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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 ≤ 0.15, non-biofilm producer; 0.15 < OD ≤ 0.30, weak biofilm producer; 0.30 < OD ≤ 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.

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