Interpretive summary

Antibacterial activity and immunomodulatory effects on a bovine mammary epithelial cell line exerted by nisin A-producing *Lactococcus lactis* strains.

Malvisi et al. Some *Lactococcus lactis* strains produce antibacterial molecules known as bacteriocins. In the present study, the cell free supernatants (CFSs) of 25 *L. lactis* broth cultures were tested for antibacterial activity against 29 mastitis pathogens. We demonstrated that the CFSs from two strains contained nisin A, a class I bacteriocin, and exerted antibacterial activity. The treatment of a mammary epithelial cell line with such CFSs or with the lactococcal live cultures induced some interesting immunomodulatory activities on the cells. Therefore, these *L. lactis* strains could be used for the development of alternative treatments against mastitis.

Running head: EFFECTS OF NISIN A-PRODUCING LACTOCOCCI

Antibacterial activity and immunomodulatory effects on a bovine mammary epithelial cell line exerted by nisin A-producing *Lactococcus lactis* strains.

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ABSTRACT

Twenty-nine strains of mastitis pathogens were used to study the antibacterial activity of the cell free supernatants (CFSs) of 25 strains of *Lactococcus lactis* subsp. *lactis.* Out of the tested strains, only the CFSs of *L. lactis* LL11 and SL153 were active, inhibiting and killing most of the pathogens. By means of UPLC/HR-MS, they were shown to produce nisin A, a class I bacteriocin. A variable sensitivity to nisin A-containing CFSs was observed among *Streptococcus uberis* and

Enterococcus faecalis strains. Nonetheless, *Strep. agalactiae*, *Strep. uberis* and *E. faecalis* displayed high minimum inhibitory concentration (MIC) values reaching 384 Arbitrary Units/mL (AU/mL). Interestingly, the MIC values and the bactericidal concentrations were almost identical among them for each of the two stains, LL11 and SL153. Staphylococci were on average less sensitive than streptococci, but the two CFSs inhibited and killed, at different dilutions, strains of methicillin-resistant *Staphylococcus aureus*. The immune response to nisin A-containing CFSs was tested using the bovine mammary epithelial cell line BME-UV1. Application of CFSs did not damage epithelial integrity, as demonstrated by the higher activity of N-acetyl-β-D-glucosaminidase (NAGase) and lysozyme inside the cells, in both treated and control samples. On the other hand, the increase of released NAGase after 15 to 24 h of treatment with LL11 or SL153 live cultures demonstrated an inflammatory response of epithelial cells. Similarly, a significantly higher lysozyme activity was detected in the cells treated with LL11 live culture confirming the stimulation of lysosomal activity. The treatment of epithelial cells with SL153 live culture induced a significant TNF-α down-regulation in the cells, but did not influence IL-8 expression. The control of TNF-α release could be an interesting approach to reduce the symptoms linked to clinical intramammary infections. Due to their antibacterial activity and to the stimulation of lysosomal activity of mammary epithelial cells, the *L. lactis* strains SL153 and LL11 could be of interest for the development of alternative intramammary treatments to control cow mastitis.

Keywords: dairy cow mastitis; *L. lactis*; nisin A; mammary epithelial cells; UPLC/HR-MS.

INTRODUCTION

Mastitis still represents one of the major cause of economic losses in dairy herds due to reduction of milk production and quality (Geary et al., 2014), to premature culling (Heikkilä et al., 2012) and to the costs related to antibiotic therapy and to waste milk during the withholding period after

antibiotic treatment. Also, calf feeding with discarded milk, either raw or pasteurized, raises the risk of selecting resistant fecal bacteria (Aust et al., 2013). The presence of antibiotic residues in milk can also inhibit the starter cultures used for manufacturing of cheese and yoghurt (Heap, 1982). For these reasons, the public opinion is pushing for the reduction of the use of antibiotics in veterinary field, and researchers are interested in finding new alternative approaches for treatment and control of mastitis.

Some strains of lactic acid bacteria (LAB) are known to produce bacteriocins, which are ribosomally-synthesized secreted peptides exerting antimicrobial activity either in the same species (narrow spectrum) or across the genera (broad spectrum) (Balciunas et al., 2013). Bacteria resist to their own bacteriocins by producing specific proteins that provide sequestration or competition for bacterial receptors or pump bacteriocins out through the bacterial membrane (Cotter et al., 2005). Among producer strains, LAB are the most investigated because they are allowed to be present in foods, being approved as Generally Recognized As Safe (GRAS) organisms by the U. S. Food and Drug Administration (Gaynor et al., 2006). At present, the application to the mammary gland of viable LAB cultures or bacteriocins is under investigation. Bouchard et al. (2013) demonstrated the *in vitro* ability of *Lactobacillus casei* strains to prevent *Staphylococcus aureus* adhesion and internalization in MAC-T bovine mammary epithelial cells. Other recent studies highlighted the efficacy of nisin A, a class I bacteriocin produced by *Lactococcus lactis* subsp. *lactis* (*L. lactis*), as a valid tool for the treatment of clinical or subclinical mastitis (Cao et al., 2007; Wu et al., 2007; Klostermann et al., 2008). Beecher et al. (2009) demonstrated that the administration of a live culture of *L. lactis* into the bovine mammary gland stimulated the innate immune response, as shown by higher pro-inflammatory cytokine expression by milk somatic cells in treated quarters in comparison with controls. To the best of our knowledge, studies regarding the interaction between LAB or bacteriocins and the mammary epithelial cells are still lacking in the literature. Therefore, the aims of the present study were: i) to evaluate the antibacterial activity of the cell-free culture supernatant (CFS) of 25 *L. lactis* strains against

mastitis pathogens; ii) to identify the produced bacteriocins by Ultra-Performance Liquid Chromatography/High Resolution Mass Spectrometry (UPLC/HR-MS); iii) to investigate the ability of the active *L. lactis* live cultures and the derived CFSs to modulate the immune response of mammary epithelial cells.

MATERIALS AND METHODS

Bacterial Strains

Twenty-nine strains of mastitis pathogens were selected from the culture collection of the Department of Veterinary Science and Public Health (University of Milan, Milan, Italy): *Streptococcus agalactiae* (*Strep. agalactiae*: MB343, MB386, MB422), *Streptococcus dysgalactiae* (*Strep. dysgalactiae*: MB280, MB324), *Streptococcus uberis* (*Strep. uberis*: MB300, MB705, MB707), *Enterococcus faecalis* (*E. faecalis*: MB330, MB561, MB562, MB706), *Staphylococcus aureus* (*Staph. aureus*: MB221, MB254, MB351, MB390, MB439, MB501, MB512, MB535, MB543, MB781, MB786, MB798), including methicillin-resistant *Staph. aureus* (MRSA: MB508, MB628) and *Staph. chromogenes* (MB307, MB309, MB316).

Bacterial isolates had been collected from quarter milk samples of dairy cows during mastitis control programs. All strains were from subclinical intramammary infections, 4 from clinical mastitis (MB280, MB705, MB535, MB798) and one from a quarter after antibiotic treatment (MB707). Bacteriological analysis of milk and bacterial colony identification was performed according Hogan et al. (1999). Species identification was confirmed by API System (Rapid ID 32 Strep, Biomérieux, Marcy-l'Étoile, France). Antibiotic sensitivity of each strain was tested by Kirby-Bauer disk diffusion method (CLSI, 2008). The drugs mainly used in the treatment of clinical or subclinical bovine mastitis (penicillin, ampicillin, amoxicillin/clavulanic acid, oxacillin, 1st, 3rd and 4th generation cephalosporins, norfloxacin, rifaximin, tylosin, kanamycin,

tiamphenicol, sulfamethoxazole/trimethoprim) were tested. All isolates were stored at -80 °C in Microbank Bacterial Preservation System (Thermo Fisher Scientific Inc., Waltham, MA, USA). Before use, the thawed isolates were cultured on bovine blood agar plates (5% of blood, Oxoid, Basingstoke, UK) and thereafter grown in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) for 24 h at 37 °C.

Twenty-five strains of *L. lactis* from the collection of Clerici-Sacco Group (Cadorago, Italy) were tested for antibacterial activity. They were maintained on MRS agar (Difco, Bordeaux, France) at 4 °C until use.

Partial Purification of Antibacterial Molecules

Partial purification of antibacterial molecules produced by the strains of *L. lactis*, cultivated in MRS broth, was performed by the adsorption-desorption method as described by Yang *et* al. (1992) and Baljinder *et* al. (2013). The release of bacteriocins from the cell wall is known to be complete at pH 3.0: after the addition of lactic acid, the broth cultures were centrifuged at 29,000 x *g* for 15 min and adjusted to pH 7.0. Finally CFSs were sterilized by filtration through 0.22 μm pore size cellulose acetate filter and stored at -80 °C until use. The crude or partially purified CFSs were subsequently fractionated by ultrafiltration (UF) through a 1 kDa cut-off membrane (Millipore, Darmstadt, Germany) and tested for antibacterial activity.

Antibacterial Activity of **L. lactis** *Strains*

Antibacterial activity of CFSs was tested using the minimum inhibitory concentration (MIC) assay against each mastitis pathogen isolate, according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). Serial twofold dilutions of each CFS were performed in 100 μL of BHI broth supplemented with 5% fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA),

in 96-well microplates. The antimicrobial activity of each CFS was expressed as arbitrary units/mL (AU/mL), being 1 AU defined as the reciprocal of the highest dilution that completely inhibited bacterial growth. The turbidity in the wells containing the CFS was compared with the turbidity of the control wells, as detected by the unaided eye, in accordance with CLSI procedure (2008). To test bactericidal effect of the CFSs, the minimum bactericidal concentration (MBC) was assayed, plating 10 µL of the dilution representing the MIC endpoint and of two more concentrated dilutions and then counting the colonies of growth. The MBC was defined as the lowest concentration showing ≥99% killing (CLSI, 2008).

SDS-PAGE of CFSs of* L. lactis *LL11 and SL153

After UF at 1 kDa, the permeates and the retentates of crude CFSs and partially purified CFSs were mixed with SDS-PAGE native sample buffer (Bio-Rad Laboratories, Hercules, CA). Separation was performed on a 15 % polyacrylamide (PAA) gel in TRIS–glycine–SDS buffer on SE 250 mini-vertical gel electrophoresis system (Hoefer Inc., Holliston, MA). PageRuler Plus Prestained Protein Ladder (10–250 kDa; Thermo Fisher Scientific, Waltham, MA) and Spectra Multicolor Low Range Protein Ladder (1.7-40 kDa; Thermo Fisher Scientific) were used as 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 2 h. Gels were stained with Coomassie brilliant blue G-250. The molecular weights of protein bands were estimated by calculating the relative migration distance (*rf*) values as described by Hames (1998).

For antibacterial activity test, unstained PAA gels were fixed, washed (with milliQ-treated water for 2 h at 25°C in slight agitation changing water every 15 min), aseptically placed on soft agar (1% BHI agar supplemented with 5% of fetal calf serum) and overlaid with the same medium inoculated with 10^6 cfu/mL of *Streptococcus agalactiae* MB386. This strain was chosen as an

indicator of antibacterial activity, based on the MIC results. The plates were incubated at 37 °C for 18 to 24 h and the inhibition zone of growth was evaluated.

Separation and Identification of Antimicrobial Molecules Present in the CFSs of L. lactis *Strains LL11 and SL153 by UPLC/HR-MS*

The UF retentates of the partially purified CFSs were analyzed by UPLC using an Acquity separation module (Waters, Milford, MA) with a photo diode array eλ detector (Waters). The UPLC was coupled to a high resolution Q Exactive MS (HR-MS, Thermo Scientific, San Jose, CA, USA) through an electrospray ionization source. Samples were separated on an Aeris Widepore XB-C4 column (2.1×150 mm, 3.6 µm; Phenomenex, Torrance, CA) 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 at 210 nm and subsequently analyzed by HR-MS using full scan analysis in the range 300–2000 m/z. The resolution was set at 35K. The AGC target was $5x10^5$. The maximum ion injection time was 100 ms. The MS data were automatically processed using Xcalibur 3.0 software (Thermo Scientific), and protein mass deconvolution was performed using Xtract software (Thermo Scientific).

Challenge of Bovine Mammary Epithelial Cell line BME-UV1 with CFSs or Live L. lactis *Cultures*

The continuous cell line of bovine mammary epithelial cells, BME-UV1, was grown to confluence in 6-well plates (Falcon Becton Dickinson Labware, Franklin Lakes, NJ, USA) for 58 h, as described by Zavizion et al. (1996). The cells were washed twice with Hanks' Balanced Salts

194 Solution (HBSS, Sigma-Aldrich, St. Louis, MO). Complete medium without fetal calf serum was
195 added before the challenge with CFS or live lactococcal cultures, in order to avoid possible
196 interference of the serum with the enzyme activity. The experiment was performed in duplicate.
197 Two *L. lactis* strains (LL11 and SL153) were incubated in Roswell Park Memorial Institute
198 medium (RPMI-1640) medium for 48 h and the derived CFSs were assayed with the MIC test to
199 confirm the antibacterial activity. The CFSs were then added to BME-UV1 cell-culture to a final
200 concentration of 10% in fetal calf serum-free medium. Analogously, 30 µL of a suspension (10^4
201 cfu/mL) of each lactococcal live culture were added to distinct wells, and after 4 h, 8 h, 15 h, and
202 24 h of stimulation both culture medium and epithelial cells were collected and stored separately.
203 Aliquots of $3x10^6$ BME-UV1 cells were suspended in RNA*later* (Sigma-Aldrich) and stored at -
204 80 °C until RNA extraction, while culture medium was frozen at -20 °C.
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206 ***Detection of Enzyme Activity and Cytokine Expression***
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208 Culture medium was tested for the amount of antibacterial enzymes typically involved in the
209 mammary innate immune response, i.e. N-acetyl-β-D-glucosaminidase (NAGase) and lysozyme
210 (LZ). Epithelial cells were assayed for both enzymes activities and for pro-inflammatory cytokines
211 (TNF-α and IL-8) expression.
212 NAGase and LZ were quantified in duplicate on a microplate fluorometer (Fluoroskan Ascent,
213 Thermo Fisher Scientific) using fluorescence-based procedures, as previously described (Kitchen
214 et al., 1978; Pilla et al., 2010).
215 Total RNA was extracted using Illustra Mini RNA isolation kit (GE Healthcare, Little Chalfont,
216 UK) according to manufacturer's instructions; RNA quantification and purity was estimated by a
217 spectrophotometer ND-100 (Nanodrop Technologies, Wilmington, DE). RNA was reverse
218 transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Venlo, Netherlands) and
219 quantitative real-time PCR (qPCR) was performed in triplicate, using EvaGreen fluorescent

220 detection system and the Eco Real-Time PCR System (Illumina Inc., San Diego, CA, USA). The
221 gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as
222 housekeeping gene for its stability, as demonstrated in a recent research study on the validation of
223 reference genes to be applied in bovine mastitis research (Bougarn et al., 2011). Primer sequences
224 for GAPDH and TNF-α gene were previously published by Tomasinsig et al. (2010); the primers
225 for IL-8 were from Günter et al. (2010). Oligonucleotide concentration was 100 nM for GAPDH
226 and TNF-α, or 200 nM for IL-8. PCR amplifications were performed using the following
227 conditions: *Taq* polymerase activation at 95 °C for 3 min, 45 cycles of denaturation at 95 °C for
228 10 s, and annealing/extension at 62 °C (TNF-α) or at 59 °C (IL-8) for 15 s. The specificity of the
229 reaction was verified post-amplification by melt curve analysis. For relative quantification of
230 mRNA, the Ct values of the target gene transcripts were normalized against the reference gene
231 GAPDH, and the results were expressed as normalized fold expression relative to the control (not
232 stimulated cells).
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234 ***Statistical Analysis***
235
236 The statistical analysis of the data was performed using the software SPSS Statistics (version 21.0;
237 IBM, Armonk, NY). For the analysis, the results of NAGase and LZ activity in the first two time
238 points (4 h and 8 h) were gathered and considered as early observations; the other time-points (15
239 h and 24 h) were considered as late observations. For pro-inflammatory cytokine mRNA, only the
240 4 h observations were considered as early ones, because it is expressed in the first hours after
241 stimulation. Means were compared by Two-way ANOVA and Scheffè post-hoc test. Time and
242 treatment variables were used as main effects, and NAGase, LZ and cytokine expression as the
243 dependent variables. One-way ANOVA was applied when the time variable was not significant
244 and also to analyze the differences between intracellular and extracellular localization of enzymes.
245 Results were considered as statistically significant at P values <0.05.

RESULTS

Antibiotic sensitivity patterns of mastitis isolates, determined by agar plate diffusion, showed that all strains were resistant to at least one of the tested antibiotics (Table 1). All *Strep. agalactiae* strains were resistant to oxacillin and most of them were also resistant to rifaximin and macrolides. *Streptococcus dysgalactiae* and *Strep. uberis* were mostly resistant to macrolides but susceptible to the other tested drugs. On the contrary, *E. faecalis* evidenced a broad resistance to oxacillin, penicillin, macrolides and tiamphenicol. When considering *Staph. aureus*, most strains were resistant to penicillin and ampicillin, but sensitive to the other drugs. Both MRSA were also resistant to sulfamethoxazole/trimethoprim and, obviously, to oxacillin.

Out of 25 *L. lactis* strains, only the CFSs of LL11 and SL153 were active against mastitis pathogens (Table 2). Nevertheless, a variable sensitivity, expressed as both MIC and MBC, was observed not only among species, but also among different strains belonging to the same bacterial species (Table 2). Identification of the antibacterial molecules produced by these two *L. lactis* strains was performed in partially purified CFSs obtained by the adsorption-desorption method as described in Materials and Methods. The activity of these partially purified CFSs against the indicator strain (*Strep. agalactiae* MB386) was maintained, indicating that the antimicrobial compound was successfully recovered. After UF at 1 kDa, the permeates of the partially purified LL11 and SL153 CFSs lost their antibacterial activity, in contrary to the two UF retentates that were further subjected to the SDS-PAGE analysis. For both CFSs, a narrow protein band at about 4 kDa was revealed (Supplementary Figure S1). The retentates of partially purified CFSs of *L. lactis* LL11 and SL153 were subsequently fractionated by UPLC/HR-MS. The chromatograms showed the presence of a major peak at 11.8 min retention time for both CFSs (Supplementary Figure S2 A and B). The accurate mass of the molecules corresponding to both chromatographic peaks was 3353.55 Da (Supplementary Figure S3). Accordingly, the molecule was assigned to the

peptide nisin A (Piper et al., 2011). Further UPLC/HR-MS analysis of standard nisin A (Sigma-Aldrich) confirmed this assignment (Supplementary Figure S2 C).

The live *L. lactis* cultures and the derived CFSs were further investigated for their ability to modulate the immune response of mammary epithelial cells. To this purpose, the stabilized line of bovine mammary epithelial cells BME-UV1 was used as an *in vitro* model, because it produces pro-inflammatory cytokines and antibacterial molecules upon stimulation (Tomasinsig et al., 2010). The NAGase and LZ activities were investigated in both the intracellular and extracellular compartments. Significant differences between the compartments were observed in controls for both NAGase and LZ ($P \leq 0.001$), with higher amounts of the enzymes stored in the cells. When considering NAGase, the figure did not change in all treated samples, while for LZ the intracellular activity remained significantly higher only in the samples treated with *L. lactis* SL153 bacteriocins or live culture. After the application of LL11, an increase of released enzyme was detected, but no decrease in the intracellular activity (Figure 1). Time and treatment (CFSs or live cultures of *L. lactis* LL11 and SL153) did not significantly influence the intracellular NAGase and LZ activities during 24 h of incubation. In the extracellular compartment, in the early observations, NAGase activity was higher when the cells were treated with the live LL11 strain, rather than with its CFS ($P=0.049$). After 15 to 24 h of incubation, the cells treated with LL11 or SL153 live cultures showed a 5-fold increase in the release of NAGase ($P=0.002$ and $P=0.001$, respectively) in comparison with both bacteriocin-treated cells and controls (Figure 2).

A completely different activity pattern described the extracellular LZ. In the early observations, LZ activity showed a significant increase when cells were treated with CFSs, but not with live cultures of LL11 or SL153 strains ($P=0.02$ and $P=0.001$, respectively). Thereafter, in the late observations, a maximum LZ activity was detected in of BME-UV1 cells stimulated with LL11 live culture ($P<0.001$; Figure 3).

Overall, the expression of pro-inflammatory cytokines by BME-UV1 cells in response to nisin A-containing CFSs or live lactococcal cultures was similar to that of untreated cells (Figure 4). The

only difference (P=0.047) was observed with SL153 culture, which caused a marked decrease in TNF-α expression in comparison with the control. The measurements at different time-points showed considerably stable values throughout the 24 h observations. The only exception, even though not significant, was the relative expression of IL-8, which showed a 7-time increase over the untreated cells at 15 h post treatment with LL11 culture.

DISCUSSION

Even though a variable sensitivity to nisin A-containing CFSs was observed among *Strep. uberis* and *E. faecalis* strains, *Strep. agalactiae*, *Strep. uberis* and *E. faecalis* displayed high MIC values, i.e. high sensitivity to CFSs, reaching 384 AU/mL. This result is even more worth to be noted, when considering the pattern of antibiotic resistance demonstrated by the tested strains. Indeed, most *Strep. agalactiae*, *Strep. dysgalactiae*, *E. faecalis* and *Staph. aureus* strains showed multiple resistance to the drugs generally used in the treatment of dairy cow intramammary infections. Interestingly, the MIC values and the bactericidal concentrations were almost identical among them for each of the two stains, LL11 and SL153. Such finding is explained by the mode of action of class I bacteriocins. Nisin A forms a complex with the cell wall precursor lipid II, thus inhibiting the biosynthesis of the cell wall. After aggregation, the complexes include other peptides and form a pore in the bacterial membrane, thus causing the death of the microorganism (Moll et al., 1999). In this study, staphylococci were on average less sensitive than streptococci, but the two tested methicillin-resistant *Staphylococcus aureus* strains (MRSA) were inhibited or killed at a 1:1 or 1:4 dilution, respectively. The result is worth of interest, because these microorganisms represent a major threat to human health, due to the development of multiple antimicrobial resistance.

On the side of mammary cells, the application of nisin A-containing CFSs or LL11 and SL153 live cultures did not damage epithelial integrity, as demonstrated by NAGase and LZ release. The amount of intracellular enzymes was significantly higher than that of released enzymes, both in

controls and in treated cells. The unique exception was the increase in the secretion of LZ, when LL11 was applied, even if with no decrease in the intracellular activity was detected: as a consequence, the significance between the LZ activity in the two compartments was lost. The enzymes are physiologically produced and stored in the cells and are secreted when an inflammatory stimulus occurs (Kitchen et al., 1978; Ebling et al., 2001). On the other hand, the noticeable increase of released NAGase after 15 to 24 h of treatment with LL11 or SL153 live cultures demonstrated an inflammatory response by epithelial cells. The enzyme is usually considered an indicator of dairy cow mammary inflammation and immunity response activation, since its amount increases in milk from clinical and subclinical mastitis (Åkerstedt et al., 2012). Similarly, the significantly higher LZ activity detected in the cells treated with LL11 live culture confirmed the stimulation of lysosomal activity, while intracellular enzyme remained stable. It is well known that the activity of NAGase and LZ increase during an inflammation (Sarikaya et al., 2006), and that the content of both enzymes in healthy mammary quarters is not correlated with milk somatic cell counts, but rather to age and lactation stage of cows (Piccinini et al., 2007).

As further demonstrated by Bruckmaier (2005), udder tissues play a pivotal role as a primary source of innate immunity factors, expressing pro-inflammatory cytokine and antibacterial enzymes after challenge with *Escherichia coli* lipopolysaccharide. The treatment with SL153 live culture induced a significant TNF-α mRNA expression down-regulation in the cells, but did not influence IL-8 expression. Although pro-inflammatory cytokines stimulate the host innate immune defences, which are fundamental against infections, TNF-α causes the typical signs of clinical inflammation. Therefore, the control of TNF-α release could be an interesting approach to reduce the symptoms linked to clinical intramammary infections. Furthermore, the cytokine was shown to suppress lactogenic function of the glands after infusion (Alluwaimi, 2004), by an autocrine effect inhibiting the synthesis of caseins by MEC (Shea-Eaton et al., 2001). The mRNA expression of IL-8, the other pro-inflammatory cytokine considered, did not significantly change over the study, showing high variability between the 2 repetitions of the experiment. These results seem to

confirm the anti-inflammatory properties of *L. lactis* SL153 live culture, when applied onto the mammary epithelial cells.

CONCLUSIONS

The identification of only two strains with antibacterial activity, out of 25 LAB tested, indicated that the strains applicable to the dairy cow mastitis are quite rare. *L. lactis* LL11 and even more SL153 resulted of interest for the development of alternative intramammary treatments, due to their antibacterial activity and to the stimulation of lysosomal enzymes of mammary epithelial cells. Indeed, the nisin A-containing CFSs could kill different mastitis pathogens, including MRSA strains. Moreover, the application of the CFSs to a mammary epithelial cell line stimulated the secretion of antibacterial enzymes NAGase and LZ by the cells, without causing either damage to them or any adverse inflammatory reaction. Further *in vivo* studies are required, to investigate potential clinical side effects and the cure rate of mastitis following the administration of a preparation as that described above.

REFERENCES

Åkerstedt, M., M. Forsbäck, T. Larsen, and K. Svennersten-Sjaunja. 2011. Natural variation in biomarkers indicating mastitis in healthy cows. J. Dairy Res. 78:88–96.

Alluwaimi, A. M. 2004. The cytokines of bovine mammary gland: prospects for diagnosis and therapy. Res. Vet. Sci. 77:211–222.

Aust, V., K. Knappstein, H. J. Kunz, H. Kaspar, J. Wallmann, and M. Kaske. 2013. Feeding untreated and pasteurized waste milk and bulk milk to calves: effects on calf performance, health status and antibiotic resistance of faecal bacteria. J. Anim. Physiol. Anim. Nutr. 97:1091–1103.

Balciunas, E. M., F. A. Castillo Martinez, S. D. Todorov, B. D. Gombossy de Melo Franco, A. Converti, and R. Pinheiro de Souza Oliveira. 2013. Novel biotechnological applications of bacteriocins: A review. Food Control 32:134-142.

Baljinder, K., B. Praveen, M. Bharti, C. Ashish, K. Balvir and G. Neena. 2013. Antimicrobial spectrum of Anti-*Gardnerella vaginalis* bacteriocin producing *Lactobacillus fermentum* HV6b against bacterial vaginosis associated organisms. Am. J. Biochem. Mol. Biol. 3:91-100.

Beecher, C., M. Daly, D. P. Berry, K. Klostermann, J. Flynn, W. Meaney, C. Hill, T. V. McCarthy, R. P. Ross, and L. Giblin. 2009. Administration of a live culture of *Lactococcus lactis* DPC 3147 into the bovine mammary gland stimulates the local host immune response, particularly IL-1β and IL-8 gene expression. J. Dairy Res. 76:340-348.

Bouchard, D. S., L. Rault, N. Berkova, Y. Le Loir, and S. Even. 2013. Inhibition of *Staphyloccoccus aureus* invasion into bovine mammary epithelial cells by contact with live *Lactobacillus casei*. Appl. Environ. Microbiol. 79:877-885.

Bougarn, S., P. Cunha, F. B. Gilbert, F. Meurens, and P.Rainard. 2011. Validation of candidate reference genes for normalization of quantitative PCR in bovine mammary epithelial cells responding to inflammatory stimuli. J. Dairy Sci. 94:2425-2430.

Bruckmaier, R. M. 2005. Gene expression of factors related to the immune reaction in response to intramammary *Escherichia coli* lipopolysaccharide challenge. J. Dairy Res. 72:120-124.

Cao, L. T., J. Q. Wu, F. Xie, S. H. Hu, and Y. Mo. 2007. Efficacy of nisin in treatment of clinicalmastitis in lactating dairy cows. J. Dairy Sci. 8:3980-3985.

Clinical and Laboratory Standards Institute. 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standards (M31-A3). CLSI, Wayne, PA, USA

Cotter, P. D., C. Hill, and R. P. Ross. 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. 3:777-788.

Ebling T. L., L. K. Fox, K. W. Bayles, G. A. Bohach, K. M. Byrne, W. C. Davis, W. A. Ferens, and J. K. Hillers. 2001. Bovine mammary immune response to an experimental intramammary infection with a *Staphylococcus aureus* strain containing a gene for staphylococcal enterotoxin C1. J. Dairy Sci. 84:2044-2050.

Gaynor, P. M., R. Bonnette, E. Garcia Jr., L. S. Kahl, and L. G. Valerio Jr. 2006. FDA's approach to the GRAS provision: a history of processes. Accessed Sept. 30, 2015. http://www.fda.gov/food/ingredientspackaginglabeling/gras/ucm094040.htm.

Geary, U., N. Lopez-Villalobos, B. O'Brien, D. J. Garrick, and L. Shalloo. 2014. Estimating the impact of somatic cell count on the value of milk utilising parameters obtained from the published literature. J. Dairy Res. 81:223-232.

Günther, J., S. Liu, K. Esch, H. J. Schuberth, and H. M. Seyfert, 2010. Stimulated expression of TNF-α and IL-8, but not of lingual antimicrobial peptide reflects the concentration of pathogens contacting bovine mammary epithelial cells. Vet. Immunol. Immunopathol. 135:152–157

Hames, B. D. 1998. Gel Electrophoresis of Proteins: A Practical Approach, 3rd ed., Oxford: Oxford University Press.

Heap, H. A. 1982. Sensitivity of starter cultures to penicillin and streptomycin in bulk-starter milk**.** N. Z. J. Dairy Sci. Technol. 17:81-86.

Heikkilä, A. M., J. Nousiainen, and S. Pyörälä. 2012. Costs of clinical mastitis with special reference to premature culling. J. Dairy Sci. 95:139-150.

Hogan, J. S., R. N. Gonzales, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 1999. Laboratory Handbook on Bovine Mastitis, revised edition. National Mastitis Council Inc. Madison WI, p. 222.

Kitchen, B. J., G. Middleton, and M. Salmon. 1978. Bovine milk N-acetyl-ß-D glucosaminidase and its significance in the detection of abnormal udder secretions, J. Dairy Res. 45:15-20.

Klostermann, K., F. Crispie, J. Flynn, R. P. Ross, C. Hill, and W. Meaney. 2008. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. J. Dairy Res. 75:365-37.

Moll, G. N., W. N. Konings, and A. J. Driessen. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie van Leeuwenhoek 76:185-198.

Piccinini, R., E. Binda, and M. Belotti. 2007. Evaluation of milk components during whole lactation in healthy quarters. J. Dairy Res. 74:226–232.

Pilla, R., V. Daprà, A. Zecconi, and R. Piccinini. 2010. Hygienic and health characteristics of donkey milk during a follow-up study. J. Dairy Res. 77:392-397.

Piper, C., C. Hill, P. D. Cotter, and R. P. Ross. 2011. Bioengineering of a Nisin A-producing *Lactococcus lactis* to create isogenic strains producing the natural variants Nisin F, Q and Z. Microbial Biotechnology 4:375-382.

Sarikaya, H., G. Schlamberger, H. H. D. Meyer, and R. M. Bruckmaier. 2006. Leukocyte populations and mRNA expression of inflammatory factors in quarter milk fractions at different somatic cell score levels in dairy cows. J. Dairy Sci. 89:2479-2486.

Shea-Eaton, W. K., P. P. Lee, and M. M. Ip. 2001. Regulation of milk protein gene expression in normal mammary epithelial cells by tumor necrosis factor. Endocrinology 142: 2558-2568.

Tomasinsig, L., G. De Conti, B. Skerlavaj, R. Piccinini, M. Mazzilli, F. D'Este, A. Tossi, and M. Zanetti. 2010. Broadspectrum activity against bacterial mastitis pathogens and activation of mammary epithelial cells support a protective role of neutrophil cathelicidins in bovine mastitis. Infect. Immun. 78:1781-1788.

Wu, J., S. Hu, and L. Cao. 2007. Therapeutic effect of nisin Z on subclinical mastitis in lactating cows. Antimicrob. Agents Chemother. 9:3131-3135.

Yang, R., M. C. Johnson, and B. Ray. 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. 58:3355-3359.

Zavizion B, M. van Duffelen, W. Schaeffer, and I. Politis. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. In Vitro Cell Dev. Biol. Anim. 32 (3):138-148.

456 Table 1. Antibiotic susceptibility patterns of mastitis pathogens: in each column, the number of sensitive (S) or resistant (R) strains is reported.

Antibiotic	*Strep. agalactiae* (*n*=3)		*Strep. dysgalactiae* (*n*=2)		*Strep. uberis* (n=3)		*E. faecalis* (n=4)		*Staph. aureus* (n=14)		*Staph. chromogenes* (n=3)	
	S	R	S	R	S	R	S	R	S	R	S	R
Penicillin	3		2		3		2	2	5	8	2	1
Ampicillin	3		2		3		3	1	5	8	2	1
Amoxicillin/clavulanic acid	3		2		3		4		11	2	3	
Oxacillin		3	2		3		1	3	11	2	3	
1st generation Cephalosporin	3		2		3		3	1	13		3	
3rd generation Cephalosporin	3		2		3		4		13		3	
4th generation Cephalosporin	3		2		3		4		11	2	3	
Norfloxacin	3		2		3		4		12	1	3	
Rifaximin	1	2	2		3		2	2	13		3	
Tylosin	1	2		2	2	1	2	2	12	1	2	1
Spiramycin	1	2		2	2	1	2	2	13		2	1
Tiamphenicol	3		2		3		2	2	12	1	3	
Sulfamethoxazole/trimethoprim	3		2			3		4	11	2	3	

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458 Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)
459 values showed by *L. lactis* LL11 and SL153 culture cell-free supernatants against mastitis pathogens,
460 expressed as Arbitrary Units/mL (AU/mL).

Mastitis pathogen	MIC (AU/mL)		MBC (AU/mL)	
	LL1	SL153	LL11	SL153
Strep. agalactiae (MB343, MB386, MB422)	256	384	256	384
Strep. dysgalactiae MB280	1.5	1	not killed	1
Strep. dysgalactiae MB324	1	2	not killed	2
Strep. uberis MB300	8	8	4	8
Strep. uberis MB705	64	256	64	128
S. uberis MB707	16	32	8	32
E. faecalis MB330	2	2	2	2
E. faecalis MB561	64	128	32	128
E. faecalis MB562	128	128	64	128
E. faecalis MB706	64	256	32	256
Staph. aureus MB221	1	8	1	4
Staph. aureus MB254	2	12	1	8
Staph. aureus MB351	16	24	8	24
Staph. aureus MB390	64	256	24	256
Staph. aureus MB439	24	64	16	64
Staph. aureus MB501	16	96	12	96
Staph. aureus MB512	24	64	16	64
Staph. aureus MB535	6	12	6	12
Staph. aureus MB543	32	64	16	64
Staph. aureus MB781	64	64	32	32
Staph. aureus MB786	4	12	2	12
Staph. aureus MB798	32	48	16	48
MRSA MB508	1	2	not killed	1
MRSA MB628	2	4	1	4
Staph. chromogenes MB307	8	16	8	16
Staph. chromogenes MB309	48	64	24	64
Staph. chromogenes MB316	12	48	12	48

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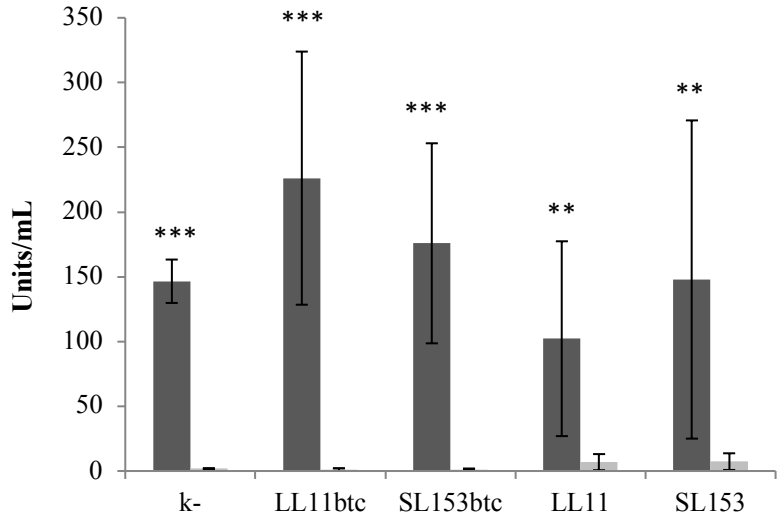
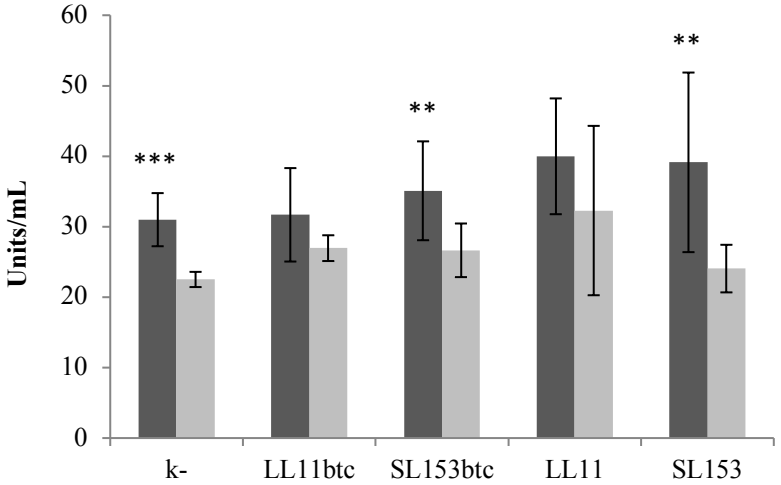
Figure 1. Intracellular activity (dark grey bars) and extracellular release (light grey bars) of NAGase (A) and lysozyme (B) in untreated (k-), *L. lactis* LL11 or SL153 CFS-treated (btc) or live culture-treated cells. Data are shown as means ± standard deviations (SD) of all measures throughout the 4 time points of the experiment. The mean values of NAGase and lysozyme activity were calculated from a total of 40 measurements each, in duplicate.



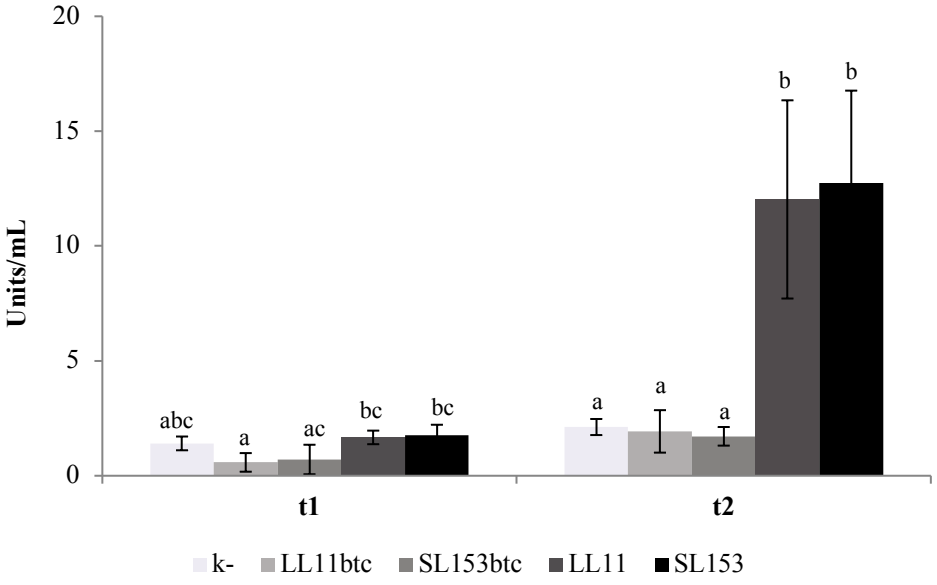
A)



B)

**P ≤ 0.01; ***P ≤ 0.001

Figure 2. Extracellular NAGase activity in BME-UV1 cells treated with *L. lactis* LL11 or SL153 CFS (btc), or live culture, in the early observations (t1) and late observations of the experiment (t2). The mean values were calculated from a total of 20 measurements, in duplicate.



[a, b, c] Different letters indicate significant difference in enzyme production between treatments, $P<0.05$.

Figure 3. Extracellular lysozyme activity in BME-UV1 cells treated with *L. lactis* LL11 or SL153 CFS (btc), or with LL11 or SL153 live culture, in the early observations (t1) and late observations of the experiment (t2). The mean values were calculated from a total of 20 measurements, in duplicate.

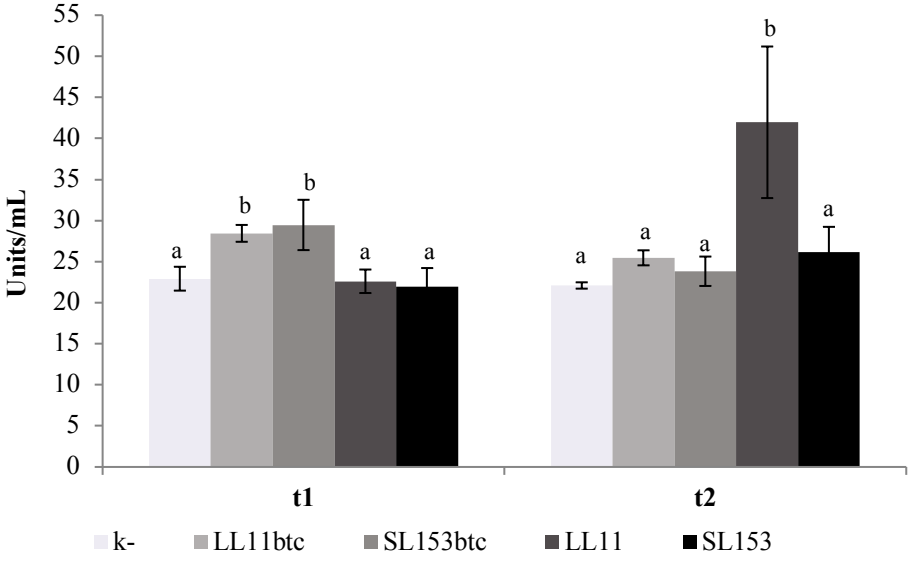
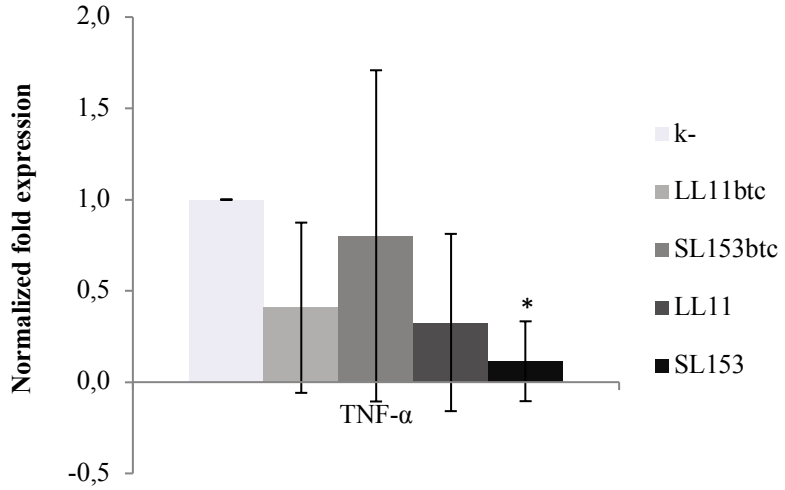


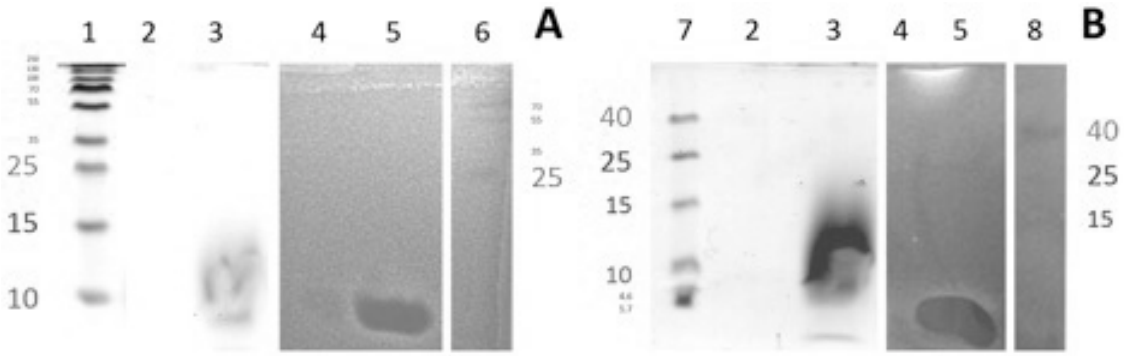
Figure 4. Normalized expression of TNF-α gene in BME-UV1 untreated (k-) and BME-UV1 cells treated with *L. lactis* LL11 or SL153 CFS (btc), or with LL11 or SL153 live culture. Data are shown as means ± standard deviations (SD) of all measures throughout the 4 time points of the experiment. The mean values were calculated from a total of 20 measurements in duplicate.



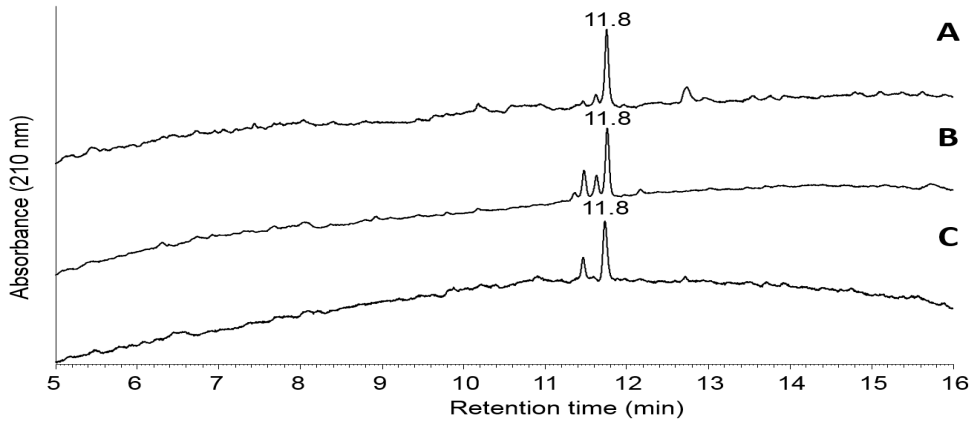
* means a statistically significant (P=0.047) difference between BME-UV1 cells treated with *L. lactis* SL153 live culture and the control.

[a, b] Different letters indicate significant difference in enzyme production between treatments, P<0.05.

Supplementary Figure S1. SDS-PAGE (lanes 2–3) and antibacterial activity test (lanes 4–5) of fractionated CFSs of *L. lactis* LL11 (A) and *L. lactis* SL153 (B) indicating the presence of antibacterial activity band against indicator strain *Streptococcus agalactiae* MB386 (lanes 5). Lanes 1 and 6: PageRuler™ Plus Prestained Protein Ladder, 10–250 kDa (Thermo Fisher Scientific, Waltham, MA, USA); 2: 1 kDa UF permeate; 3: 1 kDa UF retentate; 4: absence of antibacterial activity of 1 kDa UF permeate; 5: antibacterial activity of 1 kDa UF retentate; 7 and 8: Spectra™ Multicolor Low Range Protein Ladder, 1.7–40 kDa (Thermo Fisher Scientific).



Supplementary Figure S2. UPLC chromatograms of the retentates of partially purified CFSs of *L. lactis* LL11 (A), *L. lactis* SL153 (B) and of a nisin A standard (C) indicating the presence of a major peak at 11.8 min retention time.



Supplementary Figure S3. HR-MS spectra and deconvoluted HR-MS spectra (inserts) of a major peak (Supplementary Figure S2, at 11.8 min retention time) for retentates of partially purified CFSs of *L. lactis* LL11 (A) and *L. lactis* SL153 (B) indicating an accurate mass of a nisin A (3353.55 Da) and of its oxidized form (3369.55 Da).