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Screening of bacteriocin associated genes of *Streptococcus uberis* strains

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ABSTRACT

A wide variety of contagious and environmental bacteria can cause bovine mastitis worldwide. Antibiotic therapy is currently used for the treatment of the disease, although its intensive use leads to the emergence of resistant strains. Bacteriocins arise as potential antibacterial option for mastitis treatment. The aim of this work was to analyze bacteriocin associated genes as *Streptococcus uberis* ubericin A (*ubaA*), ubericin A immunity protein (*ubaI*), uberolysin A (*ublA*), Lantibiotic nisin-U (*nsuA* and *nsuB*) in 68 *S uberis* strains. Furthermore, the ability of the strains to inhibit important mastitis pathogens was assayed. Results showed that genes were present in combination and all the strains carried at least one gene. Seven bacteriocion associated gene patterns were identified. *S. uberis* strains were able to inhibit different mastitis pathogens and the greatest inhibition was observed in CNS strains. The results obtained provide new insights on antibacterial activity produced by *S. uberis* strains against different mastitis pathogens and could contribute to the development of strategies to treat intramammary infections.

1. Introduction

Bovine mastitis is an infectious disease that affects dairy cows causing reduction in milk production. A wide variety of contagious and environmental bacteria can cause the disease. In order to prevent the disease different procedures are currently utilized, such as post milking teat disinfection, adequate maintenance of milking equipment, antibiotic therapy and culling of chronically infected animals. While the antibiotic therapy is effective and it has a positive impact on dairy herds, the intensive use leads to the emergence of resistant bacterial strains (Sharma et al., 2017). One of the biggest challenges of the dairy industry is to reduce the use of antibiotics in dairy production searching for alternative control methods (Pyörälä, 2002). Bacteriocins arise as potential antibacterial option for the treatment of mastitis.

Streptococcus uberis is a pathogen recovered from the natural environment of dairy cows and produces different types of bacteriocin such as Lantibiotic nisin U, uberolysin A, *Streptococcus uberis* ubericin A, ubericin A immunity protein which have been biochemically and genetically characterized (Wirawan et al., 2006, 2007; Heng et al., 2007).

Previous studies on bacteriocins against mastitis pathogens have been carried out by different researchers (Cao et al., 2007; Wu et al., 2007; Guan et al., 2017). *S. uberis* is usually found in the dairy cattle environment where it could be competing with other pathogens (Pieterse and Todorov, 2010). Nevertheless, no studies to screen bacteriocin associated genes of *S. uberis* isolated from dairy farms have been reported. Thus, the need for further studies on bacteriocins produced by *S. uberis* strains with inhibitory activity against the most prevalent pathogens associated with bovine mastitis arises. Considering this, the aim of this work was to analyze bacteriocin associated genes of *Streptococcus uberis* ubericin A (*ubaA*), ubericin A immunity protein (*ubaI*), uberolysin A (*ublA*), Lantibiotic nisin-U (*nsuA* and *nsuB*) in 68 *S uberis* strains. Furthermore, the ability of the *S. uberis* strains to inhibit important mastitis pathogens was assayed.

2. Materials and methods

2.1. Bacterial isolates

Sixty eight *S. uberis* and fifteen Gram positive and Gram negative mastitis pathogens (*Staphylococcus aureus* (SA-317, SA-318, SA-319, SA-320), *Enterococcus faecalis* (EF-218), *S. agalactiae* (SAG-632), *S. dysgalactiae* (SD-633), *Escherichia coli* (EC-135, EC-136, EC-137, EC-138)), Coagulase-negative staphylococci (CNS) (CNS-546, CNS-547, CNS-548) and *Corynebacterium* spp. (CO-405) were used in this study. All the isolates were collected from subclinical mastitis cases in different herds located in the central dairy region of Argentina. All the isolates

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were collected according to the recommendations of the National Mastitis Council methods (National Mastitis Council (NMC), 2004) without causing stress or harm to the animals.

Milk samples were streaked onto Brain Heart (BH) agar (Laboratorios Britania S.A., Argentina) subcultured on blood agar (supplemented with 5% sheep blood) and then on Mannitol Salt Agar, Tripticase Soy Agar (Laboratorios Britania S.A, Argentina) supplemented with 1% Tween 80 and MacConkey Agar (Laboratorios Britania S.A., Argentina). Identification was performed according to standardize principles and practices (Procop and Koneman, 2017). Colony morphology, pigmentation, and hemolytic characteristics were observed at 48 h. Colonies were subjected for gram staining and biochemical test for the identification of the isolates. Differentiation between staphylococci and other Gram-positive cocci was based on catalase test, mannitol fermentation test, coagulase test. Catalase test was used for the differentiation of Gram positive cocci. Negative catalase isolates were studied for growth at 45 °C and in 6.5% NaCl, Camp reaction, esculin hydrolysis, sodium hippurate hydrolysis, arginine hydrolysis, growth in inulin, mannitol, raffinose, salicin, and sorbitol (Reinoso et al., 2010). From the samples in which growth occurred in MacConkey Agar, lactose fermenting colonies were selected for biochemical tests for enterobacteria such as, triple sugar iron, indole production, lysine decarboxylation, citrate Simmons and urea production.

S. uberis, E. faecalis, S. agalactiae and *S. dysgalactiae* strains were additionally confirmed using molecular techniques as 16S rDNA RFLP (Jayarao et al., 1992; Khan et al., 2003). Furthermore, *S. uberis* strains were previously genotypically characterized by using pulsed-field gel electrophoresis (PFGE) and PFGE profiles were identified in separate studies (Reinoso et al., 2015; Moliva et al., 2017).

The handling of animals was conducted according to the guidelines of the Ethics Committee of Rio Cuarto University (CoEdI). The present study complies with all regulations.

2.2. PCR amplification

DNA extraction was carried out from colonies grown in Triticase Soy agar (TSA) for 18 h at 37 °C. The colonies were resuspended in 1 ml of 1X TE buffer (10 mM Tris - 5 mM EDTA; pH 7.8), centrifugated and resuspended in 1X TE buffer again. Samples were treated with 50 mg/ml of lysozyme and lysed with 20% w/v of SDS in 50 mM Tris - 20 mM EDTA; pH 7.8, 20 mg/ml of proteinase K and 10 mg/ml of RNAse. Protein precipitation was performed with the addition of NaCl 5M. The extracted supernatant was mixed with chloroform-isoamyl alcohol (49: 1 vol/vol). DNA was precipitated by the addition of 2.5 volumes of 100% ethanol and resuspended in buffer TE 1X (10 Mm Tris - 1 mM EDTA; pH 7.5). The DNAs were stored at –20 °C until use.

Bacteriocins associated genes of *ubaA*, *ubaI*, *ublA*, *nsuA* and *nsuB* were investigated by PCR. Specific oligonucleotide primers were designed for this study with PRIMER3 software (http://frodo.wi.mit.edu/primer3/) using each gene sequence deposited in GenBank. All the oligonucleotides were synthesized by Promega Corporation (Promega, Madison, WI, USA). PCR reactions were standardized for the detection of each virulence-associated gene. Primer sequences, thermocycler programs, and the corresponding references are summarized in Table 1. The assay was repeated at least twice. in addition, bacteriocin associated gene patterns were determined to reveal bacteriocin gene variability in the fifteen *S. uberis* strains.

2.3. Inhibitory activity

Inhibitory activity by deferred-antagonism method was performed according to Tagg and Bannister (1979).

Each *S. uberis* strain culture growth of about 1 cm wide was made longitudinally in the center of a Todd-Hewitt (TH; Britania, Argentina) agar plate and incubated in microaerophilia at 37 °C for 18 h. Then, bacteria were inactivated inverting the plate on a filter paper disc embedded with chloroform during 30 min. Paper filter was removed, and the plates were left in a gas extractor hood for 15 min.

Bacterial suspension of each indicator strain cultured in TH broth was suspended in sterile distilled water to reach an optical density equivalent to 3×10^8 according to the McFarland standard (Baron and Finegold, 1990) and 10 μl streaked perpendicularly to the central culture growth line of each *S. uberis* using a calibrated loop. Inhibitory activity was determined by the lack of growth of the indicator strains around the inoculum after 18 h of incubation at 37 °C. The assay was repeated three times on different occasions.

Inhibition activity against three or four indicator isolates was categorized as narrow spectrum and inhibition activity against five or more indicator isolates was categorized as broad spectrum.

2.4. Plasmid extraction

Plasmid extraction was performed according to Anderson and McKay (1983). Briefly, pelleted cells of *S. uberis* grown in TSB media were resuspended in 6.7% sucrose-50 mM Tris-1 mM EDTA, pH 8.0 were lysed with 50 mg/ml of lysozyme and sodium dodecyl sulfate (20%o [wt/vol] in 50 mM Tris-20 mM EDTA, pH 8.0). Plasmid DNA alkaline denaturalization was carried out with OHNa 2N. Protein precipitation was performed with the addition of NaCl 5M. Supernatant was mixed with chloroform-isoamyl alcohol (49: 1 vol/vol) and plasmid DNA was precipitated by the addition of 1 volume of isoamyl alcohol. Plasmids DNA were resuspended in buffer TE 1X (10 Mm Tris - 1 mM EDTA; pH 7.5) and stored at –20 °C until use. Plasmids were visualized by electrophoresis in 0.8% agarose.

3. Results

In the present study, the presence of five bacteriocin associated genes in 68 *S uberis* strains was determined by PCR assays. Results revealed that all strains assayed carried at least one gene. In addition, not all genes were present in the strains, although combination of bacteriocin associated genes were found. Fig. 1 shows gene amplification products of *ubaA*,

Table 1
Primers and programs used in the amplification of bacteriocin associated genes.

Target Gene	Primer Sequence 5′-3′		Programe	Accession number	Predict size bp
ubaA	ATCGGTGGCAAAACTGTAAA	GCCCGTTCATGATGGAATTA	93 °C 3m, (93 °C 1m, 50 °C 1m, 72 °C 1.30 m) x 30, 72 °C 5m	EF203953	115 bp
ubaI	CTTTGCATGCTCAAGGGAAT	CATAGCGGATATTGGAAATCG	94 °C 2m, (93 °C 1m, 52 °C 1m, 72 °C 1.30 m) x 30, 72 °C 8 m	EF203953	216 bp
ublA	GGGATAGCCTCAGGTACTGC	AGCTGAGGCTGAAACTGCTC	93 °C 3m, (93 °C 1.30m, 57 °C 30 s, 72 °C 1.30 m) x 30, 72 °C 5 m	DQ650653	129 bp
nsuA	TGAAGATTTTAATTTGGATCTCATCA	TGACAACCACAGGTTGCAGT	94 °C 3m, (93 °C 1m, 51 °C 1.30 m, 72 °C 1.30 m) x 30, 72 °C 8 m	DQ146939	150 bp
nsuB	TCCCCATATGATCTGGCAAT	CTGATTATCAACCCGCGAAT	94 °C 2m, (93 °C 1.20m, 52 °C 1m, 72 °C 1.30 m) x 30, 72 °C 5 m	DQ146939	374 bp

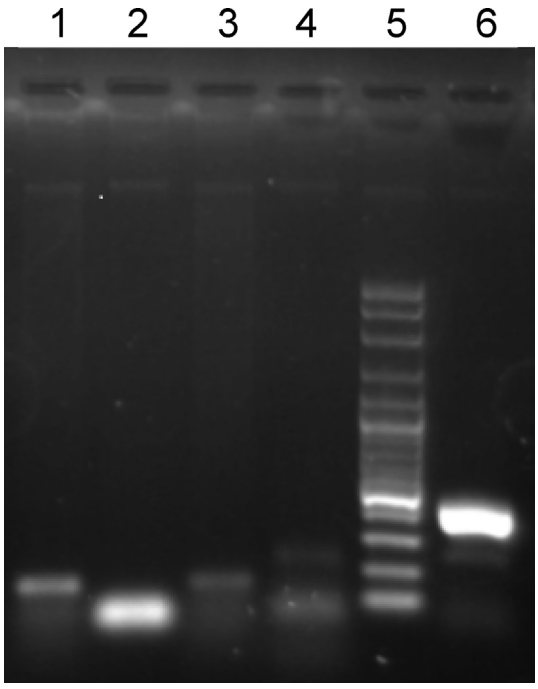

Fig. 1. Typical amplicons of *S. uberis* bacteriocin associated genes. Lane 1: *ublA* gene; lane 2: *ubaA* gene; lane 3: *nsuA* gene; lane 4: *ubaI* gene and *ubaA* gene; lane 5: 100 bp molecular weight marker (Promega) and lane 6: *nsuB* gene. Please see Supplementary Fig. 1 for original gel.

ubaI, *ublA*, *nsuA* and *nsuB*.

ubaI gene was the most common bacteriocin associated gene in the examined strains (86.76%; 59/68), whereas *ubaA* gene was found in 52.94% (36/68) of the strains. *ublA* gene, was found at in the 82.35% (56/68), indicating that most of the strains harbored this gene. In addition, *nsuA* and *nsuB* genes were harbored by 27.9% (19/68) and 33.82% (23/68) of the strains, respectively.

Seven bacteriocin associated gene patterns were identified. Pattern I (*ubaI*+, *ubaA*+, *ublA*+) was the most prevalent in four *S. uberis* strains (SU8, SU58, SU200, SU213). Pattern VI, all bacteriocin associated genes positive, was identified in two strains (SU151, SU210). The remaining 8 strains showed different patterns (Table 2).

Genotypic relationships among 68 *S uberis* isolates were previously assayed by PFGE (Reinoso et al., 2015). Macro restriction analysis revealed thirty-seven PFGE profiles. Among them, eleven strains (SU8, SU50, SU58, SU90, SU106, SU150, SU151, SU177, SU200, SU210 and SU213) yielding more than three bacteriocin associated genes were selected for their action against fifteen mastitis pathogens by deferred-antagonism method (Table 2). Two strains (SU8 and SU58) had the same bacteriocin pattern and PFGE profile.

Table 2
PCR amplification of bacteriocin genes in the selected *S. uberis* strains.

S. uberis strains	*ubaI*	*ubaA*	*ublA*	*nsuA*	*nsuB*	Bacteriocin associated gene pattern	PFGE profile
SU8	+	+	+	-	-	I	A
SU50	+	-	+	+	+	II	Q
SU58	+	+	+	-	-	I	A
SU90	+	+	+	-	+	III	O
SU106	-	+	+	-	+	IV	D
SU150	+	+	+	+	-	V	P
SU151	+	+	+	+	+	VI	O
SU177	+	+	-	-	+	VII	F
SU200	+	+	+	-	-	I	N
SU210	+	+	+	+	+	VI	C
SU213	+	+	+	-	-	I	K

(+) Presence; (-) Absence.

In addition, it was found that the eleven *S. uberis* strains carried plasmids. All plasmids had a high molecular weight (Fig. 2). PCR assay of plasmids isolated from each *S. uberis* strain showed that no plasmid yielded PCR products of the bacteriocin associated genes.

Results showed that SU200 and SU210 expressed a narrow spectrum showing inhibition activity against three indicator isolates and yielded PCR amplicons for three and five bacteriocin associated genes, respectively (Tables 2 and 3). SU213 also showed a narrow spectrum showing inhibition activity against four isolates and yielded three bacteriocin associated genes. On the other hand, eight strains (SU8, SU50, SU58, SU106, SU90, SU151, SU150 and SU177) expressed a broad-spectrum action inhibition activity against five or more than five indicator strains tested. SU8, SU50 and SU177 strains showed inhibition activity against seven and six isolates and yielded PCR products for three and four bacteriocin associated genes. SU58 showed inhibition activity against five isolates and yielded PCR products for three bacteriocin associated genes. SU90 showed inhibition activity against ten of the indicator strains and yielded four bacteriocin associated genes. SU151 showed inhibition activity against eleven of the indicator strains and yielded five bacteriocin associated genes. Whereas SU106 and SU150 strains, showed inhibitory activity against thirteen indicator isolates and yielded three and four amplicons of the genes, respectively.

Overall, results showed that CNS were the species that showed greater susceptibility. In addition, the remaining isolates as *S. aureus, E. coli, E. faecalis* and *Corynebacterium* spp. were also inhibited by different strains of *S. uberis*. On the other hand, *S. agalactiae* SAG-632 and *S. dysgalactiae* SD-633 were also assayed, although no inhibition was observed.

4. Discussion

Bacteriocin associated genes as *ubaI, ubaA, ublA, nsuA* and *nsuB* in 68 *S uberis* strains were examined in this work. Additionally, the ability of the strains to inhibit important mastitis pathogens was assayed. To our knowledge this is the first study that investigates the presence of *S. uberis*

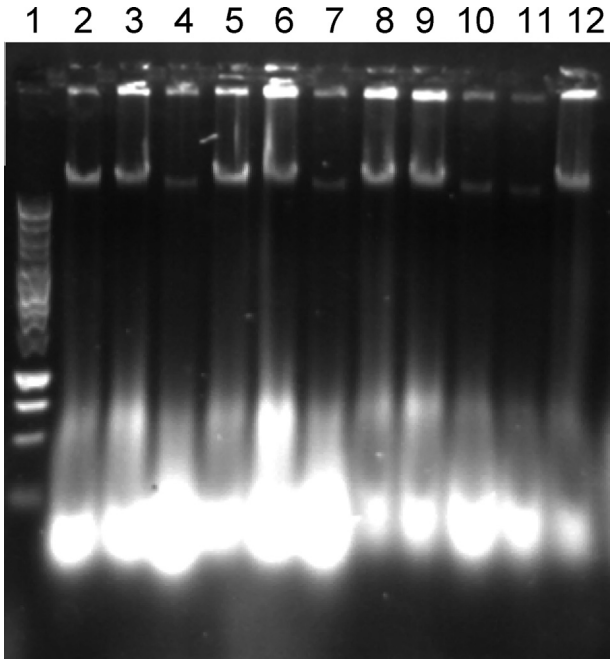

Fig. 2. Plasmid isolates from *S. uberis* strains in 0.8 % agarose gel. Lane 1: 1 kb DNA marker; lanes 2 to 12: SU8, SU50, SU58, SU90, SU106, SU150, SU151, SU177, SU200, SU210 and SU213 strains. Please see Supplementary Fig. 2 for original gel.

Table 3
Inhibitory activity of *S. uberis* strains against mastitis pathogens.

Indicator strains	*Streptococcus uberis* strains										
	SU8	SU50	SU58	SU90	SU106	SU150	SU151	SU177	SU200	SU210	SU213
E. coli EC-135	++	+	+	++	+	+	+	++	-	-	++
E. coli EC-136	-	-	-	++	++	+	+	-	-	-	-
E. coli EC-137	-	-	-	++	+++	+++	++	++	-	-	-
E. coli EC-138	-	-	-	-	++	+++	-	-	-	-	-
S. aureus SA-317	+++	++	-	+++	++	+++	+	+	-	-	-
S. aureus SA-318	-	-	++	-	+	++	+	-	++	-	-
S. aureus SA-319	-	-	-	-	+	+	-	-	++	-	++
S. aureus SA-320	-	-	-	+	++	++	+	-	-	-	-
CNS-546	++	++	+	++	++	+++	++	+++	++	-	++
CNS-547	+	++	+++	++	+++	++	++	+	-	+	-
SCN-548	++	++	++	++	+++	+++	+	++	-	+	-
E. faecalis EF-218	++	++	-	++	++	++	++	-	-	-	-
Corynebacterium spp CO-405	++	-		+	++	+	+	-	-	+	++
S. agalactiae SAG-632	-	-	-	-	-	-	-	-	-	-	-
S. dysgalactiae SD-633	-	-	-	-	-	-	-	-	-	-	-

-: no zone; +: zone ≤10 mm; ++: zone between <10 mm ≤ 20 mm; +++: zone >20 mm.

bacteriocin associated genes in a high number of strains and the bacteriocin associated gene patterns.

A high percent of the *S. uberis* strains assayed yielded *ubaI* and *ublA* genes. The *uba* locus, which includes *ubaA* and *ubaI* genes, was characterized in *S. uberis* strain E by Heng et al. (2007). These authors reported that *ubaI* gene was also present in *S. uberis* strain O140J, although 0140J strain lacks *ubaA gene*. In this work, *ubaA* was yielded by 34 of the *S. uberis* strains assayed. As far as we know there are no screening studies on *uba* locus of *S. uberis* strains. Uberolisin locus, which comprises *orf1, ublA, ublB, ublC, ublD* and *ublE* genes was characterized by Wirawan et al. (2007) in *S. uberis* strain 42. These authors reported that *ublA* could be amplified by PCR in 18% of 45 *S uberis* tested (Wirawan et al., 2007), including *S. uberis* 0140J. Moreover, results obtained in this study showed that *ublA* gene could be amplified in most of the strains indicating that the gene is present in *S. uberis* strains tested. Nisin U, closely similar to nisin A from *Lactococcus lactis*, was described by Wirawan et al. (2006) in *S. uberis* strain 42. The locus contains 11 open reading frames. In this work, *nsuA* and *nsuB* genes were harbored by a low percent of the strains. Similar results were obtained by Wirawan et al. (2006) who reported that 22% of the strains were positive to *nsuA* gene tested by dot blot and colony PCR.

Previously, DNA macrorestriction analysis of the 68 *S uberis* strains was assessed by PFGE (Reinoso et al., 2015; Moliva et al., 2017). Eleven *S. uberis* strains, yielding more than three bacteriocin associated genes, were selected from thirty-seven PFGE profiles. Plasmid extraction and antimicrobial activity were carried out with the 11 selected strains. No bacteriocin associate gene in plasmids could be amplified, suggesting that bacteriocin genes were localized in the chromosome. These results are in accordance with those reported in the literature (Heng et al., 2007; Wirawan et al., 2006, 2007).

Bacteriocins can provide an alternative to antibiotics in the treatment of intramammary infections due to their antibacterial activity, stability and low toxicity (Cotter et al., 2013). A study reported the use of nisin for mastitis (Cao et al., 2007). Nevertheless, this is the first report that investigated the presence of bacteriocin associated genes produced by *S. uberis* strains. Results showed that *S. uberis* strains yielded bacteriocin genes in different combinations. In the present study, the *S. uberis* strains assayed, showed inhibition against different mastitis pathogens. According to previous reports (Heng et al., 2007; Wirawan et al., 2006) ubericin A and nisin U showed no inhibition activity against *S. aureus*, whereas uberolysin inhibited one of the two *S. aureus* strains tested (Wirawan et al., 2007).

Furthermore, the strains assayed in this work were able to inhibit different mastitis pathogens and the greatest inhibition was observed in CNS strains. As CNS strains are considered emerging pathogens (De Visscher et al., 2017), bacteriocins produced by *S. uberis* could contribute to the development of strategies to treat intramammary infections caused by different pathogens focused on CNS.

In addition, the Gram-negative inhibitory spectrum detected in this study is important since bacteriocins are more effective against Gram-positive bacteria. The outer membrane of Gram negative, acts as a barrier against some antimicrobials, detergents and dyes (Gyawali and Ibrahim, 2014). However, *S. uberis* strains could inhibit Gram-negative strains.

The results obtained provide new insights on antibacterial activity produced by *S. uberis* strains against different mastitis pathogens and could contribute to the development of strategies to treat intramammary infections.

Declarations

Author contribution statement

Mirta Lasagno: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Maria de los Angeles Navarro: Performed the experiments. Melina Moliva: Performed the experiments; Analyzed and interpreted the data. Elina Reinoso: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Additional information

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