until use.

1.2. Antimicrobial activity test: spot-on-lawn assay

Antibacterial activity of supernatants was screened with the *spot-on-lawn* assay (Saavendra *et* Sesma, 2011). All LAB supernatants extracted with the method described above from all strains of LAB were tested. LAB strains were maintained on MRS agar at

4°C until use. For this test Brain Heart Infusion (BHI) agar plates supplemented with 5% of Foetal Calf Serum (FCS) were applied. *S. agalactiae* MB386 was used as indicator strain for CFS activity. A suspension containing 10^6 UFC/mL *S. agalactiae* MB386 was prepared and spread onto the plate surface. Five µL of each LAB CFS was spotted on the plate and then incubated at 37°C for 18-24h. The presence of a clear zone of growth inhibition indicated bacteriocin activity.

2. ANTIBACTERIAL ACTIVITY AGAINST MASTITIS PATHOGENS

2.1. Bacterial strains

Antibacterial activity of bacteriocin-producing strains was tested against six different species of mastitis pathogens, including methicillin resistant strains of *Staphylococcus aureus*. Except for one S. aureus strain obtained from American Type Culture Collection (ATCC, Manassas, VI, USA) all bacterial strains had been isolated from clinical and subclinical mastitis. All tested strains are listed in the table 1.

Table 1. Bacterial strains used in the antimicrobial activity tests and their origin.

Species	Strain	Source
Streptococcus agalactiae	MB90	mastitic milk
	MB98	mastitic milk
	MB386	subclinical mastitis
Streptococcus uberis	MB705	clinical mastitis
	MB707	post-treatment mastitis
	MB300	subclinical mastitis
Streptococcus dysgalactiae	MB280	mastitic milk
	MB324	subclinical mastitis
Enterococcus faecalis	MB330	subclinical mastitis
	MB561	subclinical mastitis
	MB562	subclinical mastitis
	MB706	subclinical mastitis
Staphylococcus aureus	29213	ATCC 29213
	MB221	subclinical mastitis
	MB254	subclinical mastitis
	MB351	subclinical mastitis
	MB390	subclinical mastitis
	MB439	subclinical mastitis
	MB501	subclinical mastitis
	MB512	subclinical mastitis
	MB535	clinical mastitis
	MB543	subclinical mastitis

	MB781	subclinical mastitis
	MB786	subclinical mastitis
	MB798	clinical mastitis
Methicillin-resistant Staphylococcus aureus (MRSA)	MB508	subclinical mastitis
	MB628	subclinical mastitis
Coagulase-negative staphylococci (CNS)	MB307	subclinical mastitis
	MB309	subclinical mastitis
	MB316	subclinical mastitis

For bacteriological analysis of milk, 10 mL of quarter milk was aseptically collected from the teats previously cleaned and disinfected and after the discard of first squirts. Samples were refrigerated until arrival at laboratory facilities. Ten µL of each sample was spread onto blood agar plates (5% bovine blood) and incubated at 37°C for the bacteriological analysis as previously described (Oliver et al., 2004). Plates were evaluated after 24 and 48 h, and colonies were isolated and identified by biochemical tests following Hogan et al. (1999) and confirmed by API System (Rapid ID 32 Strep, Biomérieux City, Country). The somatic cell count of each sample was performed with Bentley Somacount 150 (Bentley Instruments City, Country). All isolates were stored at -80°C in Microbank Bacterial Preservation System (Thermo Fisher, City, Country). Immediately after thawing, the isolates were subcultured on blood agar plate (5% of blood, Oxoid) and thereafter grown in BHI broth for 24 h at 37°C. Antibiotic sensitivity of each strain was tested by Kirby-Bauer disk diffusion method. The drugs most widely used in the treatment of bovine mastitis (penicillin, ampicillin, amoxicillin/clavulanic acid, oxacillin, 1^{st}, 3^{rd} and 4^{th} generation cephalosporins, norfloxacin, nafcillin/penicillin/streptomicin, rifaximin, tylosin, kanamicin, tiamphenicol, sulfamethoxazole/trimethoprim) were tested.

2.2. Minimum inhibitory concentration (MIC) assay

Bacteriostatic activity of cell-free supernatants (CFS) containing bacteriocins (sample A) was tested using MIC assay against each pathogen strain, according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Serial two-fold dilutions of each supernatant were performed in 100µL of BHI broth supplemented with 5% Fetal Calf Serum (FCS), in 96-well microplates. For each pathogen, a 10^6 cfu/mL inoculum was prepared, then 10 µL of bacterial inoculum was added to each well and the plates were

incubated for 24h at 37°C. In addition, for each test a sterility control was performed in a separate well and checked for any growth after incubation. The antimicrobial activity of each supernatant was expressed as arbitrary units/mL (AU/mL). One AU was defined as the reciprocal of the highest serial twofold dilution that completely inhibited bacterial growth (Todorov *et* al., 2011).

The sensitivity of three strains (*S. agalactiae* MB386*, S.uberis* MB707 and *S.dysgalactiae* MB280) to a nisin solution containing 150 µg/mL of pure nisin was also evaluated and compared to the results obtained with CFS, in order to estimate the amount of bacteriocin in the supernatants.

2.3. Minimum bactericidal concentration (MBC) assay

To test bactericidal effect of bacteriocins, minimum bactericidal concentration (MBC) was assayed for each bacteriocin against each pathogen strain. Ten µL of each dilution representing the MIC endpoint of two more concentrated dilutions were plated onto blood agar plates (5% bovine blood) and incubated overnight at 37°C. Each dilution was plated in duplicate and colonies of growth were counted. The MBC was defined as the lowest concentration showing ≥99% killing (CLSI, 1999) and the minimum dilution of bacteriocins that killed the pathogens was expressed in AU/mL (see above).

3. IDENTIFICATION OF ANTIBACTERIAL MOLECULES

3.1. Partial purification of antibacterial molecules

Partial purification of antibacterial molecules produced by active strains of *Lactococcus* spp. was performed by the adsorption-desorption method as described by Yang *et* al. (1992) and Baljinder *et* al. (2013). Briefly, after growth, the culture broths were adjusted to pH 6.5, the cells were deactivated by heating at 70°C for 25 min, and left for for 3h at 4°C to allow the adsorption of bacteriocins onto the bacterial cell walls. Subsequently, bacterial cells were harvested by centrifugation at 15,000g for 15 min at 4°C, washed with 5 mM sodium phosphate buffer (pH 6.5) and resuspended in 100 mM NaCl at pH 2.0 to allow the bacteriocins to be desorbed. After 1h incubation at 4°C, cell suspensions were centrifuged at 29,000g for 20 min and then the obtained pre-purified extracts (sample B) were filter-sterilized by passing through a 0.2 µm pore size cellulose acetate membrane. The extracts were subsequently fractionated by ultrafiltration through a 1 kDa molecular

weight cut-off (MWCO) ultrafiltration membrane (Millipore, Darmstadt, Germany) and tested for antibacterial activity against the indicator strain by *spot-on-lawn* test as indicated above (see chapter 1.2).

3.2. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and antibacterial activity test in gel

Cell-free supernatant (sample A) and partially purified bacteriocin extract (sample B) ultrafiltration (1 kDa) permeates and retentates were mixed with SDS–PAGE native sample buffer. Separation was performed on a 15 % polyacrylamide (PAA) resolving gel in TRIS–glycine–SDS buffer on SE 250 mini-vertical gel electrophoresis system (Hoefer Inc., Holliston, MA, USA). PageRuler^{TM} Plus Prestained Protein Ladder (mol wt 10–250 kDa; Thermo Fisher Scientific Baltics; Vilnius, Lithuania) and Spectra Multicolor Low Range Protein Ladder (mol wt 1,7-40 kDa; Thermo Fisher Scientific Baltics; Vilnius, Lithuania) were used as a protein molecular weight markers. The electrophoresis was run at 23°C and 50 V for the first 0.5 h and then at 100 V for another 2 h. Gels were stained with Coomassie brilliant blue G-250. The molecular weights of obtained protein bands were estimated by calculating the relative migration distance (*rf*) values (Hames, 1998). For antibacterial activity test PAA gel was fixed with 50% methanol and 10% acetic acid in milliQ-treated water for 15 min at 25°C, washed in milliQ-treated water for 2 h at 25°C changing water every 15 minutes, then aseptically placed on *S. agalactiae* soft agar (BHI agar (1%) supplemented with 5% of FCS) and overlaid with the same soft agar, but inoculated with 10^6 cfu/mL of the indicator strain. The plates were incubated at 37°C for 18–24 h and the inhibition zone of growth was observed.

3.3. Ultra Performance Liquid Chromatography/Electrospray Ionization-High Resolution-Mass Spectrometry (UPLC/ESI-HR-MS) analysis

Partially purified bacteriocin extract (sample B) ultrafiltration (1 kDa) retentates were fractionated by ultra-performance liquid chromatography (UPLC) carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) and analyzed with a photo diode array (PDA) eLambda detector (Waters) and a high resolution Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (HR-MS, Thermo Scientific, San Jose, CA, USA) interfaced through an electrospray ionization (ESI) source. Samples were separated on an Aeris WIDEPORE XB-C4 column (2.1 × 150 mm, 3.6 µm) (Phenomenex, Torrance, CA, USA) kept at 40 °C. The eluents were: 0.1% trifluoroacetic acid (TFA) in milliQ-treated water

(solvent A) and 0.1% TFA in acetonitrile (solvent B). The UPLC separation was performed by using a linear elution gradient (10% to 55% of solvent B in 13.7 min) at a flow rate of 0.2 mL/min. Proteins in the eluate were detected with a PDA detector at 210 nm and subsequently analyzed by MS using full scan analysis in the range 300–2000 m/z. The resolution was set at 140K. The AGC target was 5e5. The maximum ion injection time was 100 ms. The MS data were automatically processed using Xcalibur software (Thermo Scientific), and protein mass deconvolution was performed using Xtract software (Thermo Scientific).

3.4. Lactococcus lactis *subsp.* lactis *SL208 genome sequencing*

Whole DNA of *L. lactis* subsp. *lactis* SL208 was extracted using the DNeasy blood and tissue kit (Qiagen, I) following the manufacturer instructions and was subjected to quality control. After library preparation with the Nextera XT sample preparation kit (Illumina), sequencing was performed using the Illumina MiSeq platform with a 2 × 250 paired-end run. Reads were analyzed and quality checked using FastQC (Andrews, 2010) and a specifically designed python script. Genome assembly was performed using Mira4 (Chevreux et al., 1999), while genome annotation was performed using the PROKKA software (version 1.1). NCBI Protein Basic Local Alignment Search Tool (BLAST) was used to identify protein amino acids sequences, while the alignment of Lactococcin G, Enterocin C2 and SL208 Lactococcin-like protein was performed using the ClustalW software alignment tool (version 2.1).

4. INTERACTION OF BACTERIOCINS OR LIVE LACTOCOCCAL CULTURES WITH MAMMARY EPITHELIAL CELLS

4.1. Bovine mammary epithelial cell culture

BME-UV1 cells were cultured at 37°C with 5% CO2 in the following medium: 50% Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DM/F12, Gibco), 30% RPMI 1640 (Gibco), 20% NCTC 135 (Gibco), containing 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 10 µg/ml L-ascorbic acid, 1 µg/ml hydrocortisone, 1 µg/ml insulin, 5 µg/ml transferrin, and 0.5 µg/ml progesterone. The cells were grown to confluence in 6-wells plates for 58 h in complete medium supplemented with 10% Fetal Calf Serum (FCS). To evaluate the role of serum addiction, the cells were grown to confluence and maintained for 4, 8, 24, 34 and 58 h with 10%, 5% or without FCS; at each

time point both culture medium and cells were collected and tested for enzymes production. To test the effect of the treatments, the 58h old cells were washed twice with Hanks' Balanced Salts Solution (HBSS) and maintained in the complete medium without FCS until the addiction of CFSs or live lactococcal cultures (see below). Trypan blue solution (0.4%, Sigma-Aldrich) was used to assess cell viability: 20 µL of cells were suspended in 80 µL of diluted solution (Trypan blue/PBS, 1/5) and the rate of viable cells was calculated using a Burker cell counting chamber.

4.2. Challenge with bacteriocins and live cultures of Lactococcus lactis *subsp.* lactis

Each preparation containing LL11 or SL153 bacteriocin was obtained after 48h incubation of lattococci in RPMI medium. Antibacterial activity of such CFSs was assayed with the MIC test to measure the antibacterial activity. For the evaluation of enzymes and cytokines production by epithelial cells, CFSs were added to 6-wells cell culture plate to a final concentration of 10% in FCS-free medium. Moreover, to test the stimulation of epithelial cells by lactococcal live cultures , 30 µL of a suspension (10^4 cfu/mL) of each bacteriocin producing-lactococcus strain (LL11 or SL153 or SL208) were added to distinct wells and after 4h, 8h, 15h, 24h of stimulation both culture medium and cells were collected separately and stored at -20°C. Bacterial growth and antimicrobial activity of extracellular compound were also assayed at such time points. Aliquots of 150 µL containing 3 x 10^6 BME-UV1 cells were suspended in 750 µL of RNA*later* (Sigma-Aldrich) and stored at -80 °C until RNA extraction. Production of antibacterial enzymes was tested on both epithelial cells and culture medium, while pro-inflammatory cytokine expression was assayed on the cells of each well.

4.3. N-acetil-β-D-glucosaminidase and Lysozyme assays

The amount of N-acetil-β-D-glucosaminidase (NAGase) and Lysozyme (LZ) were assayed in both supernatants and cells using a fluorescence-based procedure. In order to disrupt the cell membrane and allow the complete release of enzymes, the cells were shaken with 150-212 µm glass beads (Sigma-Aldrich) and then harvested by centrifugation. Trypan blue solution (0.4%, Sigma-Aldrich) was used to assess cell viability according to the manufacturer's instructions.

NAGase activity was assessed in duplicate by a fluorometric assays according to Kitchen et al. (1984), on a microplate fluorometer (Fluoroskan Ascent, Thermo Labsystem, FL). Each

sample was incubated with 4-methylumbelliferyl-N-acetyl-β-D-glucosamidine (MUAG, Sigma-Aldrich) as a substrate. NAGase activity was measured as fluorescence increment on a microplate florimeter (Fluoroskan Ascent, Thermo Labsystem, FL) using 355 nm excitation and 460 nm emission. The concentration was calculated against a standard curve with a range of 0-200 µM of 4-methylubelliferone (4-MU, Sigma-Aldrich). Activity was expressed as pmol 4-methylubelliferone released/min at 25 °C catalysed by 1 µl of solution.

LZ was assessed in duplicate by (EnzChek Lysozyme Kit, Invitrogen, CarlsbadCA, USA), which is based on the lysis of *Micrococcus lysodeycticus* labelled with fluorescine. LZ activity was assayed by measuring fluorescence increment after 30 min of incubation at 37°C on a microplate fluorimeter at 494nm excitation and 518 nm emission (FluoroskanAscent, Thermo Labsystem, FL, USA), against a standard curve with a range of 8-500 units. One unit of LZ is defined as the quantity of enzyme that produces a decrease in turbidity of 0.0001 OD units per min at 450 nm measured at pH 7.0 (25 °C) using 0.3 mg/mL.

4.4. Real-time RT-PCR for mRNA quantification

After thawing, cells were centrifuged at 5000 x *g* for 18 min, to remove RNA*later* and then washed once with PBS. Total RNA was extracted using Illustra Mini RNA isolation kit (GE healthcare Europe) according to manufacturer's instructions, and stored at -20°C. RNA quantification and purity was estimated using spectrophotometer ND-100 (Nanodrop Technologies inc. USA). For RNA retrotranscription, QuantiTect Reverse Transcription Kit (Qiagen, I) was used and 0,1 µg of RNA was reverse transcribed following the recommended procedure in a final volume of 20 µL. A negative control without enzyme was added to exclude DNA contaminations in RNA. Quantitative real-time PCR (qPCR) was performed using EvaGreen fluorescent detection system and the Eco Real-Time PCR System (Illumina Inc., USA). In each reaction, 3 µL of cDNA equivalent to 3 ng of total RNA were amplified in a 15 µL mixture containing 100nM reverse and forward primer each (GAPDH and TNFα) or 200nM each (IL-8). In each plate, GAPDH was run as housekeeping gene and a negative and positive control were included. Each sample was processed in triplicate.

The primers for GAPDH, TNFα and IL-8 are listed in the table 2.

Table 2. Primer sequences with respective melting temperature (Tm) and product size.

Target cDNA	Primer (5'-3')	Tm (°C)	Product size	Source
TNFα	F:CTGGTTCAGACACTCAGGTCCT R:GAGGTAAAGCCCGTCAGCA	62.1 58.8	183 bp	Tomasinsig *et al.* (2010)
IL-8	F:CCTCTTGTTCAATATGACTTCCA R:GGCCCACTCTCAATAACTCTC	56.26 57.81	189 bp	Günter *et al.* (2010).
GAPDH	F:CCTGGAGAAACCTGCCAAGT R:GCCAAATTCATTGTCGTACCA	59.4 55.9	214 bp	Tomasinsig *et al.* (2010)

The thermal-cycling program was different for TNFα or IL-8 in the annealing/extension step: initial denaturation/activation (95°C for 3min), 45 cycles of denaturation (95°C for 10 s), annealing/extension (62°C for 15 s for TNFα, or 59°C for 15 s for IL-8) and melting curve analysis.

At the cycle number at witch fluorescence signal intersected with the threshold, the Cq value of a sample was set and used for quantification. The specificity of the reaction was checked post-amplification by melt curve analysis using high resolution melting analysis (HRMA). In the Eco Real-Time PCR System (Illumina Inc., USA) fluorescence data were registered every 0,1°C, from 55°C to complete denaturation of DNA at 95°C.

4.4.1 Data Analysys of Real-Time RT-PCR

For relative quantification of mRNA, the Cq values of the target gene transcripts were normalized against the reference gene GAPDH, and the results were expressed as normalized fold expression relative to the control (not stimulated cells).

Because the amplification efficiencies of the target and the reference genes were not equal the normalization of the samples to a single reference gene was made with the Pfaffl method (Pfaffl, 2001) that does not assume equal or 100% amplification efficiency

and incorporates the experimentally determined efficiencies of the target and reference genes to correct for any differences.

The calculations for this method are shown below:

$$\Delta Cq = AVE\ Cq_{(ref\ sample)} - AVE\ Cq_{(unk\ sample)}$$

$$Quantity = (Efficiency)^{\Delta Cq}$$

$$RQ = Quantity_{target\ assay} \div Quantity_{reference\ assay}$$

5. STATISTICAL ANALYSIS

The analysis of data has been performed with the software SPSS Statistics version 21.0 (IBM, USA). Means were compared by Two-way ANOVA (Analysis of Variance) and Scheffè post-hoc test. Time and treatment variables were used as main effects and the NAGase, Lysozyme and cytokine expression as the dependent variables. One-way ANOVA was applied when the time variable was not significant and also to highlight differences among intracellular and extracellular localization of enzymes. Results were considered as statistically significant at *P* values <0.05.

RESULTS AND DISCUSSION

1. SCREENING OF LAB STRAINS FOR ANTIBACTERIAL ACTIVITY

Sixty-five strains from the collection of Centro Sperimentale del Latte were tested for their antibacterial activity against *Streptococcus agalactiae* MB386 by *spot-on-lawn* assay. This pathogen was used as indicator strain, because *S. agalactiae* is known to exhibit high sensitivity to bacteriocins (Wu et al., 2007) and the 3 strains tested (MB386, MB90, MB98) showed high and comparable sensitivity. As bacteriocin production by LAB is a growth-associated process (Cintas et al., 2011) starting at early stationary phase, and reaching its peak toward the end of this phase (De Vuyst and Vandamme, 1991), the selection of the best medium to grow the bacteria allowed the optimisation of CFS antibacterial activity (Table 1.1). The acid-extraction method was found to maximize the amount of bacteriocins released in the CFSs allowing the release of peptide from producer cell surfaces as suggested by Yang et al. (1992) and thus the potency of CFSs. MRS medium allowed the highest bacterial proliferation and exerted high antibacterial activity, which was enhanced after the acidification method. Furthermore a commercial medium assured a more constant and better characterized composition. Cell-free culture supernatants (CFS) obtained by bacteriocin producers cultivated in acidified MRS medium were used to screen the LAB strains for antibacterial activity. Among the 65 strains, only 3, belonging to the species *Lactococcus lactis* subsp. *lactis* (LL11, SL153 and SL208), showed antibacterial activity against *S. agalactiae* MB386. *Lactococcus lactis* subsp. *lactis* LL11 strain had been isolated from bovine milk, while SL153 and SL208 came from natural starter for dairy processing. This result is in disagreement with a previous paper (Riley and Wertz, 2002) which proposed that 99% of all bacteria may produce at least one bacteriocin. Even though different culture conditions, pH, temperature and stress factors could interfere with bacteriocin production (Drider et al., 2006). Biosynthesis of bacteriocins occurs in bacterial cells when anti-competitors activity is necessary (e.g. against invasion of other strains or species into a niche) and these proteins have also a role in quorum sensing process, mediating microbial interaction (Drider *et* al., 2006). These characteristics, together with the three-component regulation of genes activation that needs a threshold level requirement for the auto-induction activation (Balciunas et

al., 2013), make hard the comprehension of basic mechanisms of bacteriocin production or failure.

Table 1.1 MIC values of cell-free culture supernatants extracted from different media and from acidified MRS medium against reference strain *Streptococcus agalactiae* MB386.

Medium (broth)	***Lactococcus lactis* subsp. *lactis* LL11 UFC/mL**	**Diameter of inhibition zone (mm) *Spot-on-lawn* assay**	**MIC (AU/mL)**
TGE1	$1x10^9$ UFC/mL	10.0	128
TGE2	/	0.0	1.5
GYB2	$1x10^8$ UFC/mL	2.0	6
GYB1	$2x10^7$ UFC/mL	0.0	1.5
SG	$1x10^9$ UFC/mL	11.5	128
MRS	$2x10^9$ UFC/mL	14.0	128
Acidified MRS	$2x10^9$ UFC/mL	15.0	256

2. ANTIBACTERIAL ACTIVITY AGAINST MASTITIS PATHOGENS

Antibiotic sensitivity patterns of mastitis isolates, determined by plate diffusion method, showed that all strains were resistant to at least one of antibiotics tested. *E. faecalis* evidenced a particular resistant pattern: three strains were resistant to oxacillin and demonstrated intermediate susceptibility to fluoroquinolones. In addiction 2 out of 4 were also resistant to penicillin, macrolides and tiamphenicol and one of them also showed no sensitivity to first generation cephalosporin.

All *S. agalactiae* strains were resistant to oxacillin and 2 out of 3 strains were also resistant to rifaximin and macrolides. In addition, macrolides were not effective on one *S. uberis* and both *S. dysgalactiae* strains.

A clear resistance to penicillin and ampicillin was evidenced by *S. aureus* with overall 8 resistant strains, 2 of them also resistant to amoxicillin/clavulanic acid. Two strains were MRSA and showed also resistance to fourth generation cephalosporin (Table 2.1).

Antimicrobial activity of the 3 active CFSs was evaluated against the pathogens. Among *streptococcal* strains, the most sensitive species to antibacterial activity of CFS were *S. agalactiae*, *E. faecalis* and *S. uberis* which showed the highest MIC values reaching 384 and 256 AU/mL. While the 3 *S. agalactie* strains (MB90, MB98, MB386) demonstrated an identical sensitivity to all CFS, the different strains of *E. faecalis* and *S. uberis* showed an evident difference in their antimicrobial susceptibility to the same bacteriocin. In particular *E. faecalis* strains displayed up to 7-fold higher sensitivity in the MIC assay when tested against SL153 CFS with the values ranging 2-256 AU/mL. *E. faecalis* strain MB330 demonstrated a marked resistance to all bacteriocins tested showing a MIC of 2 AU/mL when tested with LL11 CFS and SL153 CFS, and no sensitivity against SL208 CFS. *S. uberis* reached 5 dilution differences among strains when tested against SL153 CFS. The two *S. dysgalactiae* strains were the most resistant to LL11 and SL153 CFSs, and showed MIC values of 32 and 4 AU/mL when tested against SL208 CFS.

Among staphylococcal strains high sensitivity was showed by *S. aureus* MB390 that reached a MIC value of 256 AU/mL in the presence of SL153 CFS. The lower inhibition levels was observed for *S. aureus* ATCC29213, MB221, MB254 and MB786 strains and for the two MRSA strains tested. Overall, no resistant strains were evidenced with the MIC test aainst LL11 and SL153 CFSs. To the contrary 53% of *S. aureus* and one of the 3 coagulase negative staphylococci (CNS) strains were resistant to SL208 CFS. As well as *S. uberis* and *E. faecalis*, *S. aureus* and CNS demonstrated a large variability in the sensitivity to lactococcal bacteriocins (see tables 2.2 and 2.3).

Lactococcus lactis subsp. *lactis* SL153 showed the highest inhibitory activity against the pathogens in the MIC test, while SL208 CFS displayed the lowest antibacterial activity since 10 pathogens were resistant. Only *S. aureus* MB290 and both *S. dysgalactiae* strains (MB280 and MB324) were equally or even more sensitive to SL208 CFS compared to SL153 CFS. To the contrary, SL153 and LL11 CFSs were always active. The MBC values overlapped the respective MIC values or differed by a maximum of a dilution for all strains (Tables 2.2 and 2.3).

Although antibiotics still represent the election treatment for mastitis, the biggest challenge in the modern dairy industry is the reduction of the antibiotics use in food production animals (Bradley, 2002). Furthermore, the dairy industry could benefit from the development of safe antimicrobial agents and bacteriocins could be an attractive

alternatives to antibiotics (Pieterse and Todorov, 2010) avoiding withdrawal period and residues in milk.

A considerable variability of bacteriocin sensitivity was observed among both bacterial species and different strains belonging to the same species. It should be noticed that the antibacterial activity was observed also against tose species which are well known to develop antimicrobial resistance, such as *E. faecalis* and *S. aureus* (Piper et al., 2009). Nevertheless, *E. faecalis* together with *S. agalactiae* showed the highest MIC values, indicating a noticeable sensitivity to bacteriocins. It should be pointed out that, despite the differences in sensitivity, bacteriocins were able to inhibit and to kill the pathogens at the same concentration, confirming bactericidal activity of bacteriocins.

Sensitivity of 3 different *Streptococcus* spp. strains to nisin was also tested. The pathogen strains were selected among those showing no resistance to any bacteriocin preparation and belonged to 3 different species, *S. agalactiae* MB386*, S. uberis* MB707*, S. dysgalactiae* MB280. They were used to evaluate the antibacterial activity of pure nisin (150 µg/mL). The potency of this preparation was 16-64 times higher than that of bacteriocin extracts against *S. agalactiae*; 8-32 times against *S. uberis* and the same for SL208. For LL11 and SL153, it was 64 times higher against *S. dysgalactiae* (Table 2.4). The comparison of the inhibitory activity of nisin solution with the three active CFSs suggested that a small amount of bacteriocin was contained in our preparations. Nevertheless, their antibacterial activity confirmed the well-known efficacy of bacteriocins even at very low amounts, which characteristic could be advantageous in therapeutic use.

Table 2.1 Antibiotic susceptibility pattern of strains tested: the number of resistant (R) or sensitive (S) strains are indicated in the corresponding columns.

Antibiotic	*S. agalactiae* (*n*=3)		*S. dysgalactiae* (*n*=2)		*E. faecalis* (*n*=4)		*S. uberis* (*n*=3)		*S. aureus* (*n*=13)		CNS (*n*=3)	
	R	S	R	S	R	S	R	S	R	S	R	S
Penicillin		3		2	2	2		3	8	5	1	2
Ampicillin		3		2	1	3		3	8	5	1	2
Amoxicillin/clavulanic acid		3		2		4		3	2	11		3
Oxacillin	3			2	3	1		3	2	11		3
1st generation cephalosporin		3		2	1	3		3		13		3
3rd generation cephalosporin		3		2		4		3		13		3
4th generation cephalosporin		3		2		4		3	2	11		3
Norfloxacin		3		2		4		3	1	12		3
Rifaximin	2	1		2	2	2		3		13		3
Nafcillin/Penicillin/Streptomycin		3		2		4		3		13		3
Tylosin	2	1	2		2	2	1	2	1	12	1	2
Spiramycin	2	1	2		2	2	1	2		13	1	2
Tiamphenicol		3		2	2	2		3	1	12		3
Sulfamethoxazole/trimethoprim		3		2	4		3		2	11		3

Table 2.2 MIC and MBC values of LL11, SL153, SL208 CFSs against streptococcal strains expressed as AU/mL.

2.2 LL11

Microorganism	MIC values	MBC values
S. agal. (MB386, MB90, MB98)	256.0	256.0
E. faec. MB330	2.0	2.0
E. faec. MB561	64.0	32.0
E. faec. MB562	128.0	64.0
E. faec. MB706	64.0	32.0
S. uberis MB705	64.0	64.0
S. uberis MB707	16.0	8.0
S. uberis MB300	8.0	4.0
S. dysgal. MB280	1.5	not killed
S. dysgal. MB324	1.0	not killed

2.2 SL153

Microorganism	MIC values	MBC values
S. agal. (MB386, MB90, MB98)	384.0	384.0
E. faec. MB330	2.0	2.0
E. faec. MB561	128.0	128.0
E. faec. MB562	128.0	128.0
E. faec. MB706	256.0	256.0
S. uberis MB705	256.0	128.0
S. uberis MB707	32.0	32.0
S. uberis MB300	8.0	8.0
S. dysgal. MB280	1.0	1.0
S. dysgal. MB324	2.0	2.0

2.2 SL208

Microorganism	MIC values	MBC values
S. agal. (MB386, MB90, MB98)	64.0	64.0
E. faec. MB330	res	not killed
E. faec. MB561	8.0	4.0
E. faec. MB562	16.0	16.0
E. faec. MB706	8.0	4.0
S. uberis MB705	64.0	64.0
S. uberis MB707	4.0	2.0
S. uberis MB300	4.0	4.0
S. dysgal. MB280	32.0	16.0
S. dysgal. MB324	4.0	4.0

Table 2.3 MIC and MBC values of LL11, SL153, SL208 CFSs against staphylococcal strains expressed as AU/mL.

2.3 LL11

Microorganism	MIC values	MBC values
S. aureus (ATCC 29213)	2.0	not killed
S. aureus MB221	1.0	1.0
S. aureus MB254	2.0	1.0
S. aureus MB351	16.0	8.0
S. aureus MB390	64.0	24.0
S. aureus MB439	24.0	16.0
S. aureus MB501	16.0	12.0
S. aureus MB512	24.0	16.0
S. aureus MB535	6.0	6.0
S. aureus MB543	32.0	16.0
S. aureus MB781	64.0	32.0
S. aureus MB786	4.0	2.0
S. aureus MB798	32.0	16.0
MRSA MB508	1.0	not killed
MRSA MB628	2.0	1.0
CNS MB307	8.0	8.0
CNS MB309	48.0	24.0
CNS MB316	12.0	12.0

2.3 SL153

Microorganism	MIC values	MBC values
S. aureus (ATCC 29213)	8.0	8.0
S. aureus MB221	8.0	4.0
S. aureus MB254	12.0	8.0
S. aureus MB351	24.0	24.0
S. aureus MB390	256.0	256.0
S. aureus MB439	64.0	64.0
S. aureus MB501	96.0	96.0
S. aureus MB512	64.0	64.0
S. aureus MB535	12.0	12.0
S. aureus MB543	64.0	64.0
S. aureus MB781	64.0	32.0
S. aureus MB786	12.0	12.0
S. aureus MB798	48.0	48.0
MRSA MB508	2.0	1.0
MRSA MB628	4.0	4.0
CNS MB307	16.0	16.0
CNS MB309	64.0	64.0
CNS MB316	48.0	48.0

2.3 SL208

Microorganism	MIC values	MBC values
S. aureus (ATCC 29213)	res	not killed
S. aureus MB221	res	not killed
S. aureus MB254	res	not killed
S. aureus MB351	2.0	1.0
S. aureus MB390	256.0	128.0
S. aureus MB439	2.0	1.0
S. aureus MB501	2.0	1.0
S. aureus MB512	2.0	2.0
S. aureus MB535	res	not killed
S. aureus MB543	6.0	6.0
S. aureus MB781	1.0	1.0
S. aureus MB786	res	not killed
S. aureus MB798	res	not killed
MRSA MB508	res	not killed
MRSA MB628	res	not killed
CNS MB307	res	not killed
CNS MB309	2.0	not killed
CNS MB316	2.0	not killed

Table 2.4 MIC expressed as arbitrary units (AU/mL) of cell-free supernatants and pure nisin solution (150 µg/mL) against three sensitive *Streptococcus* spp. strains.

Strain	LL11	SL153	SL208	Nisin
S. agalactiae MB386	256.0	384.0	64.0	4096.0
S. uberis MB707	16.0	32.0	4.0	256.0
S. dysgalactiae MB280	1.5	1.0	32.0	32.0

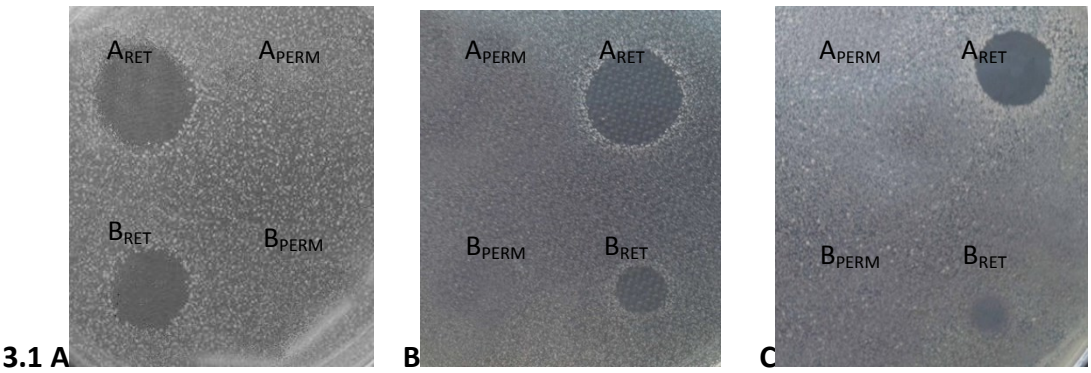
3. IDENTIFICATION OF ANTIBACTERIAL MOLECULES

Identification of antibacterial molecules produced by *L. lactis* subsp. *lactis* strains LL11, SL153 and SL208 was performed in partially purified bacteriocin extracts obtained by the adsorption-desorption method (sample B). First, the activity of these extracts was verified by the *spot-on-lawn* test. It gave positive results indicating that the antimicrobial compound was successfully extracted and purified applying this method. Subsequently, the purified bacteriocin extracts were fractionated by ultrafiltration through 1 kDa molecular weight cut-off (MWCO) ultrafiltration membrane. The partially purified extract 1 kDa ultrafiltration permeates of *Lactococcus lactis* subsp. *lactis* strains LL11, SL153 and SL208 did not show any antibacterial activity against the indicator strain (*S. agalactiae* MB386) as elucidated by *spot-on-lawn* test (Figure 3.1 A,B,C). To the contrary, the retentates A and B of all three strains maintained their antimicrobial activity indicating that the molecules, conferring antibacterial activity to these strains, are larger than 1 kDa (Figure 3.1). Ultra-filtered (1 kDa) retentates were further subjected to the SDS–PAGE analysis.

Separation of retentates A by SDS–PAGE highlighted two protein bands for each of the three samples: the smaller one of approximate molecular mass of 4 kDa and the large over-loaded spot with the approximately double lower mass limit (Figure 3.2). Retentates B did not evidentiate any band on SDS–PAGE probably due to the low concentrations of purified molecules, not detectable by Coomassie staining. Nevertheless both, sample A retentates and, to a lesser extent, sample B retentates showed the corresponding zones of inhibition after separation by the SDS–PAGE (under non-reducing conditions) when overlaid with *S. agalactiae* indicator strain in soft agar (Figure 3.2) confirming the presence of antibacterial molecules larger than 1 kDa.

Partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentates were subsequently fractionated by ultra-performance liquid chromatography (UPLC) and analyzed with photo diode array (PDA) detector and a high resolution mass spectrometer (HR-MS). Analysis of LL11 and SL153 sample B 1 kDa ultrafiltration retentates highlighted the presence of a major peak at 11.8th min in PDA (at 210 nm) chromatogram (Figure 3.3). The exact masses of the molecules corresponding to those peaks of LL11 and SL153

extracts were found to be 3353.55 Da (Figure 3.4). It is interesting to note that other two forms, differing in 16 Da and 32 Da from the main form, were present (Fig. 3.4 B and Supplementary material Figure S2), which could be attributed to the protein with one or two oxidized methionines, respectively. According the exact mass, these molecules were attributed to the Nisin A (Piper et al., 2011) and its two forms with oxidized Met^{17} and Met^{21}. *L. lactis* subsp. *lactis* strain SL208 Sample B 1 kDa ultrafiltration retentate highlighted the presence of two major peaks: at 11.8^{th} min (peak 1) and at 6.1^{th} min (peak 2) (Figure 3.3). The exact mass of the molecule corresponding to the strain SL208 partially purified extract 1 kDa ultrafiltration retentate chromatographic peak 1 was found to be 2900.25 Da (Supplementary material Figure S3), while the SL208-peak 2 indicated a molecule with the exact mass of 3867.13 (Supplementary material Figure S4). The exact masses of the molecules corresponding to the peak 1 and peak 2 were as those reported for Lacticin 481 (Piard et al., 1993) and Enterocin C2 (Maldonado-Barragán et al., 2009), respectively.



3.1

Figure 3.1 *Spot-on-lawn* test of bacteriocin extracts of *Lactococcus lactis* subsp. *lactis* strains: LL11 (A), SL153 (B) and SL208 (C) against *S. agalactiae* MB386. A_{PERM}, sample A (CFS) 1 kDa ultrafiltration permeate; A_{RET}, sample A 1 kDa ultrafiltration retentate; B_{PERM}, sample B (pre-purified bacteriocin extract) 1 kDa ultrafiltration permeate; B_{RET}, sample B 1 kDa ultrafiltration retentate.

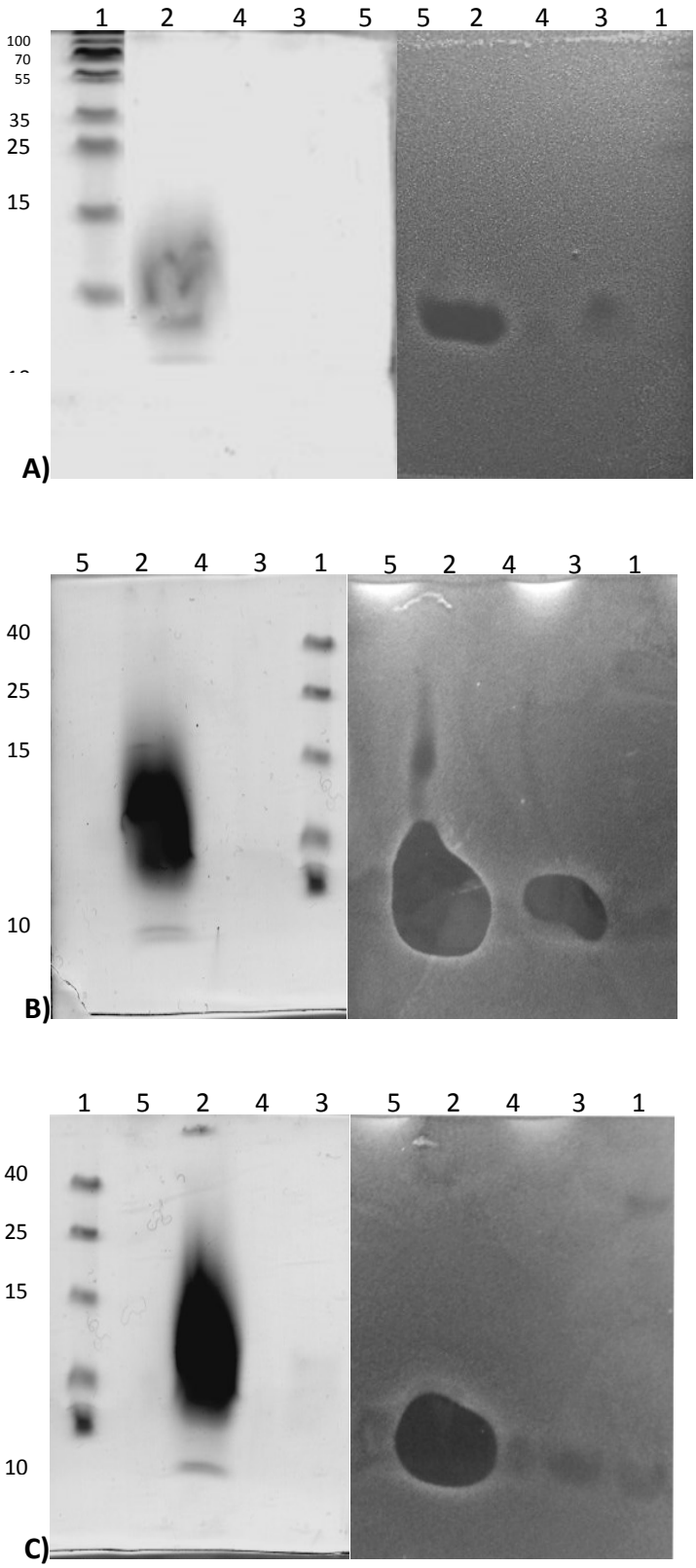


Figure 3.2 SDS–PAGE separation of fractionated (UF 1 kDa) bacteriocin extracts (left) and their inhibitory activity against *S. agalactiae* MB386 (right). *Lactococcus lactis* subsp. *lactis* strains: A) LL11; B) SL153; C) SL208. Lanes: 1, protein molecular weight ladder; 2, retentate A; 3, retentate B; 4, permeate A; 5, permeate B.

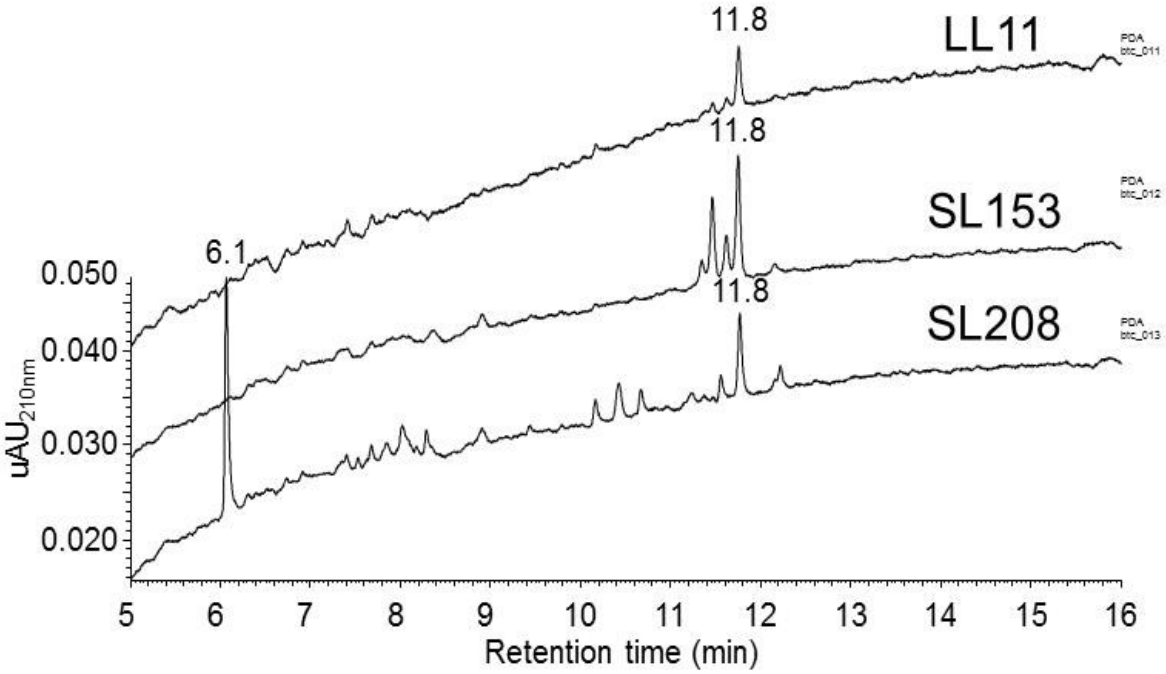


Figure 3.3 PDA (210 nm) chromatograms of *L. lactis* subsp. *lactis* strain LL11, SL153 and SL208 partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentates fractionated by UPLC.

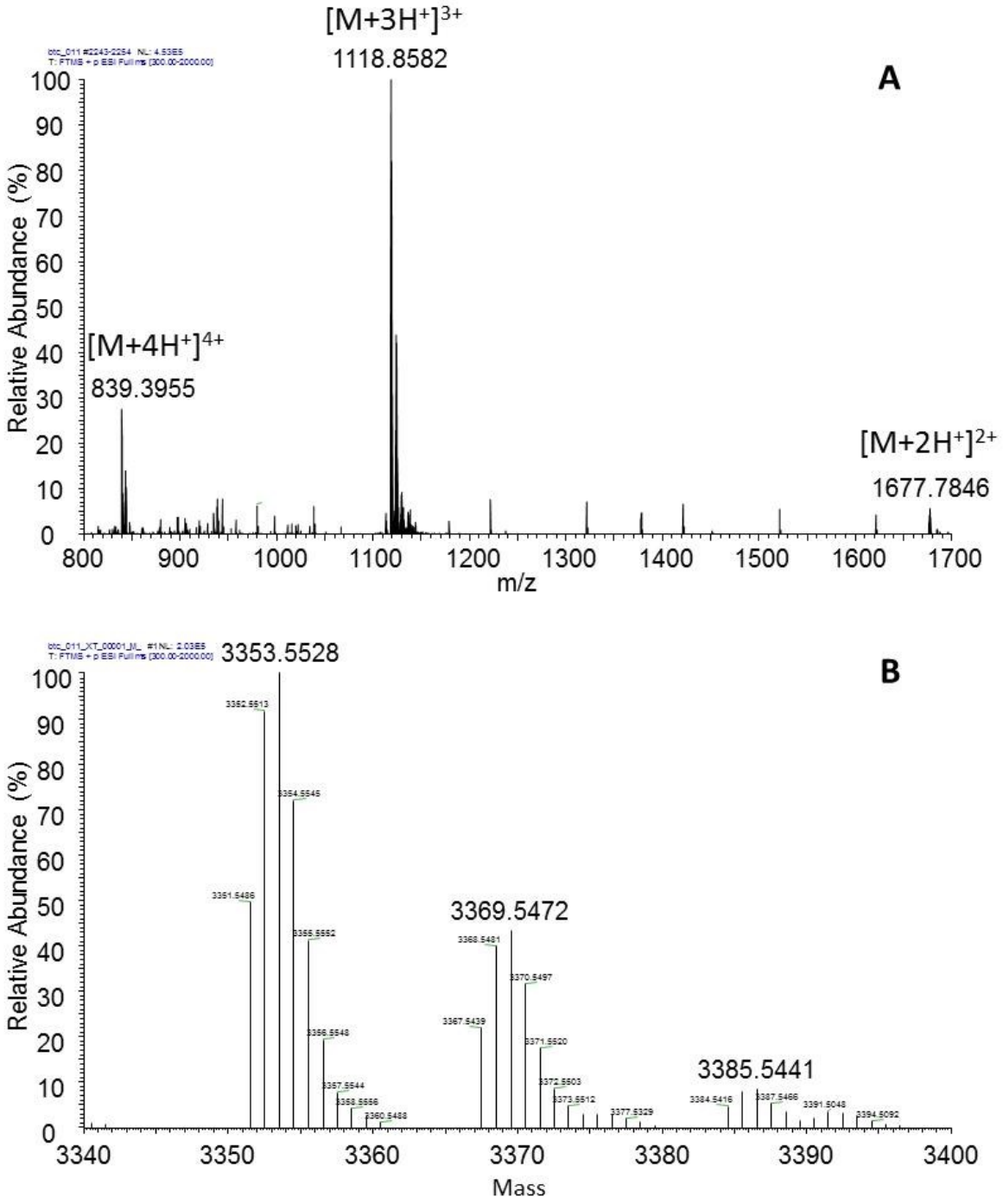


Figure 3.4 HR-MS spectrum (A) and deconvoluted MS spectrum (B) of *L. lactis* subsp. *lactis* LL11 partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentate peak eluting at 11.8 min.

In two recent studies, the efficacy of nisin in the treatment of clinical and subclinical mastitis was compared with that obtained with antibiotic administration. Clinical mastitis caused by *S. aureus* or *S. agalactiae* (Cao et al., 2007) and subclinical mastitis by *S. aureus*, coagulase-negative staphylococci, *S. agalactiae*, *S. dysgalactiae* or *S. uberis* were considered (Wu et al, 2007). Both studies concluded that administration of nisin was effective in the treatment of mastitis caused by several mastitis pathogens in lactating dairy cows. Nisin was also tested for its efficacy in a post-dipping formulation: Ambicin® N showed a reduction of *S. aureus* (62%), *S. agalactiae* (99%) and *S. uberis* (67%) in experimentally challenged teat surfaces (Sears et al., 1992). In our study, a considerable variability of bacteriocin sensitivity was observed both among the species and in the different strains belonging to the same species. According to results obtained by Cao et al. (2007) none of the strains tested were resistant to nisin-containing CFS. Nevertheless, of 13 *S. aureus* strains, 60% were resistant to β-lactams, 15% showed resistance to fourth generation cephalosporin and 15% were MRSA and were also resistant to amoxicillin enhanced by clavulanic acid. Our results are also in agreement with Wu et al. (2007), who observed the same high variability in the MIC of *S. aureus* after nisin treatment. Furthermore, we confirmed the sensitivity of MRSA strains to nisin (Piper et al., 2009) in bovine isolates. Despite variability of sensitivity intra species, the high MIC values reached against *S. agalactiae, E. faecalis and S. uberis* confirmed the marked sensitivity of streptococci to nisin (Klostermann et al., 2009). Considering the antibacterial activity of the two nisin producer strains LL11 and SL153, a general higher activity could be observed for the SL153 CFS, indicating higher amount of nisin produced. The comparison of sensitivity observed for both streptococcal and staphylococcal strains suggested a dose-dependent sensitivity for the pathogens. Nevertheless, *Streptococcus dysgalactiae* MB280 and MB324 strains had surprisingly the capability to resist to nisin activity. To the contrary, due to the higher sensitivity to SL208 CFS, we hypothesized the presence of Enterocin C2, which is a class II bacteriocin with a different mode of action. Thus, it could be explained why a completely different pattern of susceptibility was shown for all the pathogens when exposed to SL208 CFS. Sparo et al. (2006) observed a sensitivity of mastitis isolates to enterocin MR99 and concluded that this bacteriocin could offer a therapeutic alternative to antibiotics in mastitis treatment.

3.1. L. lactis *subsp.* lactis *SL208 genome sequencing, identification and analysis of bacteriocin-related genes*

As demonstrated above, *L. lactis* subsp. *lactis* SL208 antibacterial extract analysis showed the presence of two potentially antibacterial proteins with exact masses corresponding to those of already known bacteriocins: Lacticin 481 and Enterocin C2. In order to verify the presence or the absence of Lacticin 481 and Enterocin C2-encoding genes in *L. lactis* subsp. *lactis* SL208, a complete genome sequencing was performed: 1,533,479 paired sequences were generated with a mean length of 184 bases per read. Genome was assembled resulting in 113 large contigs (>1000 nt), a statistic value associated with contig lengths (N50) of 133,223 and an average coverage of 64.53×, for a total of 2,657,411 bp, with a G+C content of 64.17%. After genome annotation, 2,657 protein-coding genes were obtained.

The presence of bacteriocin-encoding genes was further investigated (Table 3.1.1). DNA sequence analysis revealed the presence of Lacticin 481 (Lactococcin-DR)-encoding gene (PROKKA_01892; Table 3.1.1). The calculated exact mass of the protein encoded by this gene, corresponded to the protein peak (Peak 1) exact mass identified by UPLC/HR-MS analysis (2900 Da) and was attributable to the previously observed average mass of Lacticin 481 (Piard et al., 1993; Uguen et al., 2005).

Table 3.1.1 Bacteriocin-encoding genes (and corresponding proteins with calculated monoisotopic masses) found after genome sequencing of *L. lactis* subsp. *lactis* SL208. Signal peptides (SP) are indicated in red and putative SP in grey.

No.	Contig	Proteins and their amino acid sequences	Calculated monoisoto-pic mass
1	C4	>PROKKA_01529 Lactococcin-like family Pfam:PF04369.7 protein = hypothetical protein [*Lactococcus lactis*], WP_021214955, /note="Lactococcin-like family; pfam04369" MKNQLNFEVV SDEELLTTSG GQNSQQGEGG GYGSSNDTWG G	4277 Da
2	C4	>PROKKA_01535 Lactococcin-like family Pfam:PF04369.7 protein = hypothetical protein [*Lactococcus lactis*], WP_021214958, /note="Lactococcin-like family; pfam04369" MKNQLNFEVV SDEELMTING GQNMSMTDGG FEWVYAGGKP WFRIV	5124 Da
3	**C4**	>PROKKA_01540 Lactococcin-like family Pfam:PF04369.7 protein = hypothetical protein [*Lactococcus lactis*], WP_023163574, /note="Lactococcin-like family; pfam04369" MENQLNFEVI IDEELEKISG GYLPIPDMPG WRGQSTPWWW SLKQSNFSDA YSSFYNATH	**3937 Da (without predicted SP)**
4	C4	>PROKKA_01547 Lactococcin-like family Pfam:PF04369.7 protein = hypothetical protein [*Lactococcus lactis*], WP_003132257, /note="Lactococcin-like family; pfam04369" MENRLNFEAI SDDELAKIVG GGYPNNQSMN DVLHWLNGHN DGNPKQLPKW MGGLG	3788 Da (without predicted SP)
5	C8	>PROKKA_02390 bacteriocin, lactococcin 972 family = bacteriocin [*Lactococcus lactis*], WP_025016835, /note="Bacteriocin (Lactococcin_972)" MQTKKLLVST LILATLGGTL LQVSPVFAIN RSTYSQGSTN DKKYGMGAYA AYWNSYGNHW AEVTYGDKYG GRVVSVHANQ QAYAWLNTRW AEPATFYHSN GWVGTRSW	9474 Da (without predicted SP)
		Similar to: bacteriocin lactococcin 972 family Lactococcin 972, lcn972, O86283: MKTKSLVLAL SAVTLFSAGG IVAQAEGTWQ HGYGVSSAYS NYHHGSKTHS ATVVNNNTGR QGKDTQRAGV WAKATVGRNL TEKASFYYNF W	7377 Da (without SP)
6	**C43**	**>**PROKKA_01892 Lactococcin-DR (= Lacticin 481, lctA, P36499) MKEQNSFNLL QEVTESELDL ILGAKGGSGV IHAbuIAHEANM NAWQFVFDhbAA S	2900 Da (without SP; post-translationally modified)

Five other bacteriocin-encoding genes were found in the genome of the *L. lactis* subsp. *lactis* strain SL208. Surprisingly, none of these genes showed annotation similarity to Enterocin C2 encoding gene (*entC2*). Enterocin C2 (Acc. No. ACJ54160: region_name= "LcnG-beta", note= "Lactococcin G-beta; pfam11632"; part of *Enterococcus faecalis* strain C9901 plasmid pENTC, EU862242) is a non-modified plasmid-encoded protein synthetized as pre-bacteriocin with a consensus double-glycine leader sequence (Table 3.1.2). Pre-protein (consisting of a signal peptide of 24 amino acids and a mature protein of 35 amino acids) monoisotopic mass was calculated to be 6447 Da, while mature bacteriocin Enterocin C2 (the length of 35 amino acids) calculated monoisotopic mass was 3867 Da. Experimentally measured exact mass of Enterocin C2 was found to be 3867 Da (Maldonado-Barrágan et al., 2009) which corresponded to the exact mass measured for a *L. lactis* subsp. *lactis* SL208 sample B UF 1 kDa retentate peak 2 protein.

Table 3.1.2 Amino acid sequence of Lactococcin G- β subunit, Enterocin C2 and *L. lactis* subsp. *lactis* SL208 PROKKA_01540 pre-peptides deduced from their coding DNA sequences (signal peptides are indicated in red and putative predicted signal peptide in grey). Molecular weights of mature proteins (MW mp) are indicated.

Protein	Amino acid sequence	MW mp	Accession number
Lactococcin G β subunit	*MKNNNNFFKGMEIIEDQELVSITGG*KKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI	4110 Da	ACR43770
Enterocin C2	*MKNIKNASNIKVIEDNELKAITGG*GPGKWLPWLQPAYDFVAGLAKGIGKEGNKNKWKNV	3867 Da	WP_ 032492334
Lactococcin-like protein (PROKKA_01540)	*MENQLNFEVI IDEELEKISGGYLPIP*DMPGWRGQSTPWWWSLKQSNFSDAYSSFYNATH	3937 Da	WP_ 023163574

In addition, none of the other five bacteriocin-related genes, identified after genome sequencing of *L. lactis* subsp. *lactis* SL208, had a similar calculated mass corresponding to that measured for the protein of peak 2 by UPLC/HR-MS (3867 Da). Due to *entC2* plasmidic localization, the possibility that incomplete sequencing of plasmid and thus the

lack of matching with the reference gene should be considered. However, the examination of the bacteriocin-encoding genes highlighted the presence of a gene (PROKKA_01540; Table 3.1.1) belonging to lactococcin-like family, which putatively encodes a protein with a calculated mass of 3937 Da. Detailed analysis of this sequence showed that PROKKA_01540 gene potentially encodes a peptide of 59 amino acids, containing the double-glycine leader sequence that probably, after processing, could give a mature peptide of 33 amino acids. Summing up, after the potential cleavage of the leader peptide (calculated with provisional models) the mature protein would be of 3937 Da, thus 70 Da larger than that corresponding to SL208 peak 2. Post-translational modifications or amino acid replacements could explain the difference of molecular weight found through UPLC/HR-MS analysis and only then the 3867 Da protein identified in the antibacterial extract of *L. lactis* subsp. *lactis* SL208 peak 2 could be potentially attributed to the PROKKA_01540 mature transcript but not to any other SL208-encoded protein (taking into account that the genome sequencing coverage was sufficient to reject the hypothesis of an incomplete coverage). Furthermore, the bacteriocin Enterocin C2 is highly homologous to the other lactococcin-like family proteins (Balla et al., 1999) and, as demonstrated in this study, also to the protein encoded by PROKKA_01540 (Figure 3.1.1).

Figure 3.1.1 Amino acids sequence alignment of Lactococcin G, Enterocin C2 and SL208 Lactococcin-like protein (ClustalW 2.1 multiple sequence alignment). *, identical amino acids.

```
Lactococcin G ACR43770         MKNNNNFFKGMEIIEDQELVSITGGKKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI 60
Enterocin C2 WP_032492334      MKNIKNASN-IKVIEDNELKAITGGGPGKWLPWLQPAYDFVAGLAKGIGKEGNKNKWKNV 59
                               *** :*  : :::***:** :****    **.*::***:*: *:.**  *****:****:

Lactococcin G ACR43770          MKNNNNFFKGMEIIEDQELVSITGG--KKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI- 60
SL208 Lactococcin-like protein  MENQLNF----EVIIDEELEKISGGYLPIPDMPGWRGQSTPWWWSLKQSNFSDAYSSFYNATH 59
WP_023163574                     *:*: **    *:* *:** .*:**     .  .* . :  :  .: :. :.:. .. ::

Enterocin C2 WP_032492334      MKNIKNASNIKVIEDNELKAITGG-GPGKWLP-WLQPAYDFVAGLAKGIGKEGNKNKWKNV- 59
SL208 Lactococcin-like protein ---MENQLNFEVIIDEELEKISGGYLPIPDMPGWRGQSTPWWWSLKQSNFSDAYSSFYNATH 59
WP_023163574                      ::*  *::** *:**: *:**  *   :* *   :  :  .* :.  .:. .. :: .
```

They are a class II bacteriocins, and many of these bacteriocins had shown to share part of their sequences, indicating the fundamental role of these regions for bacteriocin activity (Nissen-Meyer et al., 1992). Finally, peak 2 purification, confirmation of its

antibacterial activity and a detailed proteomic analyses are further necessary in order to attribute it to any of the *L. lactis* bacteriocins.

Summing up, the investigation of bacteriocin-related genes of *L. lactis* subsp. *lactis* SL208 genome demonstrated the presence of genes encoding Lacticin 481 and one more protein with likely antibacterial properties not yet characterized among the species *L. lactis* antibacterial molecules. The emergence of antibiotic resistant strains make the discovery of new antibacterial molecules very attractive for researchers (Dicks et al., 2011). Furthermore, due to the safety and use in food grade products, the species *L. lactis* is considered advantageous to harbor bacteriocin-encoding genes (Rodriguez et al., 2003). On the other hand, despite the activity of SL208 CFS against streptococcal strains, the lower activity against *S. aureus* and CNS made the SL208 bacteriocins less attractive for further application in bovine mastitis.

4. INTERACTION OF BACTERIOCINS OR LIVE LACTOCOCCAL CULTURES WITH MAMMARY EPITHELIAL CELLS

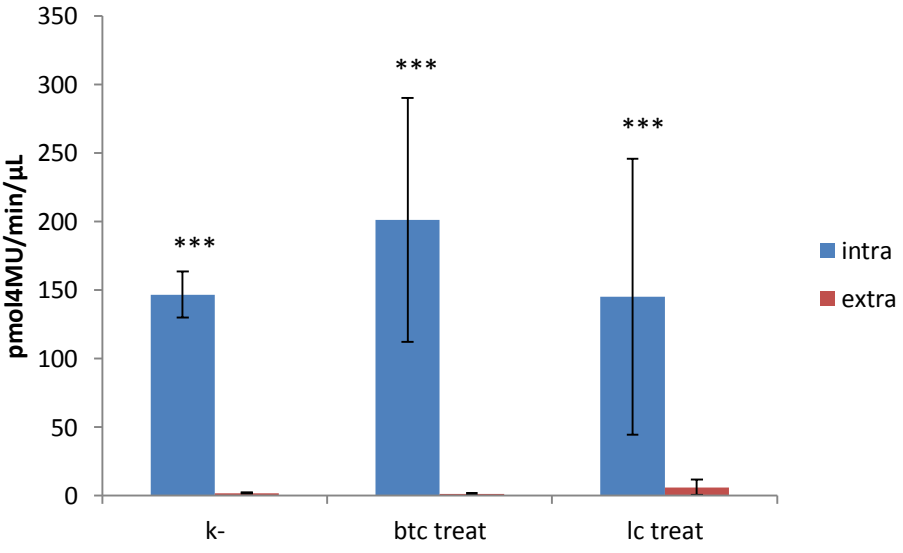
Before to stimulate BME-UV1 cells, the influence of fetal calf serum (FCS) on N-acetil-β-D-glucosaminidase (NAGase) and Lysozyme (LZ) activity was analyzed: the monolayers were maintained for 24h in the medium with or without FCS and then intracellular and extracellular activity of both enzymes were measured. The results showed that FCS acted as stimulus for enzymes production, in particular NAGase release was higher with increasing FCS concentrations (data not shown). On the other hand, active cellular metabolism was shown in the cells cultured without FCS, as confirmed by the comparison with intracellular levels obtained from cells cultured with FCS: similar intracellular amounts could be observed in all cultures with or without FCS. When lysozyme was considered, enzyme activity was stable in the course of observation period, demonstrating a basal LZ production by mammary epithelial cells in the presence or absence of FCS.

The production of NAGase and LZ by BME-UV1 demonstrated the capability of these cells to respond to an inflammatory stimulus, as it happens in the *in vivo* contest. In order to prevent any stimulation by medium components, we decided to add CFSs or live lattococci to BME-UV1 in the culture medium without FCS. SL208 strain was used only as

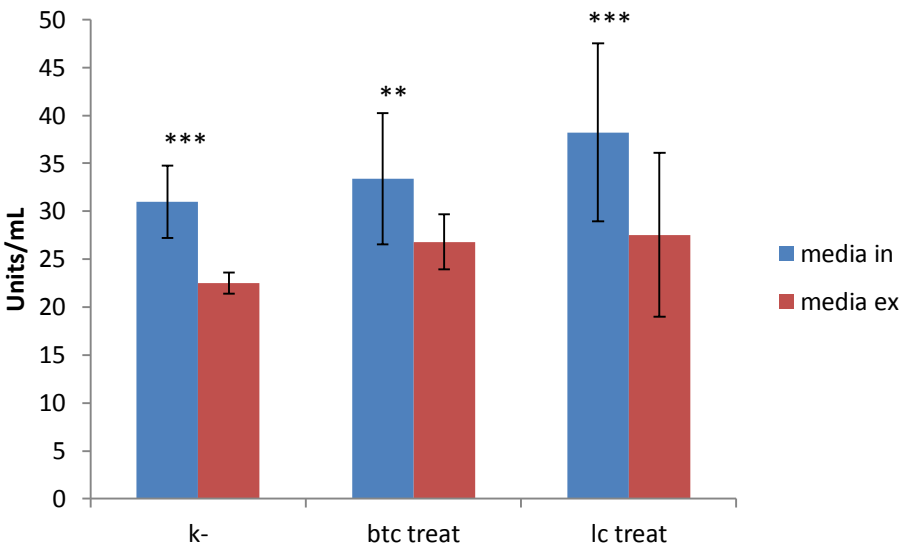
live culture, due to the lack of CFS activity against pathogens when the LAB was cultured in the cell medium. Before the addiction of LL11 and SL153 CFSs to the cells, their antimicrobial activity was assayed with the MIC test and the production of bacteriocins was confirmed by the growth inhibition of the *S. agalactiae*MB386 reference strain: LL11 and SL153 bacteriocins showed 128 and 256 AU/mL respectively.

4.1. N-acetil-β-D-glucosaminidase and Lysozyme assays

To observe the differences in enzymes production and release, NAGase and LZ activity were investigated in the intracellular and extracellular compartment. Figure 4.1 shows the comparison of extracellular release and intracellular storage of NAGase and LZ in untreated and treated cells. A statistically significant difference was observed for both enzymes between the intracellular and extracellular compartment, with higher amounts of enzymes stored in the cells. As expected, extracellular amounts of enzymes were never higher than intracellular ones, since the enzymes are produced and contained into the cells and secreted if an inflammatory stimulus occurs (Kitchen et al., 1981; Ebling et al., 2001, Zecconi and Smith, 2003). Furthermore, the higher activity of lysosomal enzymes in the intracellular compartment of treated cells suggested that cell integrity was uninfluenced by treatments.



A)



B)

Figure 4.1 Intracellular enzymes production and extracellular release in untreated (k-) CFS-treated (btc treat) and live lactococcal cultures-treated (lc treat) cells. Mean values and standard deviations of: A) NAGase (expressed in pmol 4MU/min/Ml) and B) LZ (expressed in Units/mL).

*P<0.05; **P<0.01; ***P<0.001

For the statistical analysis, the first two time points (4h and 8h) were gathered, as well as the last two (15h and 24h) in early and late observations respectively. Figure 4.2 shows the intracellular NAGase activity: the lack of statistical significance indicated that the enzyme activity remained unchanged over the time and unaffected by the treatments. Although lower amount for LL11 treated cells and a noticeable standard deviations especially in the late time of the study, mean intracellular NAGase remained always higher than the extracellular one (Figures 4.2 and 4.3). These results suggested that an active cellular metabolism was maintained by the cells during the trial.

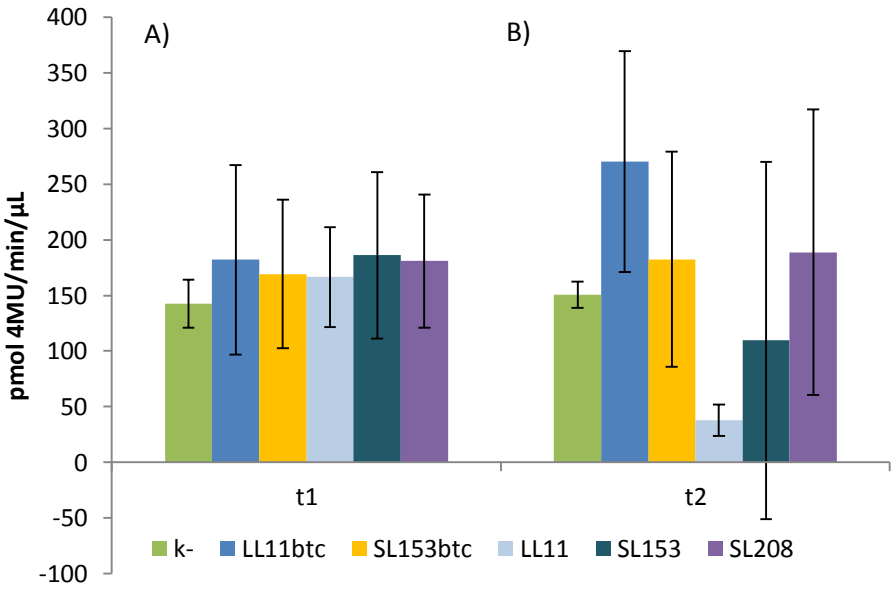


Figure 4.2 Intracellular NAGase activity in BME-UV1 cells treated with nisin containing-CFSs (LL11btc and SL153btc) or *Lactococcus* live cultures (LL11, SL153 and SL208), in the A) early observations (t1) and B) late observations (t2) of experiment.

To the contrary, excretion of NAGase was influenced by time (P<0,001) and treatments (P<0,001). Due to the statistically significant difference between the two observations, the post-hoc test was performed separately for each observation time: in the early observation no statistically significant difference was observed when treated cells were compared with the control, but a difference was demonstrated among the treatments with live culture lactococci (LL11, SL153 and SL208) and the treatments with nisin-containing CFS (LL11btc and SL153btc). In particular, the secretion of NAGase was higher when the cells were treated with the live LL11 strain, rather than with its CFS (P=0,048). SL208 live strain-treated cells showed higher amount of extracellular NAGase when compared with LL11 and SL153 CFS treatments (P=0,021 and 0,046 respectively), indicating an early stimulus for NAGase secretion by live cells but not by nisin-containing CFSs. This result was more evident after 15-24h of incubation, when the cells treated with LL11 and SL153 showed a 5-fold increment in the release of NAGase (P=0,013 and P=0,007 respectively) in comparison with both untreated cells and bacteriocin-treated cells (Figure 4.3).

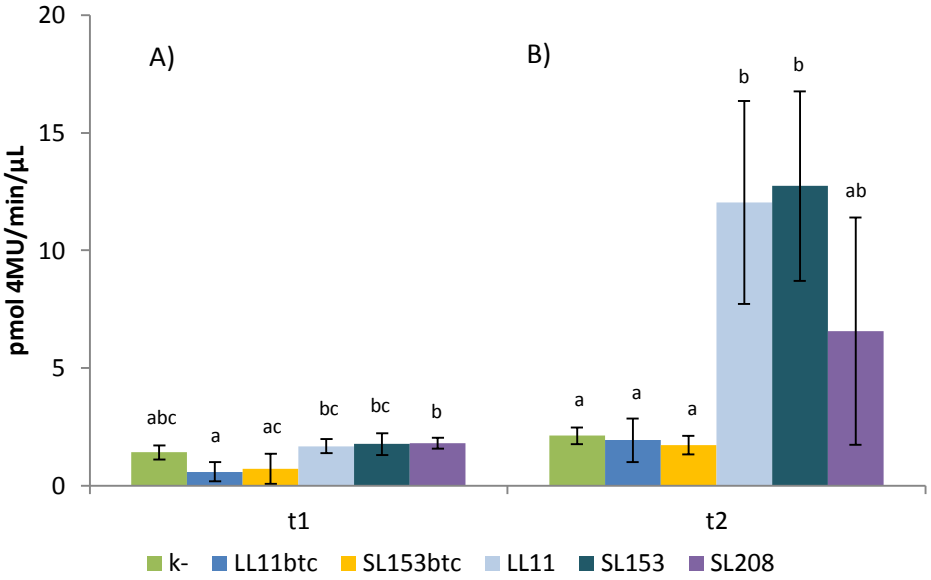


Figure 4.3 Extracellular NAGase concentration in BME-UV1 cells treated with nisin containing-CFSs (LL11btc and SL153btc) or *Lactococcus* live cultures (LL11, SL153 and SL208), in the A) early observations (t1) and B) late observations of experiment (t2).

[a, b, c] Different letters indicate significant difference in enzyme production between treatments.

These data suggested a slight inflammatory response of epithelial cells induced by live lactococcal cultures, in particular LL11 and SL153 strains.

NAGase is mainly released from neutrophils during phagocytosis and cell lysis, it is considered a marker of tissue damage (Forman et al., 1996) but its increment is also associated to an increased lysosomal activity (Bosomworth et al., 1999). Some studies demonstrated that NAGase activity in milk increased both in clinical and subclinical mastitis (Akerstedt et al.,2012), thus this enzyme can be used as an indicator of mammary inflammation and immunity response activation.

Similarly to NAGase, LZ intracellular accumulation was not influenced by the treatments or time: as showed in figure 4.4, similar amounts of intracellular enzyme were detected in the treatments and over the time. These results confirmed that cellular metabolism and lysosomal activity were maintained by the cells during the trial.

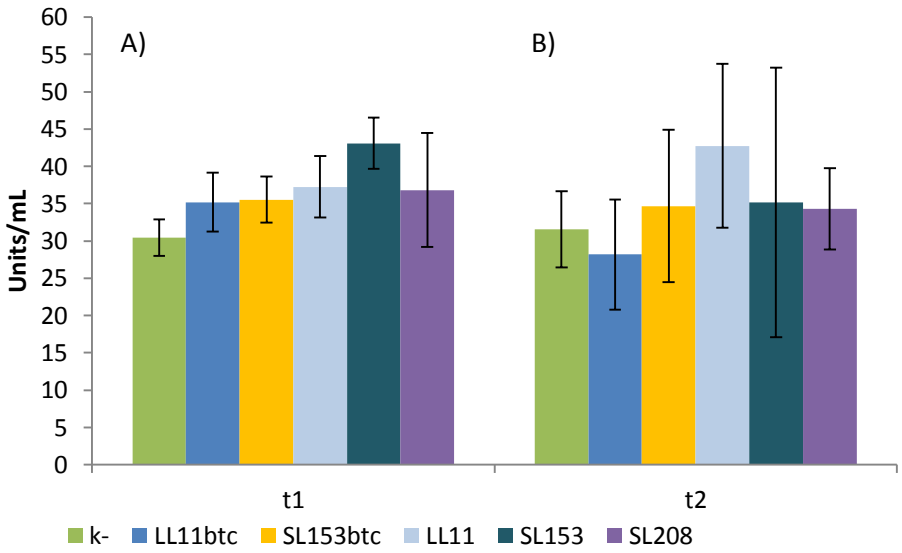


Figure 4.4 Intracellular LZ in BME-UV1 cells treated with nisin containing-CFSs (LL11btc and SL153btc) or *Lactococcus* live cultures (LL11, SL153 and SL208), in the A) early observations (t1) and B) late observations (t2) of experiment (t2).

A completely different pattern was observed for extracellular LZ, which activity was statistically significant influenced by time (P<0,001) and treatments (P<0,001). Due to the statistically significant difference between the two observations, the post-hoc test was performed separately for each observation time. In the early times (4h and 8h) of the experiment, LZ showed similar amounts among the cells treated with live lactococcal strains; unexpectedly, LZ activity incremented significantly when cells were treated with the nisin-containing CFSs. This increase respect to the control was statistically significant (P=0,021 and P=0,005 respectively). To the contrary, after 15h, the extracellular activity of this enzyme was higher in the cells treated with SL208 and LL11 live cultures. In particular, LL11 treated cells evidenced a statistically significant increment (P<0,001) in LZ activity that was almost duplicated (Figure 4.5).

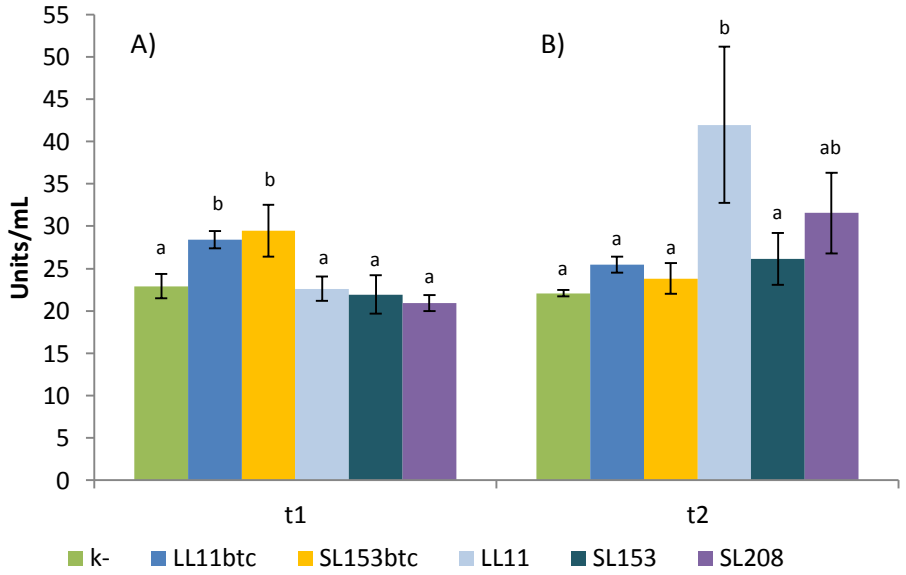


Figure 4.5 Extracellular LZ in BME-UV1 cells treated with nisin containing-CFSs (LL11btc and SL153btc) or *Lactococcus* live cultures (LL11, SL153 and SL208), in the A) early observations (t1) and B) late observations (t2) of experiment.

[a, b, c] Different letters indicate significant difference in enzyme production between treatments.

These results confirmed the stimulation of lysosomal activity by lctococcal strains and, to a lesser extent by nisin-containing CFSs, sustained by unaltered amount of both enzymes in the intracellular compartment. Although these enzymes increase their activity when an inflammation occurs (Sarikaya et al., 2006), it has been shown that in the absence of an inflammatory stimulus, the somatic cell count (SCC) is not correlated with NAGase and LZ amounts (Piccinnini et al., 2007). Thus, when SCC levels are low, the primary source of innate immunity factors could be udder tissues (Bruckmaier, 2005). Therefore, the role of epithelial cells should be considered in a preventive approach of mastitis. The stimulation of enzymes secretion due to the administration of lactococci or their antibacterial products, with consequently potential enhancement of pathogens cleaning, can be of interest for the prevention of intramammary infections.

4.2. Cytokine expression

The epithelial cells have both a sentinel and an effector role and they are well suited to exert these functions due to their abundance and exposed position to invading microorganisms (Günther et al., 2010). BME-UV1 cells express immunological and inflammatory molecules (Mazzilli and Zecconi, 2010), furthermore it has been demonstrated that they produce proinflammatory cytokines upon stimulation with antimicrobial peptides (Tomasinsig et al., 2010). TNFα is long known to elicit both local and systemic immune response (Pfeffer, 2003), while IL-8 is a key factor for recruiting effector cells of immune defense (polymorphonuclear granulocytes, PMN) into the udder (Toshihide, 2003). We examined the effect of bacteriocinogenic lactococcal-strains (LL11, SL153 and SL208) and the two nisin-containing CFS on transcriptional levels of those cytokines in BME-UV1 cells.

Figure 4.6 shows the expression of TNFα as a result of the five different treatments. BME-UV1 response to nisin-containing CFS (LL11btc and SL153btc) was different: cells treated with LL11 CFS had a negative expression in comparison with the control at all the time-points, while cells treated with SL153 CFS showed lower levels of TNFα until 15h and a positive expression at 24h of exposure. The increment of TNFα transcription levels in epithelial cells after antimicrobial peptide stimulus was previously observed in a study by Tomasinsig et al. (2010), which concluded that enhanced TNFα expression triggered the activation of other immune system components. Indeed, the moderate increment induced by SL153 CFS is more similar to modulation of immune response, than to activation of acute phase response. The differences of TNFα stimulation observed between SL153 and LL11 CFSs could be attributed to the different amount of nisin contained in the CFSs, as elucidated during pathogens sensitivity tests. For the 3 live culture treatments, a decreasing tendency was overall demonstrated, with lower levels of expression compared to the control.

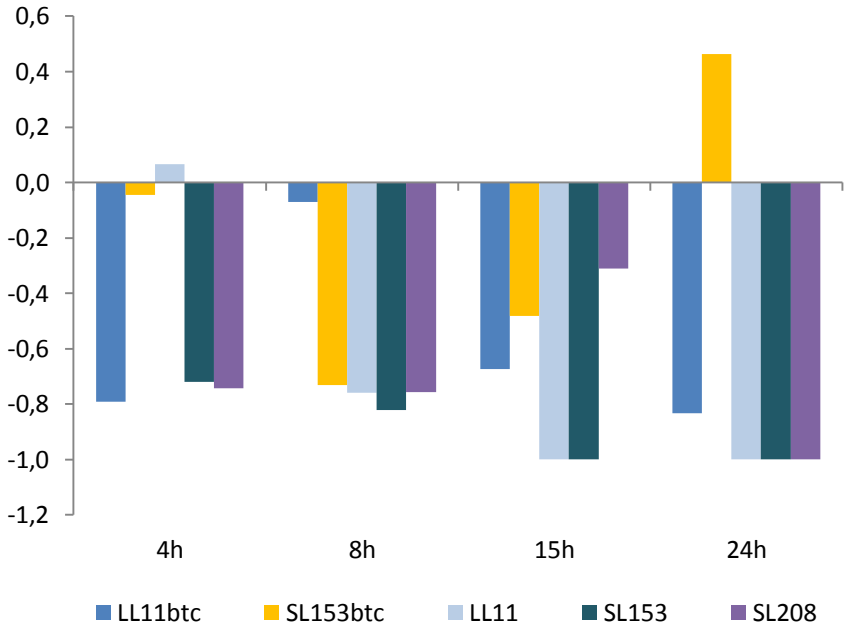


Figure 4.6 TNFα expression (normalized fold expression relative to the control) in BME-UV1 cells treated with nisin containing-CFSs (LL11btc and SL153btc) or *Lactococcus* live cultures (LL11, SL153 and SL208). The data are the means of two independent experiments displayed as difference to the control.

Since the statistical analysis did not show any significant effect of time on TNFα expression, the data were analysed grouping all time points (Figure 4.7), to observe the effect of the single treatment on the expression levels. That analysis highlighted that SL153 live culture treated cells reduced significantly the expression of TNFα in comparison with untreated cells, independently of treatment time.

These results are in agreement with Luerce et al. (2014), who showed the ability of live *Lactococcus lactis* culture to reduce TNFα response in stimulated intestinal epithelial cells and also to inhibit IL-8 production when the culture supernatant was applied. They observed also that the immunomodulatory response was strain-dependent. Moreover, Jensen et al. (2014) observed the strain-specific induction of pro-inflammatory cytokines by *Lactobacillus* species. They also suggested that live culture LABs have the ability to secrete pro-inflammatory and anti-inflammatory mediators.

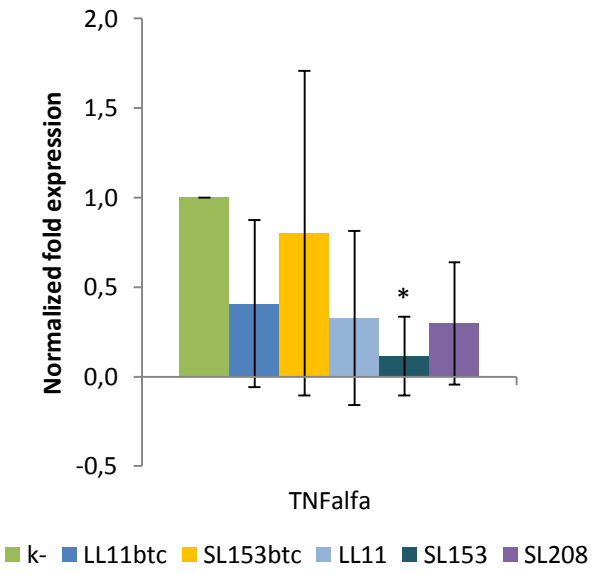


Figure 4.7 Expression levels of the TNFα gene in BME-UV1 untreated (k-) and treated cells. Data are showed as means ± standard deviations (SD) of all time point-measures.

IL-8 expression (Figure 4.8) showed a negative, but not significant trend in the cells treated with SL153 or LL11 CFSs.

Also live culture treatments demonstrated negative levels of IL-8 expression, indicating a lower expression in comparison with the control. Only LL11 treated cells showed a positive relative expression at 15h with 7 fold-changes over the untreated cells, followed by a drastic drop in the last time point measurement.

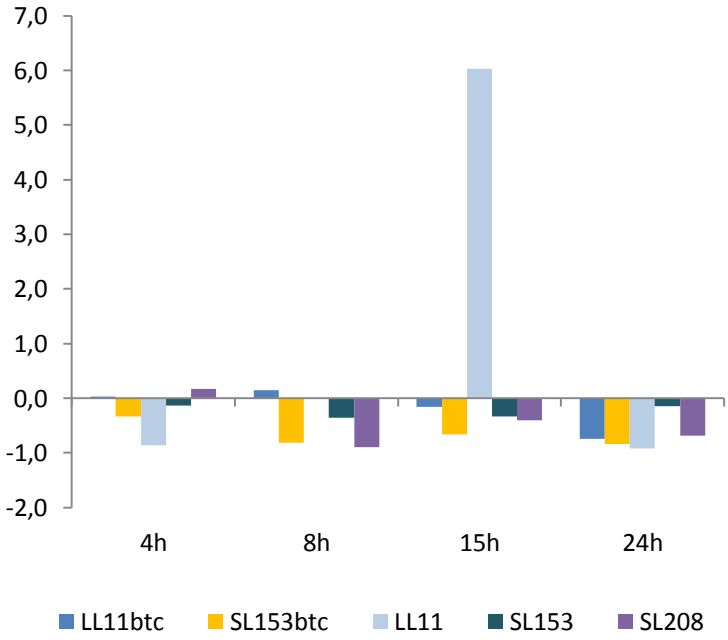


Figure 4.8 IL-8 expression (normalized fold expression relative to the control) in BME-UV1 cells treated with nisin containing-CFSs (LL11btc and SL153btc) or *Lactococcus* live cultures (LL11, SL153 and SL208). The data are the means of two independent experiments displayed as difference to the control.

When the four time points were grouped, no statistically significant differences were observed between treatments and control, with obvious high standard deviation observed in the expression of LL11 live culture-treated cells (Figure 4.9).

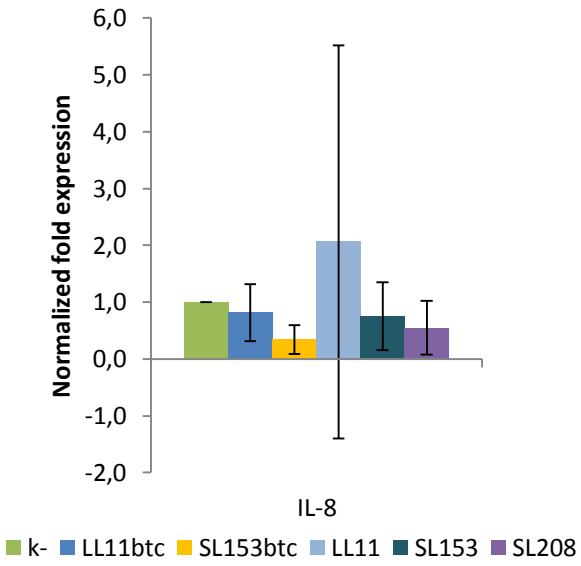


Figure 4.9 Expression levels of the IL-8 gene in BME-UV1 untreated (k-) and treated cells. Data are showed as means ± standard deviations (SD) of all time point-measures.

TNFα is a proinflammatory cytokine stimulating the acute phase reaction and the local immune response. Although these local and systemic effects are beneficial to stimulate the host innate immune defense against infections (Bannerman, 2014), TNFα causes the classical signs of inflammation and is associated with severe symptoms which characterize *E. coli* mastitis (Alluwaimi, 2004). Thus, on one hand TNFα increment could be desirable to ensure the activation of immune response, but on the other hand the control of TNFα release could be an interesting approach to reduce the symptoms linked to clinical intramammary infections. Furthermore, it was demonstrated the capability of TNFα to suppress lactogenic function of the glands after infusion (Alluwaimi, 2004). Indeed, it may be essential for the mammary gland to keep the expression of TNFα under rigid control, because it has an autocrine effect inhibiting the synthesis of caseins by MEC (Shea-Eaton et al., 2001). There is also the evidence that both bacterial and host immune factors contribute to tissue damage during mastitis with consequent epithelial cells apoptosis and decreased milk production (Zhao and Lacasse, 2008). The role of cytokines in tissue damage is under investigation and while the therapeutic potential of certain cytokines has been proposed (Alluwaimi, 2004) it has been also demonstrated that TNFα and IL-1 induce apoptosis in different cell types (Zhao and Lacasse, 2008). TNFα infusion was also evaluated to enhance intracellular killing of *S. aureus* by mammary gland-derived

neutrophils and macrophages, but this approach failed its purpose (Sanchez et al., 1994). The cytokine-mediated recruitment of neutrophils could be of interest to enhance phagocytosis, but PMN also release factors which are responsible for tissue damages. Therefore, to protect mammary tissues from oxidative stress due to PMN recruitment during mastitis, intramammary treatments often contain anti-inflammatory components (Klostermann et al., 2008).

Due to their high antibacterial activity and their ability to stimulate lysosomal metabolism, SL153 and LL11 *Lactococcus lactis* strains could be of interest for the development of intrammary probiotic treatments. In addition, SL153 strain could be advantageous for its potential anti-inflammatory properties. Furthermore nisin-containing CFSs should be considered as alternative treatments to achieve bacterial cleaning and stimulate LZ secretion without any cell damage or pro-inflammatory action.

CONCLUSIONS

Since bacteriocins produced by LAB display antimicrobial activity against a broad range of Gram-positive bacteria and, to a lesser extent, against Gram-negative bacteria, they are attracting considerable interest for their potential use as natural and non-toxic antimicrobial molecules (Cotter et al., 2005; Deegan et al., 2006). Bacteriocins may be useful in human and veterinary applications, therefore they could have an interesting development in those preparations requiring safety, residual absence and antimicrobial efficacy combination. From a clinical perspective, the emergence of drug-resistant pathogens, both in medical treatments and animal production makes the identification of novel antimicrobial tools even more important (Cintas et al., 2011). A considerable number of bacteriocins have already been described in literature but few studies have been performed concerning application of bacteriocins produced by *Lactococcus* spp. in veterinary medicine. Safety and efficacy of molecules produced by *Lactococcus* spp. encourage the efforts in the development of new formulations for mastitis prevention and therapy. Moreover, the absence of residues in the milk of treated cows meets the increasing request for reduced use of antibiotics and safety and natural food.

Accordingly to the proposed tasks of this study the following conclusions can be drawn:

1. The best culture conditions to optimize LAB bacteriocin production were defined and the acid-extraction method was shown to efficiently extract their antibacterial proteins.
2. Antibacterial *spot-on-lawn* is an optimal test to screen large numbers of strains, and to select bacteriocinogenic strains, while MIC assay can be successfully applied to determine their antibacterial potency.
3. The efficacy of *Lactococcus lactis* subsp. *lactis* LL11, SL153 and SL208 strains was demonstrated against mastitis pathogens.
4. *Lactococcus lactis* subsp. *lactis* LL11 and SL153 were found to produce an antibacterial protein attributable to Nisin A according its exact mass. The strain SL208 potentially produced Lacticin 481 and a novel molecule with likely antibacterial activity. Detailed proteomic analyses are further necessary to definitely identify this molecules.

5. Both live culture strains and their CFSs, displayed the capability to interact *in vitro* with bovine epithelial cells, demonstrating their ability to contribute to immune response modulation.

To our knowledge, this is the first study demonstrating the interaction between lactococci or bacteriocins and epithelial cells.

Further investigations focusing possible resistance development are necessary. Antibacterial peptides from *Lactococcus* spp. are likely to find a niche in the antibiotics market. Likewise the effectiveness of live culture treatments against mastitis pathogens and their interactions with the innate response open the way for the development of probiotic products for mastitis cure and prevention.

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ATTACHMENTS

Attachment A –Scientific activity

- Conte F., Piccinini R., **Malvisi M**., Foti M., Giacopello C., Fisichella V., Cascio O. Studio sull'attività antibatterica del lisozima del latte d'asina. Aspetti igienico-sanitari. LXV Convegno nazionale SISVet (Società Italiana delle Scienze Veterinarie), Vibo Valentia 7-10 settembre 2011

- **Malvisi M.**, Giardini A., Zecconi A., Piccinini R. Preliminary results of antimicrobial activity of bacteriocins secreted by lactic acid bacteria against mastitis pathogens. Poster presentation, International Scientific Conference on Bacteriocins and Antimicrobial Peptides (BAMP2012), Kosice – SK 21-23 Febbraio 2012

- **Malvisi M.**, Giardini A., Zecconi A., Piccinini R. Attivita' antimicrobica di batteriocine prodotte da *lactococcus* spp. Nei confronti di batteri patogeni della mammella bovina. Poster presentation, 3° Congresso lattiero-caseario Associazione Italiana Tecnici del Latte (AITeL), Milano 28 settembre 2012

- Conte F., Foti M., **Malvisi M.**, Giacopello C., Piccinini R. (2012) Valutazione dell'azione antibatterica del lisozima del latte d'asina. Considerazioni igienico - sanitarie. Large Animal Review 18:13-16

- **Malvisi M**., Giardini A., Zecconi A., Piccinini R. (2013) Attivita' antimicrobica di batteriocine prodotte da *Lactococcus* spp. nei confronti di batteri patogeni della mammella bovina. Scienza e Tecnica Lattiero-Casearia 64 (5-6):167-172

- Pilla R., **Malvisi M.**, Snel G.G., Schwarz D., König S., Czerny C.P., Piccinini R. (2013) Differential cell-count as an alternative method to diagnose dairy cow mastitis.J Dairy Sci. 96(3):1653-60

- Pilla R., Snel G.G., **Malvisi M**., Piccinini R. (2013) Duplex real-time PCR assay for rapid identification of Staphylococcus aureus isolates from dairy cow milk.J Dairy Res 80:223–226

- Pilla R., Bonura C., **Malvisi M**., Snel G.G., Piccinini R. (2013) Methicillin-resistant *Staphylococcus pseudintermedius* as causative agent of dairy cow mastitis. Veterinary record. Vet Rec 173(1):19

- Piccinini R., **Malvisi M.**, Cremonesi P., Capra E., Bignoli G., Castiglioni B., Valentino L., Pozzi F., Vezzoli F., Luini M. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in Italian dairy herds. Intervento presentato al convegno 3rd ASM-

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- Snel G.G. , **Malvisi M.**, Pilla R., Piccinini R. (2014) Evaluation of biofilm formation using milk in a flow cell model and microarray characterization of *Staphylococcus aureus* strains from bovine mastitis. DOI: 10.1016/j.vetmic.2014.09.020

Attachment B –Supplementary material

Figure S1. HR-MS spectrum (A) and deconvoluted MS spectrum (B) of *L. lactis* subsp. *lactis* SL153 partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentate peak eluting at 11.8 min.

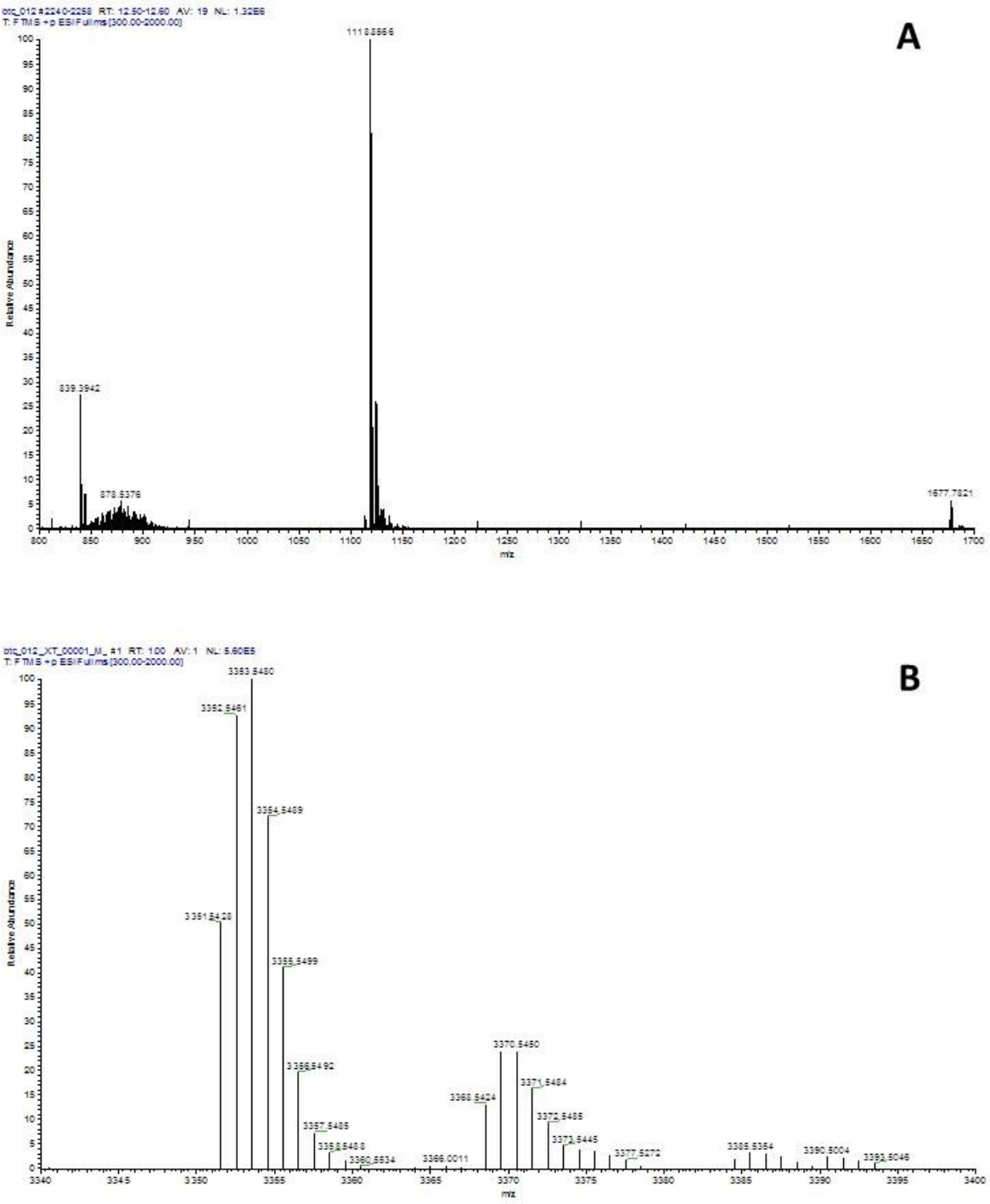

Figure S2. HR-MS spectrum and deconvoluted MS spectrum of *L. lactis* subsp. *lactis* SL153 partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentate peak eluting at 11.5 min.

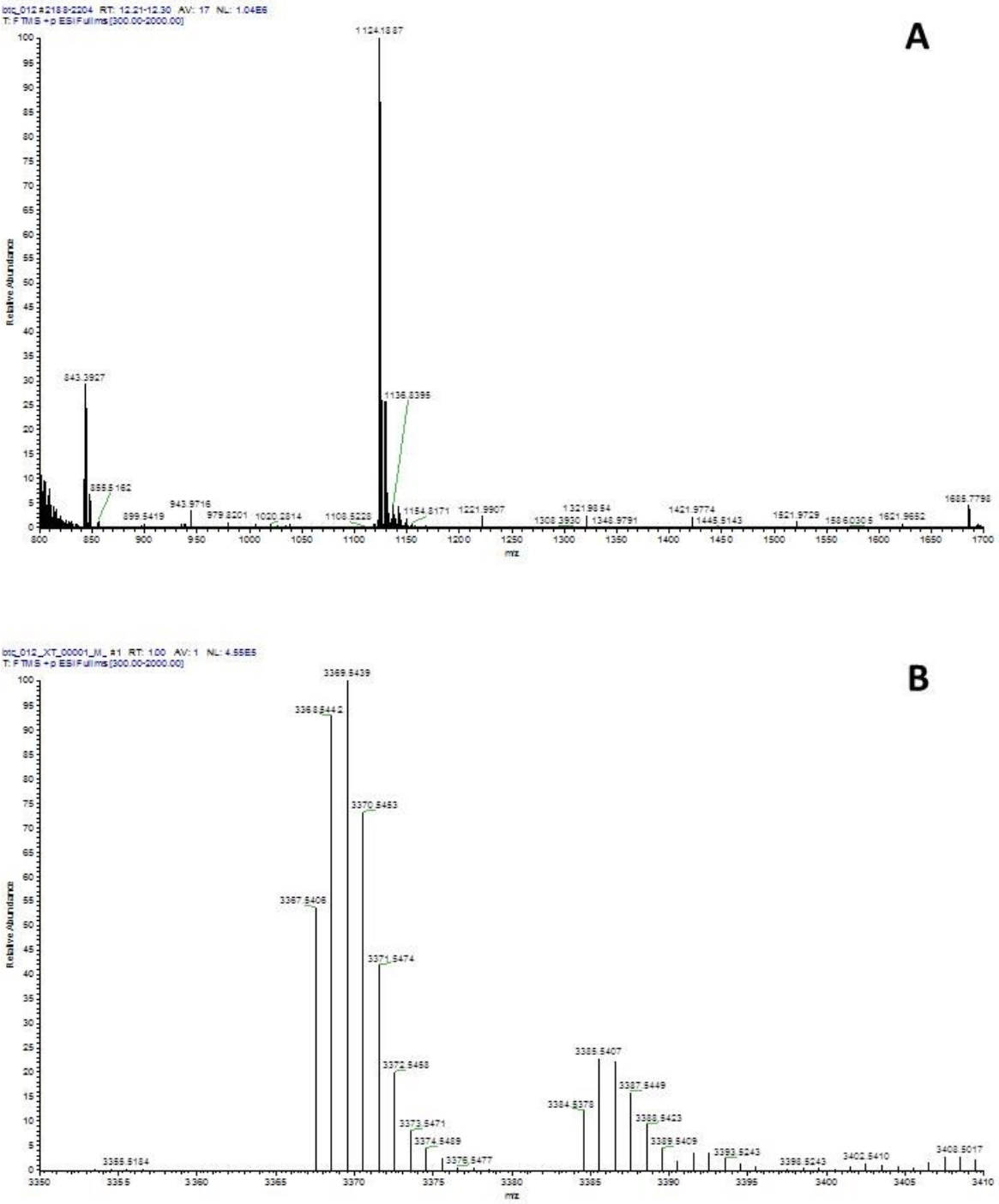

Figure S3. HR-MS spectrum (A) and deconvoluted MS spectrum (B) of *L. lactis* subsp. *lactis* SL208 partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentate peak eluting at 11.8 min.

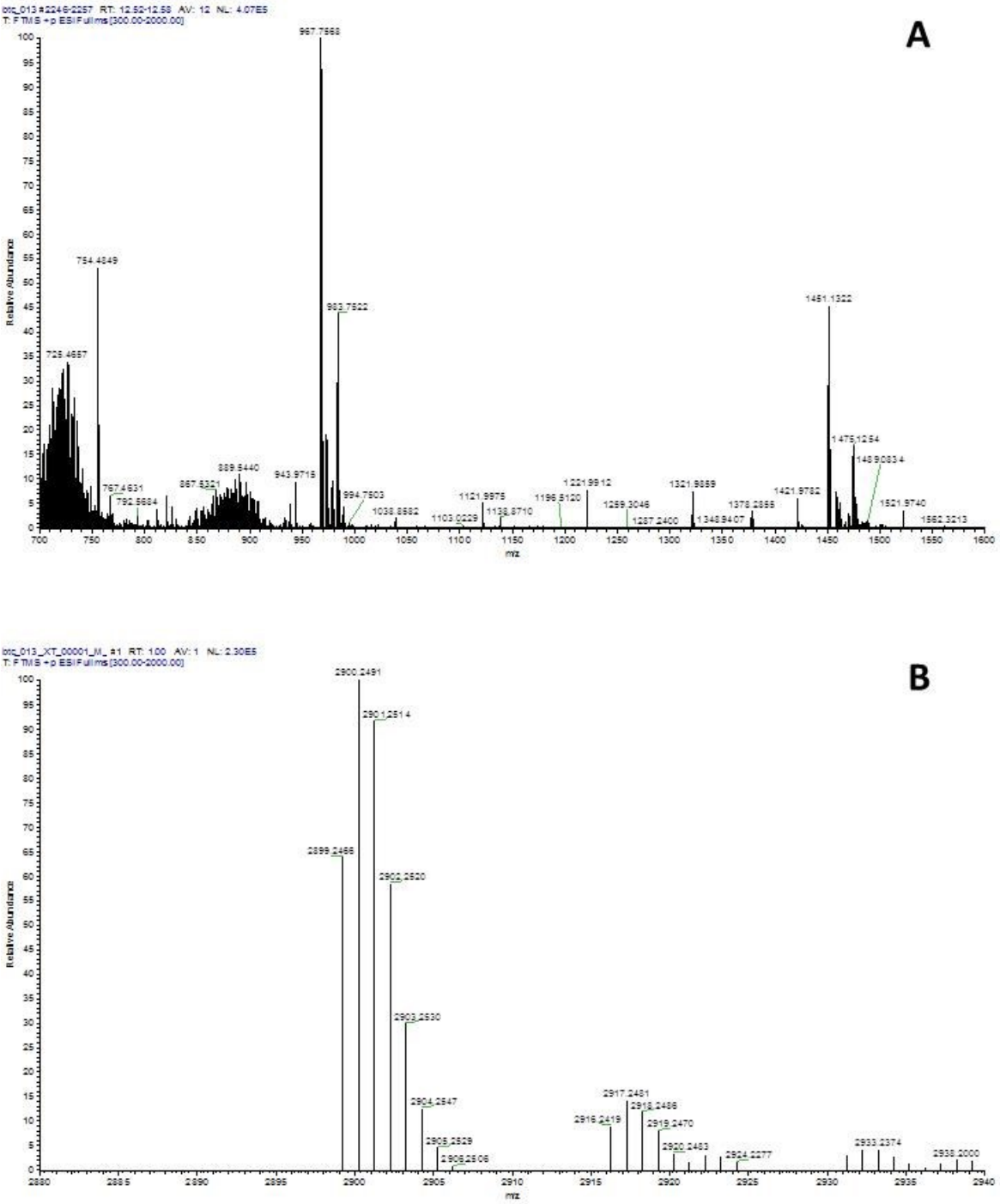

Figure S4. HR-MS spectrum (A) and deconvoluted MS spectrum (B) of *L. lactis* subsp. *lactis* SL208 partially purified bacteriocin extract (sample B) 1 kDa ultrafiltration retentate peak eluting at 6.1 min.