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 (CFS) of 25 strains of *Lactococcus lactis* ssp. *lactis*. Out of the tested strains, only the CFS of *L. lactis* LL11 and SL153 were active, inhibiting and killing most of the pathogens. By means of ultra-performance liquid chromatography/high resolution mass spectrometry, they were shown to produce nisin A, a class I bacteriocin. A variable sensitivity to nisin A-containing CFS was observed among *Streptococcus uberis* and *Enterococcus faecalis* strains. Nonetheless, *Streptococcus agalactiae*, *Strep. uberis*, and *E. faecalis* displayed high minimum inhibitory concentration values, reaching 384 arbitrary units/mL. Interestingly, the minimum inhibitory values and the bactericidal concentrations were almost identical among them for each of the 2 stains, LL11 and SL153. Staphylococci were, on average, less sensitive than streptococci, but the 2 CFS inhibited and killed, at different dilutions, strains of methicillin-resistant *Staphylococcus aureus*. The immune response to nisin A-containing CFS was tested using the bovine mammary epithelial cell line BME-UV1. Application of CFS 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 tumor necrosis factor- α downregulation in the cells, but did not influence IL-8

expression. The control of tumor necrosis factor- 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.

Key words: dairy cow mastitis, *Lactococcus lactis*, nisin A, mammary epithelial cells, ultra-performance liquid chromatography/high resolution mass spectrometry

INTRODUCTION

Mastitis still represent one of the major cause of economic losses in dairy herds due to reduction of milk production and quality (Geary et al., 2014), premature culling (Heikkilä et al., 2012), and the costs related to antibiotic therapy and 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 yogurt (Heap, 1982). For these reasons, public opinion has shifted toward reduction in 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 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

Received July 23, 2015.

Accepted November 28, 2015.

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organisms by the US 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* ssp. *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 proinflammatory 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 (1) to evaluate the antibacterial activity of the cell-free culture supernatant (CFS) of 25 *L. lactis* strains against mastitis pathogens; (2) to identify the produced bacteriocins by ultra-performance liquid chromatography/high resolution mass spectrometry (UPLC/HR-MS); and (3) to investigate the ability of the active *L. lactis* live cultures and the derived CFS 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* (MB343, MB386, MB422); *Streptococcus dysgalactiae* (MB280, MB324); *Streptococcus uberis* (MB300, MB705, MB707); *Enterococcus faecalis* (MB330, MB561, MB562, MB706); *Staphylococcus aureus* (MB221, MB254, MB351, MB390, MB439, MB501, MB512, MB535, MB543, MB781, MB786, MB798), including methicillin-resistant *Staph. aureus* (MRSA; MB508, MB628); and *Staphylococcus 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 IMI, 4 from clinical mastitis (MB280, MB705, MB535, and MB798) and 1 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, first-, third-, and fourth-generation cephalosporins, norfloxacin, rifaximin, tylosin, kanamycin, tiamphenicol, and sulfamethoxazole/trimethoprim) were tested. All isolates were stored at -80°C in Microbank Bacterial Preservation System (Thermo Fisher Scientific Inc., Waltham, MA). 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) 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 de Man, Rogosa, Sharpe 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 de Man, Rogosa, Sharpe broth, was performed by the adsorption-desorption method as described by Yang et al. (1992) and Kaur et al. (2013). The release of bacteriocins from the cell wall is known to be complete at pH 3.0, and after addition of lactic acid, the broth cultures were centrifuged at $29,000 \times g$ for 15 min at room temperature and adjusted to pH 7.0. Finally, CFS were sterilized by filtration through $0.22\text{-}\mu\text{m}$ pore size cellulose acetate filter and stored at -80°C until use. The crude or partially purified CFS were subsequently fractionated by UF through a 1-kDa cut-off membrane (Merck Millipore, Darmstadt, Germany) and tested for antibacterial activity.

Antibacterial Activity of *L. lactis* Strains

Antibacterial activity of CFS was tested using the MIC assay against each mastitis pathogen isolate according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). Serial 2-fold dilutions of each CFS were performed in $100\text{ }\mu\text{L}$ of BHI broth supplemented with 5% fetal calf serum (Gibco, Thermo Fisher Scientific) in 96-well microplates. The antimicrobial activity of each CFS was expressed as arbitrary units (AU) per milliliter, with 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 procedures (CLSI, 2008). To test bactericidal effect of the CFS, the minimum bactericidal concentration (MBC) was assayed, plating 10 μ L of the dilution representing the MIC endpoint and of 2 more concentrated dilutions and then counting the colonies of growth. The MBC was defined as the lowest concentration showing $\geq 99\%$ killing (CLSI, 2008).

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

After UF at 1 kDa, the permeates and the retentates of crude CFS and partially purified CFS were mixed with SDS-PAGE native sample buffer (Bio-Rad Laboratories, Hercules, CA). Separation was performed on a 15% polyacrylamide 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) 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 the antibacterial activity test, unstained polyacrylamide gels were fixed, washed (with milliQ-treated water (Merck Millipore) 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 *Strep. 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

The UF retentates of the partially purified CFS were analyzed by UPLC using an Acquity separation module (Waters, Milford, MA) with a photo diode array λ detector (Waters). The UPLC was coupled to a high-resolution Q Exactive MS (Thermo Scientific, San Jose, CA) through an electrospray ionization source. Samples were separated on an Aeris Widepore XB-C4 column (2.1 $\times$ 150 mm, 3.6 μ m; Phenomenex, Torrance, CA) kept at 40°C. The eluents were 0.1% trifluoroacetic acid in milliQ-treated water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The UPLC separa-

tion 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 of 300 to 2,000 *m/z*. The resolution was set at 35K; the automatic gain control (AGC) target was 5×10^5 . 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 with CFS or Live *L. lactis*

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) for 58 h, as described by Zavizion et al. (1996). The cells were washed twice with Hanks' balanced salts solution (Sigma-Aldrich, St. Louis, MO). Complete medium without fetal calf serum was added before the challenge with CFS or live lactococcal cultures to avoid possible interference of the serum with the enzyme activity; the experiment was performed in duplicate.

Two *L. lactis* strains (LL11 and SL153) were incubated in RPMI-1640 medium for 48 h and the derived CFS were assayed with the MIC test to confirm the antibacterial activity. The CFS were then added to BME-UV1 cell-culture to a final concentration of 10% in fetal calf serum-free medium. Analogously, 30 μ L of a suspension (10^4 cfu/mL) of each lactococcal live culture were added to distinct wells, and after 4, 8, 15, and 24 h of stimulation both culture medium and epithelial cells were collected and stored separately. Aliquots of 3×10^6 BME-UV1 cells were suspended in RNeasy lysis buffer (Qiagen, Crawley, UK) and stored at -80°C until RNA extraction, whereas culture medium was frozen at -20°C .

Detection of Enzyme Activity and Cytokine Expression

Culture medium was tested for the amount of antibacterial enzymes typically involved in the mammary innate immune response; that is, N-acetyl- β -D-glucosaminidase (NAGase) and lysozyme (LZ). Epithelial cells were assayed for both enzymes activities and for proinflammatory cytokines [tumor necrosis factor (TNF)- α and IL-8] expression. Both NAGase and LZ were quantified in duplicate on a microplate fluorometer (Fluoroskan Ascent, Thermo Fisher Sci-

tific) using fluorescence-based procedures, as previously described (Kitchen et al., 1978; Pilla et al., 2010).

Total RNA was extracted using Illustra Mini RNA isolation kit (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions; RNA quantification and purity was estimated by a spectrophotometer ND-100 (Nanodrop Technologies, Wilmington, DE). The RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Venlo, the Netherlands) and quantitative real-time PCR was performed in triplicate, using EvaGreen fluorescent detection system and the Eco Real-Time PCR System (Illumina Inc., San Diego, CA). The *GAPDH* gene was selected as housekeeping gene for its stability, as demonstrated in a recent research study on the validation of reference genes to be applied in bovine mastitis research (Bougarn et al., 2011). Primer sequences for *GAPDH* and *TNF- α* genes were previously published by Tomasinsig et al. (2010); the primers for *IL-8* were from Günther et al. (2010). Oligonucleotide concentration was 100 nM for *GAPDH* and *TNF- α* , or 200 nM for *IL-8*. The PCR amplifications were performed using the following conditions: *Taq* polymerase activation at 95°C for 3 min, 45 cycles of denaturation at 95°C for 10 s, and annealing or extension at 62 (*TNF- α*) or 59°C (*IL-8*) for 15 s. The specificity of the reaction was verified postamplification by melt curve analysis. For relative quantification of mRNA, the cycle threshold 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).

Statistical Analysis

The statistical analysis of the data was performed using the software SPSS Statistics (version 21.0; IBM, Armonk, NY). For the analysis, the results of NAGase and LZ activity in the first 2 time points (4 and 8 h) were gathered and considered as early observations; the other time points (15 and 24 h) were considered as late observations. For proinflammatory cytokine mRNA, only the 4-h observations were considered as early ones, because it was expressed in the first hours after stimulation. Means were compared by 2-way ANOVA and Scheffé post-hoc test. Time and treatment variables were used as main effects, and NAGase, LZ, and cytokine expression as the dependent variables. One-way ANOVA was applied when the time variable was not significant and to analyze the differences between intracellular and extracellular localization of enzymes. 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 1 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 CFS 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 2 *L. lactis* strains was performed in partially purified CFS obtained by the adsorption-desorption method as described in the Materials and Methods. The activity of these partially purified CFS 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 CFS lost their antibacterial activity, contrary to the 2 UF retentates that were further subjected to the SDS-PAGE analysis. For both CFS, a narrow protein band at about 4 kDa was revealed (Supplementary Figure S1; <http://dx.doi.org/10.3168/jds.2015-10161>). The retentates of partially purified CFS 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 CFS (Supplementary Figure S2A and B; <http://dx.doi.org/10.3168/jds.2015-10161>). The accurate mass of the molecules corresponding to both chromatographic peaks was 3,353.55 Da (Supplementary Figure S3; <http://dx.doi.org/10.3168/jds.2015-10161>). 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 S2C).

The live *L. lactis* cultures and the derived CFS 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

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

Antibiotic	<i>Streptococcus agalactiae</i> (n = 3)		<i>Streptococcus dysgalactiae</i> (n = 2)		<i>Streptococcus uberis</i> (n = 3)		<i>Enterococcus faecalis</i> (n = 4)		<i>Staphylococcus aureus</i> (n = 14)		<i>Staphylococcus chromogenes</i> (n = 3)	
	S	R	S	R	S	R	S	R	S	R	S	R
Penicillin	3		2		3		2		5		2	1
Ampicillin	3		2		3		3	1	5		2	1
Amoxicillin/clavulanic acid	3		2		3		4		11		3	
Oxacillin		3	2		3		1	3	11		3	
First-generation cephalosporin	3		2		3		3	1	13		3	
Third-generation cephalosporin	3		2		3		4		13		3	
Fourth-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		2	2	12		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	

cells BME-UV1 was used as an in vitro model because it produces proinflammatory 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, whereas 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 (CFS 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 CFS 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 proinflammatory cytokines by BME-UV1 cells in response to nisin A-containing CFS 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, 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 CFS 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 CFS) reaching 384 AU/mL. This result worth noting

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 IMI. Interestingly, the MIC values and the bactericidal concentrations were almost identical among them for each of the 2 stains, LL11 and SL153. Such a 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 our study, staphylococci were less sensitive than streptococci, on average, but the 2 tested MRSA strains were inhibited or killed at a 1:1 or 1:4 dilution, respectively. The result 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 CFS 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 no decrease in the intracellular activity was detected; consequently, the significance between the LZ activity in the 2 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). Conversely, 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, as its amount increases in milk from clinical and subclinical mastitis (Åkerstedt et al., 2011). Similarly, the significantly higher LZ activity detected in the cells treated with LL11 live culture confirmed the stimulation of lysosomal activity, whereas 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 SCC, but rather to age and lactation stage of cows (Piccinini et al., 2007).

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration (MBC) values showed by *Lactococcus lactis* LL11 and SL153 culture cell-free supernatants against mastitis pathogens, expressed as arbitrary units/mL (AU/mL)

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

As further demonstrated by Bruckmaier (2005), udder tissues play a pivotal role as a primary source of innate immunity factors, expressing proinflammatory cytokines and antibacterial enzymes after challenge with *Escherichia coli* LPS. The treatment with SL153 live culture induced a significant TNF- α mRNA expression downregulation in the cells, but did not influence IL-8 expression. Although proinflammatory cytokines stimulate the host innate immune defenses, 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 IML. Furthermore, cytokines were shown to suppress lacto-

Figure 1. Intracellular activity (dark gray bars) and extracellular release (light gray bars) of N-acetyl- β -D-glucosaminidase (NAGase; A) and lysozyme (B) in untreated (k-), *Lactococcus lactis* LL11 or SL153 cell-free supernatant-treated (btc) or live culture-treated cells. Data are shown as means $\pm$ SD of all measures throughout the 4 time points of the experiment. The mean values of NAGase and lysozyme activity were calculated from 40 measurements each, in duplicate; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 2. Extracellular N-acetyl- β -D-glucosaminidase (NAGase) activity in BME-UV1 cells treated with *Lactococcus lactis* LL11 or SL153 cell-free supernatant (btc), or live culture, in the early observations (t1) and late observations of the experiment (t2). The mean ($\pm$ SD) values were calculated from a total of 20 measurements, in duplicate. Different letters (a-c) indicate significant difference in enzyme production between treatments ($P < 0.05$).

genic function of the glands after infusion (Alluwaimi, 2004) by an autocrine effect inhibiting the synthesis of caseins by mammary epithelial cells (Shea-Eaton et al., 2001). The mRNA expression of IL-8, the other proinflammatory 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.

Figure 3. Extracellular lysozyme activity in BME-UV1 cells treated with *Lactococcus lactis* LL11 or SL153 cell-free supernatant-treated (btc), or with LL11 or SL153 live culture, in the early observations (t1) and late observations of the experiment (t2). The mean ($\pm$ SD) values were calculated from a total of 20 measurements, in duplicate. Different letters (a, b) indicate significant difference in enzyme production between treatments ($P < 0.05$).

Figure 4. Normalized expression of *TNF-α* gene in BME-UV1 untreated (k-) and BME-UV1 cells treated with *Lactococcus lactis* LL11 or SL153 cell-free supernatant (btc), or with LL11 or SL153 live culture. Data are shown as means $\pm$ 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. An asterisk (*) represents a statistically significant ($P = 0.047$) difference between BME-UV1 cells treated with *L. lactis* SL153 live culture and the control.

CONCLUSIONS

The identification of only 2 strains with antibacterial activity, out of 25 LAB tested, indicated that the strains applicable to the dairy cow mastitis are quite rare. *Lactococcus lactis* LL11 and, particularly, SL153 were 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 CFS could kill different mastitis pathogens, including MRSA strains. Moreover, the application of the CFS 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 described above.

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