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Commensal coagulase-negative *Staphylococcus* from the udder of healthy cows inhibits biofilm formation of mastitis-related pathogens

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ABSTRACT

Bovine mastitis, considered the most important cause of economic losses in the dairy industry, is a major concern in veterinary medicine. *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) are the main pathogens associated with intramammary infections, and bacterial biofilms are suspected to be responsible for the persistence of this disease. CNS from the udder are not necessarily associated with intramammary infections. In fact, some commensal CNS have been shown to have biological activities. This issue led us to screen exoproducts from commensal *Staphylococcus chromogenes* for anti-biofilm activity against different mastitis pathogens. The cell-free supernatant from *S. chromogenes* LN1 (LN1-CFS) was confirmed to display a non-biocidal inhibition of pathogenic biofilms. The supernatant was subjected to various treatments to estimate the nature of the biofilm-inhibiting compounds. The results showed that the bioactive compound > 5 KDa in mass is sensitive to thermal treatment and proteinase K digestion, suggesting its protein properties. LN1-CFS was able to significantly inhibit *S. aureus* and CNS biofilm formation in a dose-independent manner and without affecting the viability of bovine cells. These findings reveal a new activity of the udder microflora of healthy animals. Studies are underway to purify and identify the anti-biofilm biocompound and to evaluate its biological activity *in vivo*.

1. Introduction

Bovine mastitis is an inflammatory response of the udder, caused mainly by colonization by microbial pathogens. The susceptibility of cows to mastitis is affected by some factors, including the cow's age, genetic traits, and stage of lactation and nutrition (Sordillo, 2005). This disease has been associated with different levels of economic losses in dairy cattle in different countries. Argentina has been classified as the 17th main milk-producer country in the world (Tiwari et al., 2013). However, in this country, mastitis is still a serious problem, causing more than $0.99/cow/day economic losses for farmers (Vissio et al., 2015). This scenario is worryingly higher than the previously published average economic losses assessed by farmers (Huijps et al., 2008).

Currently, antibiotic therapy is the most common treatment of bovine mastitis-infected dairy cows. However, some of the serious problems associated with this therapy include the low cure rate, the bacterial resistance and the presence of antimicrobial residues in milk (Gomes and Henriques, 2016). A recently evaluated strategy to substitute the administration of antibiotics is the use of natural compounds produced by bacteria. The use of microbiota from healthy organisms has previously shown interesting results in both animals and humans (Bouchard et al., 2015; Iwase et al., 2010).

Coagulase-negative staphylococci (CNS) are a group of bacteria classified as either minor mastitis pathogens or commensal microbiota. Until recently, it was difficult to draw consistent conclusions about the relevance of CNS in bovine udder health. Some studies considered CNS as true mastitis pathogens, although most were retrieved from subclinical mastitis cases (Pyörälä and Taponen, 2009), whereas others considered CNS to be commensal bacteria with limited or absent negative effects on SCC, milk quality, and milk production (De Vliegher et al., 2012). It has been previously recognized that CNS play an important role in the establishment of the cow's microbiome, suggesting specific antibacterial activities in competition against pathogenic strains (Braem et al., 2014).

Biofilms have been proposed as an important virulence factor, involved in the development and maintenance of intramammary infections (Gomes et al., 2016). A biofilm is defined as a sessile microbial community where cells are adhered to a biotic or abiotic surface and

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embedded in a protective extracellular matrix. The biofilm lifestyle seems to play an important role during bacterial infection, providing defense against the host immune system and resistance to antimicrobial treatment (de la Fuente-Núñez et al., 2013; Scherr et al., 2014). Antibiotic therapies for biofilm-associated infections usually require high doses for prolonged times and they often fail (Wu et al., 2014). Thus, the development and discovery of new anti-biofilm agents is currently an urgent demand for clinical practice. Anti-biofilm compounds may act in prevention treatments by blocking biofilm formation or disrupting the microbial community within a biofilm (Ribeiro et al., 2016). Several microorganisms produce and release different compounds to combat pathogenic bacterial biofilms, including molecules that interfere with bacterial communication and signaling and enzymes capable of degrading the extracellular matrix components. An important feature of these microorganisms is their non-microbicidal mechanism of action, not placing an evolutionary pressure to develop bacterial resistance (Blackledge et al., 2013).

The aim of this study was to investigate the anti-biofilm potential of commensal CNS isolated from milk samples of lactating dairy cows. To this end, we determined the spectrum of action of the active bioproducts against different mastitis-causing and biofilm-forming pathogens, and investigated the cytotoxic effects of the anti-biofilm compounds, in view of possible application in udder health.

2. Materials and methods

2.1. Milk sampling and isolation procedure

Milk samples were collected from a dairy farm located in Villa María (Córdoba, Argentina). A total of 168 lactating-cows were first diagnosed according to general symptoms, signs of inflammation and the California Mastitis Test (CMT). Cows were milked every 12 h with pre-sampling disinfection of teat-ends and post-milking teat dipping with iodine disinfectant (Deiod Dip Gel 5000, Lab. Baher, Argentina). Quarter milk samples were selected across a range of CMT score, including sub-clinically infected and clinically mastitic quarters. Healthy quarters from animals with no previous history of mastitis were also included for the isolation of commensal bacteria. After diagnosis, 54 milk samples were obtained from 42 cows. Mammary gland quarters were sanitized and several streams of foremilk were removed prior to sample collection. All milk samples were collected using sterile bottles and kept refrigerated until analysis in our laboratory. Samples were vigorously mixed and an aliquot was used for bacterial culture. Sterile swabs were used to plate in agar-containing growth media, including trypticase soy agar (TSA) and brain heart infusion (BHI) as non-selective media, mannitol salt agar (MSA) for isolation of staphylococci, McConkey medium for isolation of gram-negative bacteria and CHROMagar™ Staph aureus medium for isolation and differentiation of *Staphylococcus aureus* strains. Media were prepared according to the manufacturer's instructions. Plates were incubated at 37 °C for up to 48 h. Colonies with different phenotypes were isolated, purified through successive streaking, and stored at −80 °C in 20% glycerol.

2.2. Phylogenetic identification of isolates

Total DNA was extracted from isolates by the quick-prep of genomic DNA from gram-positive bacteria (Pospiech and Neumann, 1995). Bacterial strains were grown overnight on trypticase soy broth (TSB) medium, centrifuged at 8000 × *g* for 5 min and washed twice with SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris). Pellets were lysed by thermal shock and enzymatic digestion with 10 µg/mL lysozyme (Sigma-Aldrich). Chloroform and isopropanol were used for purification and precipitation of DNA.

PCR amplification of the 16S rRNA gene was performed in a T100 Thermal Cycler (Bio-Rad, USA) using the universal eubacterial primers (27F/1492R) according to the protocol described by Weisburg et al. (1991). Concentrator™-5 (Zymo Research, USA) was used following the manufacturer's recommendations for purification of PCR products. The amplicons were sequenced using an ABI 3130xl Capillary DNA sequencer (Applied Biosystems, USA) at the Genomics and Sequencing Department of the Biotechnology Institute, INTA Castelar, Argentina. The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) and the Basic Local Alignment Search Tool (BLAST) algorithm at the National Center for Biotechnology Information (NCBI) were the databanks used to determine the identity and similarity to the nearest neighbor of the 16S rRNA gene sequences.

The nucleotide sequences identified in this study were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers KY364344-KY364364.

2.3. Biofilm production

The crystal violet assay was used to evaluate the biofilm formation ability of isolates. The staining protocol was performed according to O'Toole and Kolter (1998) with modifications. Bacterial strains were inoculated into TSB medium and incubated overnight at 37 °C. The culture was then diluted in fresh TSB, and 200 µL of 0.5 McFarland culture was transferred to a 96-well polystyrene microtiter plate. Plates were incubated for 24 h at 37 °C, supernatants were removed and adhered cells were washed three times using sterile physiological solution. Biofilms were incubated for 1 h at 60 °C until complete drying. Subsequently, 200 µL of crystal violet solution (0.1% w/v) was added to all wells. After 15 min of staining, the excess crystal violet was removed with distilled water. The fixed crystal violet was released by 97% ethanol for 30 min. An aliquot of 100 µL was then transferred to a new microtiter plate for quantification, according to the 570 nm absorbance in a Multiskan™ FC Microplate Photometer (Thermo Scientific, USA) and expressed in optical density (OD) value. In the assay, sterile TSB was used as blank control and the non-biofilm-forming *Staphylococcus epidermidis* ATCC 12228 was included as negative control. The biofilm formation was quantitatively classified based on the cut-off value as previously described by Stepanovic et al. (2007). The cut-off value ODc was defined as three standard deviations (SD) above the mean OD of the negative control *S. epidermidis* ATCC12228. According to the ODc calculated (0.15), the following classification was established: $OD \leq 0.15$, non-biofilm producer; $0.15 < OD \leq 0.30$, weak biofilm producer; $0.30 < OD \leq 0.60$, moderate biofilm producer; $OD > 0.60$, strong biofilm producer. Within strong biofilm producers, a new group comprising OD values over 1.20 was established, representing hyper-biofilm producers.

2.4. Composition of biofilm matrix

To characterize the chemical composition of pathogenic biofilms, a detachment assay was following the protocol described by Oniciuc et al. (2016) with slight modifications. Briefly, mature biofilms were washed twice with saline solution (0.9% NaCl) to remove not adhered bacteria. Washed biofilms were then treated for 4 h at 37 with: (i) 200 µL of 40 mM sodium meta-periodate (NaIO4) to −1,6-linked polysaccharides degradation, (ii) 200 µL of proteinase K (0.1 mg/mL in 20 mM Tris-HCl:100 mM NaCl, pH 7.5) to proteins degradation, or (iii) DNase I (0.5 mg/mL DNase I (Genbiotech, Buenos Aires, Argentina) in 5 mM MgCl2) to degrade eDNA. Control wells without treatment were included. After (ii) and (iii) treatments, biofilms were washed and residual biofilm was quantified according to crystal violet stain as described above. Considering unspecific reaction within crystal violet and polysaccharides, quantification of $NaIO_4$-treated biofilms was assessed by measuring optical density at 600 nm of sonicated cells suspensions (Oniciuc et al., 2016). Assays were performed in triplicate.

2.5. Preparation of cell-free supernatants (CFSs) from commensal bacteria

Commensal bacteria were grown overnight in TSB without shaking at 37 °C. For CFS preparation, cultures were centrifuged (for 15 min at 8000*g*) and supernatants were then sterilized by passage through a 0.22-μm pore size filter. The resulting CFSs were stored at 4 °C until use.

2.6. Anti-biofilm assays

The anti-biofilm activity of CFSs from commensal *S. chromogenes* LN1 and *S. haemolyticus* LN2 was tested against mastitis pathogens with corroborated abilities of biofilm formation. The test microorganisms were different field isolates and reference strains of *S. aureus* and CNS with different biofilm-forming abilities. The assay included: (1) the best biofilm-forming pathogenic isolates from this study, (2) other pathogenic bacteria previously isolated in our laboratory from subclinical and clinical mastitis milk samples, including *S. aureus* and CNS species (data not published), (3) staphylococcal reference strains, and (4) the wild-type *S. aureus* V329 strain and the mutant strain *S. aureus* V329Δ*ica*, kindly provided by Dr I. Lasa (Instituto de Agrobiotecnología, Pamplona, Spain). Strains not isolated but used in this work are listed in Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetmic.2017.05.025 (Supplementary information).

Staphylococci were cultured overnight at 37 °C in TSB medium. A volume of 150 μL of inoculum (0.5 McFarland) was transferred to a 96-well polystyrene microtiter plate. For anti-biofilm treatment, 50 μL of each CFS or the same volume of TSB as a control was mixed with the inoculum. The plates were incubated statically for 24 h. After incubation, the bacterial suspension of each well was analyzed spectrophotometrically at 600 nm to ensure that there was no microbicidal activity. Finally, the crystal violet assay was performed as described above, to quantify the inhibition of biofilm formation after the treatment.

In an effort to enhance the anti-biofilm activity, the CFSs were concentrated by lyophilization and subsequent dissolution in a smaller volume of sterile TSB medium. Different concentrations of lyophilized CFSs were used and concentrated supernatants (CFS_C) included concentrations from 0.25X to 4X. An anti-biofilm assay was conducted respecting all volume and incubation conditions previously described.

2.7. Confocal microscopic observation of biofilm

To observe the multicellular structures within the biofilms with or without the addition of *S. chromogenes* LN1 cell-free supernatant (LN1-CFS), Confocal Laser Scanning Microscopy (CLSM) was used. Treatment-sensitive pathogenic biofilms were cultured on Lab-Tek II chamber slides (Fisher Scientific, Rochester, NY, USA) under the corresponding conditions. Immediately, samples were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, USA) for 15 min in darkness (room temperature), and the dish contents were gently replaced with TSB buffer according to the manufacturer's instructions. Samples were then directly observed using a confocal laser scanning microscope (Leica DM 6000 CS) with 20× and 60× upright objectives. For each sample, a sequential scan in two channels was carried out and the corresponding xy optical sections and random images were acquired. Overlapping images and orthogonal cuts were obtained by using the Olympus FV 1000 software.

2.8. Physical and chemical analysis of bioactive LN1-CFS

A first characterization of the bioactive compounds on LN1-CFS was developed to approximate their chemical nature. For the protease treatment, Proteinase K (Promega) at a final concentration of 1 mg/mL was added to the supernatant and incubated for 1 h at 37 °C prior to the addition into the wells. TSB fresh medium with 1 mg/mL of Proteinase K (Sigma-Aldrich) was also added to the wells to corroborate that the enzyme did not impair the anti-biofilm activity. The heat stability of the anti-biofilm compounds in CFS was evaluated by incubating LN1-CFS at 100 °C for 15 min and cooling to room temperature prior to the addition into the wells. Size-exclusion filtration was carried out using a 5-kDa Vivaflow 200 PES cross-flow ultrafiltration cassette (Sartorius). Protease- and heat-treated supernatant and filtrates were tested in the anti-biofilm assay described above. Untreated CFSs served as positive controls in all cases and phosphate-buffered saline (PBS) was used as a negative control of anti-biofilm activity.

2.9. Cytotoxicity

Taking into account the potential future application of LN1-CFS in bovine udders, we studied its cytotoxic effects on bovine cell lines. Two cell lines of epithelial origin were used: the MAC-T cells, from bovine mammary alveolar cells (Huynh et al., 1991), and the MDBK cells, derived from bovine kidney (Madin and Darby, 1958). Also, the BoMac cell line (Stabel and Stabel, 1995), derived from bovine macrophages was used as immune system cells. MAC-T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 1 μg/mL hydrocortisone (Fada Pharma, 100 mg), 5 μg/mL bovine insulin (Betasint U-40), 100 U/mL penicillin-100 μg/mL streptomycin and 1/100 CTS™ GlutaMAX™-I Supplement. BoMac and MDBK cells were maintained in culture medium RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin-100 μg/mL streptomycin and 1/100 CTS™ GlutaMAX™-I Supplement. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was renewed every 48 h and the cells were subcultured once/twice a week.

For experiments, cells were seeded at 2×10^5 cells/well in 24-well plates and allowed to attach for 24 h. Bovine cells were then exposed to lyophilized CFSs in culture medium at 0.5, 1, 2, 4 and 8X concentrations for 24 and 48 h. Cells in culture medium without CFS were included in all assays as control group. Cellular viability was studied by the Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) method. Following incubation at 37 °C, the supernatant was discarded and replaced with MTT solution (0.5 mg/mL) in culture medium (without FBS). The plates were incubated for 4 h at 37 °C in darkness. The MTT solution was discarded and the blue crystals were solubilized with dimethyl sulfoxide (DMSO). The intensity was measured colorimetrically at 570 nm. Data represent the mean ± standard error of three wells per treatment and are representative of three experiments.

2.10. Statistical analysis

All experiments were performed in triplicate. One-way ANOVA, independent *t*-test, Bonferroni's post-test and Kruskal-Wallis test were used to analyze data. Mean values were considered significantly different at $P < 0.05$.

3. Results

3.1. Isolation of commensal and biofilm-forming pathogenic bacteria

Based on veterinary diagnosis and CMT, only 38 out of the 168 cows studied (22.62%) presented no evidence of inflammation in all four quarters, indicating healthy animals. Another 26.03% of healthy quarters were detected, but some inflammatory response in the other three quarters of the same animal was also observed. Mastitic quarters represented almost half of the total milk samples, with 42.65% corresponding to sub-clinically and 2.69% to clinically infected quarters. The remaining percentage (6.01%) included not-clearly diagnosed samples.

Plate culturing on different media allowed isolating both pathogenic and commensal bacteria. From 54 milk samples, only 21 isolates were obtained. The highest number of genera was recovered from sub-

Table 1
Phylogenetic affiliation and biofilm formation of the isolated strains.

Isolate	Phylogenetic affiliation		Biofilm formation	
	Closest relative (acc. number)[a]	Identity (%)	Biofilm (OD_{570nm})[b]	Biofilm formation ability[c]
LN1	*Staphylococcus chromogenes* ATCC 43764$^{(T)}$ (D83360)	100	0.33 ± 0.08	M
LN2	*Staphylococcus haemolyticus* ATCC 29970$^{(T)}$ (L37600)	99,89	1.33 ± 0.25	S
L11	*Bacillus sporothermodurans* M215$^{(T)}$ (U49079)	99,71	0.35 ± 0.03	M
L12	*Enterococcus durans* CECT411$^{(T)}$ (AJ420801)	99,89	0.61 ± 0.09	S
L14	*Enterococcus durans* CECT411$^{(T)}$ (AJ420801)	99,89	0.91 ± 0.19	S
L21	*Proteus mirabilis* ATCC29906$^{(T)}$ (ACLE01000013)	100	2.53 ± 0.45	S
L22	*Staphylococcus haemolyticus* ATCC 29970$^{(T)}$ (L37600)	99,78	1.67 ± 0.39	S
L23	*Staphylococcus haemolyticus* ATCC 29970$^{(T)}$ (L37600)	99,78	2.21 ± 0.80	S
L24	*Staphylococcus chromogenes* ATCC 43764$^{(T)}$ (D83360)	100	0.23 ± 0.05	W
L25	*Staphylococcus chromogenes* ATCC 43764$^{(T)}$ (D83360)	99,87	0.62 ± 0.12	S
L26	*Corynebacterium* sp. T2-10$^{(T)}$ (GU904669)	99	1.26 ± 0.21	S
L31	*Streptococcus uberis* JCM 5709 (LC071829)	100	0.27 ± 0.09	W
L32	*Staphylococcus aureus subsp. anaerobius* ATCC 35844$^{(T)}$ (D83355)	100	3.28 ± 0.29	S
L33	*Staphylococcus aureus subsp. anaerobius* ATCC 35844$^{(T)}$ (D83355)	100	3.77 ± 0.69	S
L34	*Enterobacter ludwigii* EN-119$^{(T)}$ (JTLO01000001)	100	0.58 ± 0.19	M
L35	*Enterobacter ludwigii* EN-119$^{(T)}$ (JTLO01000001)	99,88	0.61 ± 0.19	S
L36	*Enterobacter ludwigii* EN-119$^{(T)}$ (JTLO01000001)	99,89	0.34 ± 0.14	M
LMC1	*Enterococcus faecalis* ATCC 19433$^{(T)}$ (ASDA01000001)	100	0.11 ± 0.03	NF
LMC2	*Enterococcus faecalis* ATCC 19433$^{(T)}$ (ASDA01000001)	98,76	0.79 ± 0.28	S
LMC5	*Staphylococcus devriesei* LMG 25332$^{(T)}$ (D83355)	99,87	2.86 ± 0.49	S
LMC7	*Staphylococcus aureus subsp. anaerobius* ATCC 35844$^{(T)}$ (D83355)	100	1.78 ± 0.31	S

[a] The nearest GenBank neighbors for nearly complete 16S rRNA sequences obtained from isolates and accession numbers. The sequences were aligned with related sequences retrieved from the EzTaxon database.
[b] Each data point is composed of three independent experiments performed in quadruplicate. Standard errors are reported.
[c] Biofilm formation ability was classified as: (NF) no biofilm-forming, (W) weak biofilm-forming, (M) moderate biofilm-forming or (S) strong biofilm-forming.

clinical milk samples. The predominant bacterial genus in these samples was *Staphylococcus*, but different species of the genera *Bacillus*, *Enterococcus*, *Proteus*, *Corynebacterium*, *Streptococcus* and *Enterobacter* were also isolated. In mastitis clinically diagnosed samples, the genus *Enterococcus* was found in the same proportion as the genus *Staphylococcus*. Only two bacterial isolates were obtained from healthy animals. Both commensal bacteria, namely LN1 and LN2, were CNS closely related to *S. chromogenes* and *S. haemolyticus*, respectively. The remaining milk samples from healthy quarters were characterized as culture negative.

Table 1 shows the morphological characteristics, phylogenetic affiliation at species level and biofilm formation abilities of the isolated strains. According to the quantitative classification, more than 66% of the isolates were strong biofilm-producers, showing an OD_{570nm} over 0.60. Another 19% was classified as moderate producers, while only three isolates (14.2%) were weak producers or did not produce biofilm. Major biofilm producers were pathogenic isolates, predominantly *S. aureus* or *S. haemolyticus* (Table 1).

3.2. Effect of Staphylococcus chromogenes LN1 exoproducts on biofilm formation of pathogenic bacteria

We next tested the extracts from two different commensal bacteria for their ability to inhibit biofilm formation by *S. aureus* and CNS pathogenic strains in a 96-well microtiter plate assay. Commensal *S. chromogenes* LN1 and *S. haemolyticus* LN2 were isolated from healthy lactating-cows.

Both CFSs were tested at a concentration of 25% by volume. Under these conditions, LN2-CFS did not inhibit the biofilm formation of the strains evaluated. On the other hand, LN1-CFS significantly inhibited biofilm formation by more than a half of 31 biofilm-forming bacteria (Fig. 1). Before crystal violet assay, planktonic bacteria were transferred to a new microtiter plate and optical density (OD_{600nm}) of the treated/non-treated cultures was measured to determine the effects of LN1-CFS over bacterial growth. According to OD_{600nm} non-growth inhibition effect of LN1-CFS was confirmed (Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetmic.2017.05.025, Supplementary information).

It is interesting to note that LN1-CFS showed no anti-biofilm activity against most of the strains isolated in this study. Among these strains, only *Staphylococcus devriesei* LMC5 showed an important biofilm inhibition (86%) after the treatment. *Staphylococcus chromogenes* LN1 supernatant showed an important anti-biofilm effect (from 30 to 90% of biofilm inhibition) on CNS previously isolated in our laboratory, including *S. xylosus*, *S. haemolyticus, S. warneri* and *S. hominis*. LN1-CFS had no effect on the hyper-biofilm formers *S. epidermidis* or *S. aureus* (L32, L33, AU28, AU49, AU 50 and SCN56), whereas it led to an important reduction in the biofilm-forming abilities of the high-biofilm formers *S. aureus* V329 and *S. aureus* V329Δ*ica*. Finally, LN1-CFS also showed inhibitory effects on the biofilm formation of the reference strains *S. aureus* ATCC 8095, *S. aureus* ATCC 43300 and *S. aureus* ATCC 25904 (Fig. 1).

A putative association within biofilm chemical composition and LN1-CFS susceptibility was proposed to hint about the nature and mode of action of the anti-biofilm compound. However, obtained results after NaIO4, Proteinase K and DNase dispersion of pathogenic biofilms (Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetmic.2017.05.025, Supplementary information,) did not yield conclusive findings. It was observed a high percentage of proteins in the matrix of susceptible biofilms, but not relation was found when comparing patterns of anti-polysaccharide and anti-DNA treatments.

To enhance the anti-biofilm activity, different supernatant concentrations were evaluated on the selected strains (Fig. 2). Test strains were selected according to taxonomic identification and inhibitory effects of LN1-CFS, including CFS-sensitive and CFS-resistant *S. aureus* and CNS strains. In treatment-sensitive strains, LN1-CFS showed a dose-dependent inhibitory effect only on biofilm formation by *S. aureus* V329. Inhibition was significantly enhanced when fourfold concentrated supernatant (4X-CFS_C) was used. No significant differences were observed in biofilm inhibition by *S. devriesei* LMC5 or *S. hominis* SCN21 when different CFS_C (4X–0.25X) were used. Finally, no anti-biofilm activity was detected against treatment-resistant *S. aureus* L32 and *S. epidermidis* SCN56, even when using concentrated supernatant

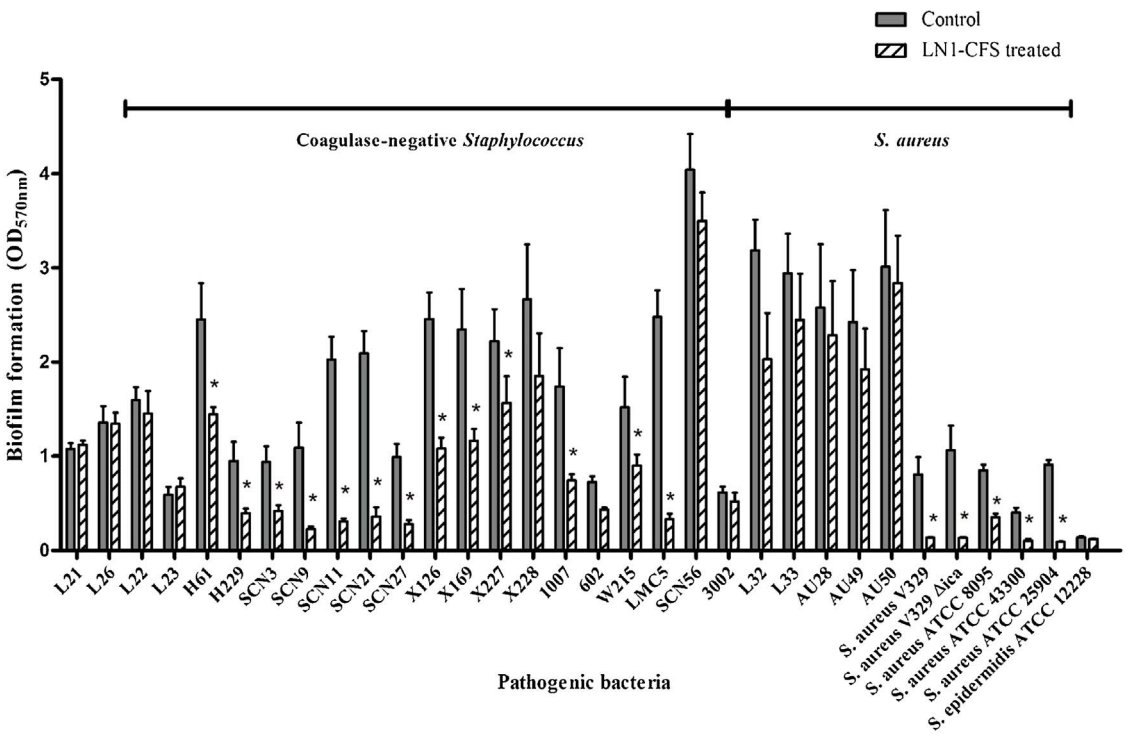

Fig. 1. Inhibition of pathogenic S*taphylococcus* biofilm formation by commensal *Staphylococcus chromogenes* LN1 (LN1-CFS). Values show means from triplicate wells. Error bars indicate range. Asterisks denote values significantly different from non-treated control ($P < 0.05$).

(Fig. 2). These results indicate that the anti-biofilm effects depend on the strain tested.

The anti-biofilm effects of LN1-CFS were also visualized onto glass surfaces by CLSM images (Fig. 3). CLSM was used to analyze the surface topography and the three-dimensional architecture of control and LN1-CFS-treated biofilms of the bacterial species selected. Cell death after LN1-CFS treatment was studied according to a red-fluorescent nucleic acid stain in bacteria with damaged membranes after addition of propidium iodide. LN1-CFS treatment clearly inhibited biofilms at a concentration of 25% vol/vol. A significant reduction in bacterial adherence to surface was observed in all three selected strains after LN1-CFS treatment. No increase of dead cells was observed after the treatment, confirming the lack of antimicrobial effects of LN1-CFS

3.3. Physico-chemical characterization of Staphylococcus chromogenes LN1 anti-biofilm compounds

To perform a preliminary characterization of the biofilm-inhibiting compound, LN1-CFS was subjected to chemical (proteinase K) and physical (thermal) treatments. The percentage of inhibition of each treated aliquot of LN1-CFS was determined against *S. devriesei* LMC5, *S. aureus* V329 and *S. hominis* SCN21 (Table 2). The heat treatment at 100 °C clearly affected the anti-biofilm activity of LN1-CFS. The molecular mass of the biofilm-inhibiting exoproducts was also approximated after an ultrafiltration using a 5-KDa membrane. The anti-biofilm activity in the extract of *S. chromogenes* LN1 was > 5 kDa in mass. In the chemical treatment, biofilm-inhibition effects were abolished after

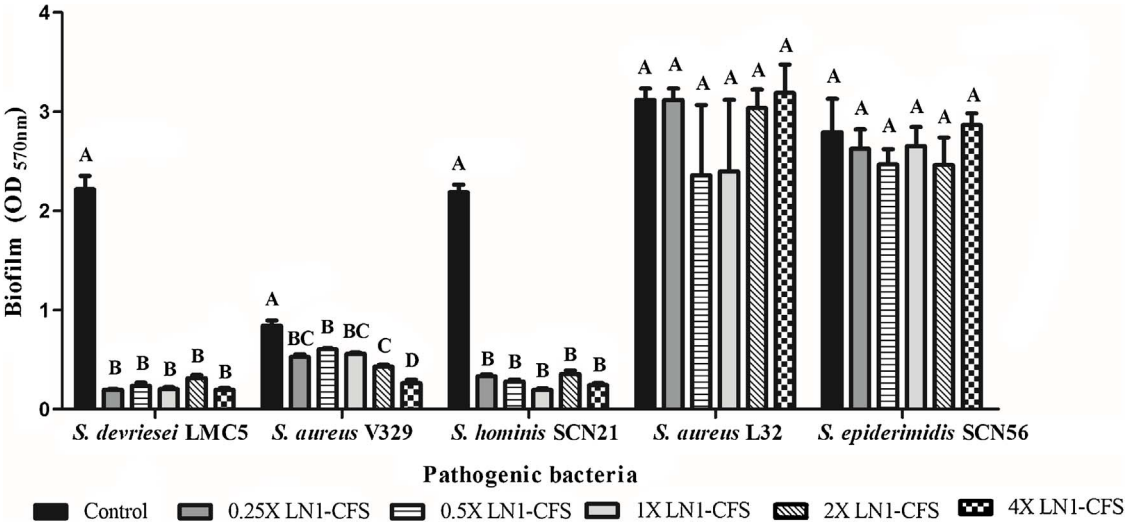

Fig. 2. Inhibition effects of increasing concentrations (0.25–4X) of LN1-CFS on biofilm formation by the treatment-sensitive *S. devriesei* LMC5, *S. aureus* V329, and *S. hominis* SCN21 strains and the treatment-resistant *S. aureus L32* and *S. epidermidis* SCN56 strains. The results are the average of three independent experiments ± SD. Means that do not share a letter are significantly different (ANOVA/Bonferroni, $P < 0.05$).

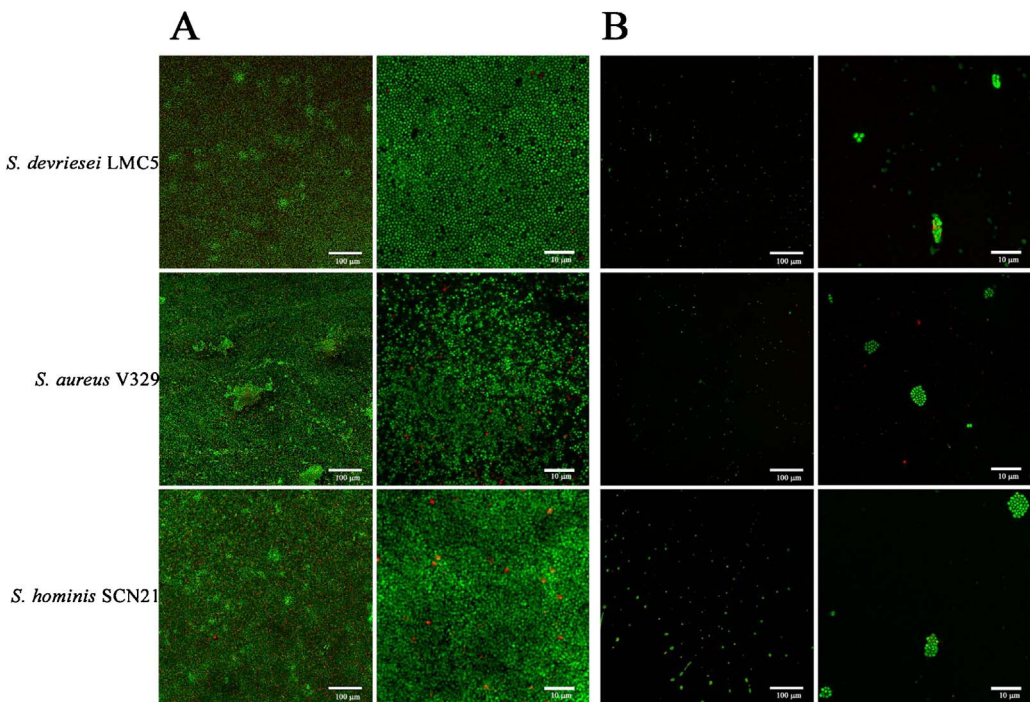

Fig. 3. Confocal Laser Scanning Microscopy (CLSM) images of biofilms formed by *S. devriesei* LMC5, *S. aureus* V329, and *S. hominis* SCN21 in the absence (**A**) or presence (**B**) of LN1-CFS. Scale bars: 100 and 10 µm.

Table 2
Effect of various treatments on the anti-biofilm activity of LN1-CFS.

Supernatant Treatments	Percentage of residual biofilm		
	S. devriesei LMC5	*S. aureus* V329	*S. hominis* SCN21
No treated	9.07 ± 0.42	23.71 ± 1.33	11.09 ± 0.82
Heated, 15 min at 100 °C	89.4 ± 0.65[a]	91.31 ± 1.26[a]	87.54 ± 1.07[a]
Proteinase K digested, 1 h at 37 °C	98.87 ± 2.54[a]	99.26 ± 1.55[a]	97.99 ± 2.76[a]
Filtrated < 5 KDa	94.89 ± 3.02[a]	98.64 ± 2.93[a]	97.39 ± 1.93[a]
Filtrated > 5 KDa	10.45 ± 0.76	21.75 ± 1.02	9.85 ± 0.62

[a]Values significantly different from non-treated supernatant ($P < 0.05$).

proteinase K digestion, indicating that protein compounds are active components of the LN1-supernantant.

3.4. Effects on the viability of bovine cells

Different supernatant concentrations of LN1-CFS were used for 24 and 48 h to evaluate if it caused cytotoxic effects on bovine cell lines by the Thiazolyl Blue Tetrazolium Bromide method. With LN1-CFS treatments of up to 1X, MAC-T, BoMac and MDBK cells exhibited normal cell viability levels and cell phenotypes (Fig. 4). Treatments with higher concentrations of LN1-CFS showed negative effects on cell viability. In this regard, macrophages and kidney epithelial cells decreased their metabolic activity when they were treated with 2, 4 and 8X LN1-CFS, whereas MAC-T cells showed more resistance to high concentrations of LN1-CSF, being affected only when the CFS was four-fold concentrated. After 48 h treatment, the trend was repeated (data not shown).

4. Discussion

Mastitis is considered the most relevant pathology in lactating cows and responsible for major economic losses in the dairy industry worldwide (Melchior et al., 2006). In Argentina, mastitis control has been significantly improved in the last years, but sub-clinical mastitis is still a serious problem (Vissio et al., 2015). The dairy farm studied was in accordance with this feature, because a high prevalence of sub-clinically infected quarters was confirmed. The identification of the bacterial isolates revealed results similar to previous reports, with CNS and *S. aureus* as the most relevant groups of minor and major mastitis–associated pathogens respectively (Dieser et al., 2013).

Due to the incidence of bovine mastitis and to the difficulties associated with its treatment and prevention, many efforts are being made to find out alternative anti-mastitis drugs or therapies (Gomes and Henriques, 2016). Concerning these novel strategies and considering the virulence properties of staphylococcal biofilms, these biofilms are interesting drug targets for the treatment of bovine mastitis. Although the use of anti-biofilm compounds in veterinary medicine has been poorly investigated, these compounds represent an innovative and promising approach in bovine mastitis research (Klein et al., 2015).

Staphylococcus chromogenes and other CNS are common inhabitants of bovine environments but not necessarily responsible for an inflammatory response of the udder (Vanderhaeghen et al., 2014; Piessens et al., 2011). Braem et al. (2014) proposed the production of antibacterial compounds as biological weapons of CNS, considering that, in contrast to lactic acid bacteria, they are not able to acidify the environment. Although commensal *S. chromogenes* has been previously confirmed to exhibit antibacterial activities (Braem et al., 2014; De Vliegher et al., 2004), the main contribution of this study was the demonstration of a new biotechnological potential of a novel strain of this species that displays anti-biofilm but not antimicrobial activity towards mastitis-related pathogens. The nonbiocidal anti-biofilm activity is an important property of biocompounds targeting bacterial virulence and would not provide selective pressure for bacterial resistance (Klein et al., 2015). This interference on biofilm formation has been proposed to have importance in medical applications, and is expected to restore the sensitivity to antibiotics and the response of the host immune system (Karwacki et al., 2013).

Staphylococcus chromogenes LN1 isolated from healthy animal releases protein active compounds > 5 kDa in mass, exhibiting a broad spectrum of biofilm inhibition against *S. aureus* and SCN pathogens. Further assays are needed to characterize the chemical structures of these biocompounds and elucidate their mechanisms of action, but these findings suggest that the commensal microbiome constitutes an underexploited source of active molecules antagonizing bacterial adhesion.

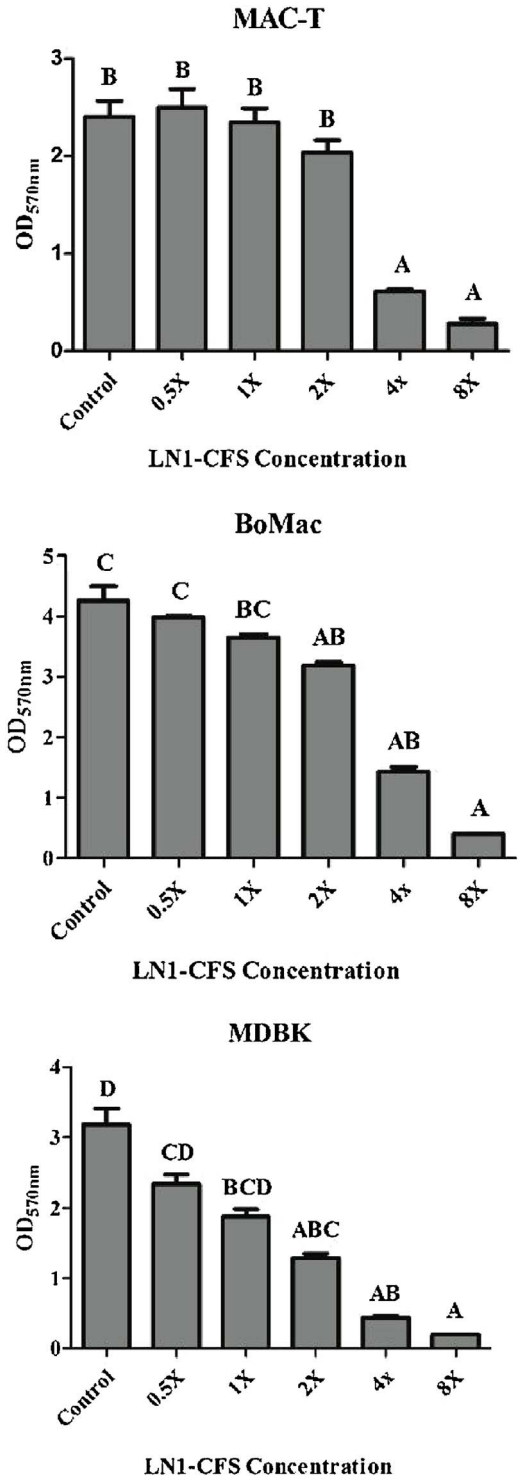

Fig. 4. Effects of LN1-CFS on the viability of bovine cells. Different LN1-CFS concentrations were used for 24 to evaluate its cytotoxic effect on MAC-T (mammary alveolar), BoMac (macrophages) and MDBK (kidney) cell lines by the Thiazolyl Blue Tetrazolium Bromide method. Values are shown as mean ± SE of OD_{570nm}. Different letters indicate significant differences (ANOVA/Bonferroni, $P < 0.05$).

Culture-dependent and -independent techniques have been used to distinguish between healthy and diseased quarters, but until now, there is scarce information about the role of the bovine mammary gland microbiota in the host-pathogen interaction (Falentin et al., 2016). The results obtained in this work are preliminary but very important to address specific research questions about the health-promoting role of commensal bacteria.

Although CNS species have become the bacteria most frequently isolated from bovine mastitis milk, some conflicting aspects about the virulence potential of CNS do not allow confirming the real importance of CNS in udder health (Vanderhaeghen et al., 2014). Isolation of bioactive *S. chromogenes* LN1 from a non-infected quarter suggests its important role in the udder microbiome in competition with pathogenic bacterial strains. CNS have been previously reported to have positive effects on lactating cows not only as antimicrobial agents against major mastitis pathogens but also as enhancers of milk production (Piepers et al., 2009).

LN1-CFS acts preventing biofilm development of the main biofilm-forming mastitis pathogens. In a static system, the biofilm formation of three selected and taxonomically diverse pathogens was almost totally inhibited, lacking the coaggregative behavior of the control untreated biofilms. As inferred by the changes detected in biofilm architecture, the treated biofilms seemingly form very small aggregates, without membrane damage or cell death indicated by the Live/Dead staining.

In most cases, the anti-biofilm properties of LN1-CFS occurred in a dose-independent manner. This is an important feature for prospective application, bioprocess scaling and cytotoxicity evaluation, since the desired results could be obtained by using a minimal concentration.

Our study demonstrates that relevant bovine cells are highly tolerant to direct contact with LN1-CFS. This feature is even more important considering that bacterial cells release not only the anti-biofilm compounds into the medium, but also the common metabolic residues that may be real responsible for the cytotoxic effects. Current therapies against bovine mastitis are administered through the intramammary or parenteral route. In the present study, mammary epithelial cells and bovine macrophages were used as representatives of the intramammary route, whereas kidney epithelial cells were used as representatives of the parenteral route. The most important finding of this study is that although the bovine cell lines used showed differential sensitivity to LN1-CFS, the anti-biofilm concentration was not cytotoxic for any of them, and that MAC-T cells exhibited the highest tolerance against LN1-CFS. Further experiments in animal models will therefore be needed to evaluate the therapeutic activity of LN1-CFS.

5. Conclusion

CFSs from commensal *S. chromogenes* LN1 showed anti-biofilm activity but no antimicrobial effect against major pathogens related to mastitis or cytotoxic activity on bovine cell lines. Considering pathogenic biofilms as important virulence factors that promote bacterial resistance to antibiotics and host immune defense evasion, anti-biofilm compounds represent a promising alternative for the treatment of mastitis. Our study provides a new perspective to evaluate the discussed role of biofilm formation in intramammary infections.

Conflict of interest

The authors report no conflicts of interest in this work.

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