Food Control 32 (2013) 313–321

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Identification of new staphylococcins with potential application as food biopreservatives

Andreza Freitas de Souza Duarte [a], Hilana Ceotto [a], Marcus Lívio Varella Coelho [a], Maria Aparecida Vasconcelos de Paiva Brito [b], Maria do Carmo de Freire Bastos [a,*]

[a] Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, CCS, Bloco I, Cidade Universitária, 21941-902, Rio de Janeiro, RJ, Brazil
[b] Embrapa Gado do Leite, Juiz de Fora, MG, Brazil

ARTICLE INFO

Article history:
Received 14 May 2012
Received in revised form
14 September 2012
Accepted 8 December 2012

Keywords:
Bacteriocins
Food preservative
Staphylococcus
Milk
Bovine mastitis
Hyicin 4244

ABSTRACT

In this study, 47 staphylococcal strains, isolated from milk of mastitic cattle from Brazilian dairy herds, were tested for antimicrobial substance (AMS) production. Fourteen strains were shown to produce AMS. Most AMS were sensitive to proteolytic enzymes, suggesting that they might be bacteriocin-like inhibitory substance (BLIS). BLIS 4059, 4231, 4244, 5409, and 5580 exhibited a broad spectrum of activity and, therefore, were selected for further studies. The five BLIS$^+$ strains were identified to the species level: 4059 and 4231 as *Staphylococcus aureus,* 4244 and 5409 as *Staphylococcus hyicus*, and 5580 as *Staphylococcus epidermidis.* Cross-immunity analysis and detection of the *aurABCD* operon by PCR and DNA/DNA hybridization revealed that strains 4059 and 4231 produce BLIS either identical or similar to aureocin A70 and encoded on plasmids with a size similar to that of pRJ6 (7.9 kb). A structural gene similar to those of epidermin and Bsa was detected in the genome of strain 5409. The BLIS$^+$ strains 4244 and 5580 produce BLIS that seem to be distinct from the best characterized staphylococcal bacteriocins described in the literature. The BLIS produced by *S. hyicus* 4244, hyicin 4244, had the ability to inhibit all 30 strains tested as targets, including food-borne pathogens, such as *Listeria monocytogenes* and *S. aureus*. Both pathogens were also inhibited in a food matrix, suggesting that hyicin 4244 has potential biotechnological application as a food biopreservative.

1. Introduction

Milk and dairy products are important components of a healthy diet, although, if consumed unpasteurized, they can also represent a health hazard due to possible contamination by pathogenic bacteria. A study released by CDC in February 2012 examined the number of dairy outbreaks in the United States between 1993 and 2006, and 60% of them were linked to raw milk products (Langer et al., 2012). Bovine mastitis is the disease that has the most significant impact on milk quality, since it is the most prevalent disease in dairy cattle worldwide (LeJeune & Rajala-Schultz, 2009). Bovine mastitis results in significant financial costs to the dairy industry due to decreased milk production and quality, discarded milk, drugs, veterinarian labor, culling, and occurrence of other diseases (Bradley, 2002; Huijps, Lam, & Hogeveen, 2008; Viguier, Arora, Gilmartin, & O'Kennedy, 2009).

Although several bacterial pathogens can cause mastitis, *Staphylococcus aureus* is generally the most important etiologic agent in mastitis of cows and female buffaloes (Kumar, 2009; Olde Riekerink, Barkema, Kelton, & Scholl, 2008; Piccinini, Borromeo, & Zecconi, 2010). Moreover, *S. aureus* is probably the most infectious agent because it causes a chronic and deep infection in the mammary glands that is extremely difficult to eradicate (Brouillette & Malouin, 2005). Coagulase-negative staphylococci (CoNS) have traditionally been considered to be minor mastitis pathogens. However, the significance of CoNS needs to be reconsidered as they have become the most common subclinical mastitis-causing agents in many countries. CoNS can cause persistent infections that can result in damage to the udder tissue, which leads to decreased milk production. *Staphylococcus simulans* and *Staphylococcus chromogenes* are currently the predominant CoNS species involved in bovine mastitis. *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* also represent important pathogens associated to this disease (Pyörälä & Taponen, 2009; Schukken et al., 2009; Simojoki, Hyvönen, Plumed Ferrer, Taponen, & Pyörälä, 2012).

Two traditional strategies used against bacterial diseases in farms are vaccination and antibiotic treatment. However, the

* Corresponding author. Tel./fax: +55 21 25608344.
E-mail addresses: mcbastos@micro.ufrj.br, mcbastos2@yahoo.com.br (M.C.F. Bastos).

0956-7135/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2012.12.008

indiscriminate use of antibiotics can contribute to the emergence of antibiotic-resistant pathogens, which can be transmitted to the consumers by milk. In addition, this treatment may contaminate the milk with residual antibiotics for some days after their administration, causing problems for consumers (Brito & Lange, 2005; Sawant, Sordillo, & Jayarao, 2005). Thus, the identification of alternative methods for controlling mastitis is essential. One of these methods could be the use of bacteriocins (Bac).

Bacteriocins are antimicrobial peptides or proteins produced by bacteria which have inhibitory or bactericidal activity against other bacteria (Heng, Wescombe, Burton, Jack, & Tagg, 2007; Jack, Tagg, & Ray, 1995). The bacteriocin-producing strains are immune to their own products and this immunity is conferred by an immune system which is generally expressed concomitantly with the bacteriocin structural genes (Bastos, Ceotto, Coelho, & Nascimento, 2009; Heng et al., 2007). Bacteriocins possess potential biotechnological application as food preservatives since they are effective against key pathogens of importance in food-borne illnesses, including *Listeria monocytogenes*, an important pathogenic bacterium common in the environment and difficult to control in foods (Gálvez, Abriouel, López, & Omar, 2007). Moreover, they are generally recognized as safe, non-toxic on eukaryotic cells and become inactivated by digestive proteases, having little influence on the gut microbiota (Gálvez et al., 2007). Additionally, the bacteriocins can be used for the treatment and prevention of some bacterial infections, including bovine mastitis (Coelho et al., 2007; Gálvez et al., 2007; Klostermann et al., 2010).

Staphylococcal strains can produce bacteriocins, known as staphylococcins. *S. epidermidis* and *S. aureus* are the most important staphylococcin producers investigated so far. In *S. epidermidis*, some staphylococcins have been characterized, such as the lantibiotics epidermin (Augustin et al., 1992), Pep 5 (Ersfeld-Dressen, Sahl, & Brandis, 1984), epilancin K7 (van de Kamp et al., 1995), and epicidin 280 (Heidrich et al., 1998), and the class II bacteriocin epidermicin NI01, which is related to aureocin A53 (Sandiford & Upton, 2012). Epidermin and its variants seem to be the most frequently produced lantibiotics in the group of CoNS, being reisolated many times from strains of *S. epidermidis* and also from other staphylococcal species (Bastos et al., 2009). Nukacin 3299 is a lantibiotic produced by *S. simulans* 3299, which is identical to nukacin ISK-1 produced by *Staphylococcus warneri* (Ceotto et al., 2010). Other well characterized staphylococcins are produced by *S. aureus*, such as aureocin A70 (Netz et al., 2001), aureocin A53 (Netz et al., 2002), and staphylococcin C55 (Navaratna, Sahl, & Tagg, 1998). Recently, a novel epidermin-like lantibiotic (Bsa) produced by *S. aureus* has been reported (Daly et al., 2010).

In the present work, bacteriocin production was investigated in *Staphylococcus* spp. strains isolated from milk of cows and female buffaloes with bovine mastitis in different Brazilian dairy herds aiming to find new staphylococcins with potential biotechnological applications. In the course of this study, a new bacteriocin-like inhibitory substance (BLIS), named hyicin 4244, was detected and its action against *S. aureus* and *L. monocytogenes* in skimmed milk was tested.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Staphylococcus spp. strains isolated from milk of either cows (35) or female buffaloes (12), all with bovine mastitis, from 33 different dairy herds located in the southeast region of Brazil, were tested for antimicrobial substance (AMS) production.

Other staphylococcal strains from previous studies were employed in the present work either as bacteriocin producers or as targets in the assay of cross-immunity, and as controls for the presence of bacteriocin-structural genes. These strains are described in Table 1.

The staphylococcal strains and the other bacteria used as targets, with exception of the lactic acid bacteria (LAB), were cultivated in either BHI (Difco, Sparks, USA) or TSB (Difco) at 37 °C for 18 h and stored in TSB with 40% glycerol (w/v) at −20 °C. The LAB strains were cultivated in MRS (Difco) at either 30 °C or 37 °C for 18 h. When necessary, the media were supplemented with agar at 1.5% (w/v) or 0.6% (w/v).

2.2. Identification of the strains

The staphylococcal strains that exhibited AMS production were identified to the species level based on Gram staining followed by the use of a commercial kit for bacterial identification (API Staph, BioMérieux, Marcy-l'Etoile, France) and conventional biochemical tests (Bannerman & Peacock, 2007). The identification of the staphylococcal strain 4244 was also confirmed by 16S rDNA sequencing as described by Fagundes et al. (2011).

2.3. Assay for antimicrobial substance production

The investigation of AMS production was performed on BHI medium using the deferred-antagonism assay as previously described by Giambiagi-deMarvaL, Mafra, Penido, and Bastos (1990). *Corynebacterium fimi* NCTC 7547, which is highly sensitive

Table 1
Staphylococcal strains previously described and used in this study.

Strains	Relevant features	Bacteriocin structural genes	Reference
S. aureus A53	Bac$^+$ (aureocin A53); pRJ9 (10.4 kb)	*aucA*	Netz et al., 2002
S. aureus A70	Bac$^+$ (aureocin A70); pRJ6 (7.9 kb)	*aurABCD*	Netz et al., 2001
S. aureus A70 Bac$^-$	Strain A70 cured of pRJ6	–	Giambiagi-deMarvaL et al., 1990
S. aureus C55	Bac$^+$ (staphylococcin C55); pC55 (32 kb)	*sacαA* and *sacβA*	Navaratna et al., 1998
S. epidermidis Tü3298	Bac$^+$ (epidermin); pTü32 (54 kb)	*epiA*	Allgaier, Jung, Werner, Schneider, & Zähener, 1986
S. epidermidis K7	Bac$^+$ (epilancin K7)	*elkA*	van de Kamp et al., 1995
S. epidermidis 5	Bac$^+$ (Pep5); pED503 (20 kb)	*pepA*	Ersfeld-Dressen et al., 1984
S. epidermidis 5 Bac$^-$	Strain 5 cured of pED503	-	Ersfeld-Dressen et al., 1984
S. epidermidis BN280	Bac$^+$ (epicidin 280); pCHO1 (>40 kb)	*eciA*	Heidrich et al., 1998
S. simulans 3299	Bac$^+$ (nukacin 3299); pRJ97 (>25 kb)	*nukA*	Ceotto et al., 2010
S. aureus COL1881	Bac$^+$ (Bsa)	*bsaA2*	Daly et al., 2010
S. aureus MB32	Bac$^+$; pSH2 (15 kb), pRJ6 (7.9 kb), pRJ5 (2.5 kb)	*aurABCD*	Ceotto et al., 2009
S. aureus MB196	Bac$^+$; pRJ15 (27 kb), pRJ10 (10.4 kb), pRJ16 (4.4 kb), pRJ17 (1.2 kb)	*aucA*	Ceotto et al., 2009

Bac$^+$, bacteriocin production; Bac$^-$, no bacteriocin production.

to staphylococcins (Bastos et al., 2009), was used as the target micro-organism in this assay.

Later, using the same procedure, 30 different Gram-positive bacteria were used as targets for determination of the spectrum of activity of each AMS: *Lactobacillus casei* ATCC 398, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *cremoris*, *L. lactis* 2084, *L. lactis* NZ 9000, *Micrococcus luteus* ATCC 4698, *Enterococcus faecium* E89, *Enterococcus faecalis* 2758, *L. monocytogenes* 11/LM, *L. monocytogenes* L1/L2a, *L. monocytogenes* 7898, *Listeria innocua* 397, *L. innocua* ATCC 33090, six *L. monocytogenes* strains (L_7, L_9, L_{12}, L_{29}, L_{30} and L_{32}) isolated from sausages and 11 staphylococcal strains isolated from cheeses ($QFrH_2$ and QM_2), salads ($5S_9$, $6H_1b$, $13H_1$ and $13S_2$), and sausages (Sal_{12}, Sal_{17}, LI_1, LI_2 and L_1N_4).

2.4. Effect of proteolytic enzymes and of sodium hydroxide

The effect of trypsin, protease XXIII, pronase, and proteinase K [Sigma–Aldrich (St. Louis, USA); 1 mg ml^{-1} prepared in Tris/HCl 50 mM, pH 8.0 − $CaCl_2$ 10 mM], and of 0.2 N NaOH on AMS activity was determined on solid medium as described by Giambiagi-deMarvaL et al. (1990).

2.5. DNA isolation and manipulations

The plasmidic DNA of staphylococcal lysates of the AMS^+ strains was isolated as described by Giambiagi-deMarvaL et al. (1990) and the plasmid profiles were analyzed by agarose gel electrophoresis [0.7% (w/v) prepared in Tris 40 mM, acetate 40 mM and EDTA 1 mM, pH 8.4].

The genomic DNA from the AMS^+ strains used in the PCR reactions and in Southern blotting was isolated as described by Potter et al. (2009). For Southern blotting, the genomic DNA (1 μg) was previously digested with the restriction enzymes *Eco*RI (Invitrogen, Carlsbad, USA) or *Hin*dIII (Invitrogen) according to the manufacturer's recommendations.

2.6. Detection of staphylococcin structural genes by PCR

PCR reactions were performed to detect, in the AMS^+ strains, the presence of the operon *aurABCD*, and of the genes *aucA*, *sacαA*/*sacβA*, *pepA*, *epiA*, *elkA*, *eciA*, *nukA*, and *bsaA*2 (the structural genes of aureocin A70, aureocin A53, staphylococcin C55, Pep5, epidermin, epilancin K7, epicidin 280, nukacin 3299, and Bsa, respectively). With exception for the latter, the primers used for amplification of each gene and the PCR conditions were essentially those previously described by Ceotto, Nascimento, Brito, and Bastos (2009). The presence of the *bsaA*2 structural gene was assessed using the primers BsaF (5′ −TTAACAGCAGAAGCTATTAAAACTACCAG- 3′) and BsaR (5′-ATGGAAAAAGTTCTT GATTTAGACG-3′) (A. Potter, unpublished data) in the same conditions, except for the the annealing temperature which was 54 °C. The amplicons were analyzed by agarose gel electrophoresis [1.4% (w/v)], using a 100-bp DNA ladder (Promega, Madison, USA) as the molecular-weight marker.

2.7. DNA/DNA hybridization assays

To detect the structural genes *aurABCD*, *aucA*, *sacαA*/*sacβA*, *pepA*, *epiA*, *elkA*, and *eciA*, Southern blot hybridizations were performed using the genomic DNA previously digested with *Eco*RI, and to detect *nukA*, the genomic DNA was digested with *Hin*dIII.

The digested genomic DNAs were separated by agarose gel electrophoresis [0.7% (w/v)] and blotted onto nylon membranes (Hybond-N; GE Healthcare, Piscataway, USA), using standard methods as described by Sambrook, Fritsch, and Maniatis (1989). The amplicon corresponding to each staphylococcin structural gene was purified by the Wizard SV Gel and PCR Clean-Up System (Promega) and used as probes. Labeling and DNA/DNA hybridizations were performed using the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

In both types of experiments (PCR and hybridization), the genomic DNA isolated from the producer strain of each staphylococcin under investigation was used as a positive control.

2.8. Pulsed-field gel electrophoresis

The genomic DNAs from strains 4059, 4231, 4244, 5409, and 5580 were typed by pulsed-field gel electrophoresis (PFGE). Bacterial suspensions were included in agarose plugs and treated as previously described by Nascimento, Santos, Gentilini, Sordelli, and Bastos (2002). Chromosomal DNA was digested with *Sma*I (New England Biolabs, Ipswich, USA). Restriction fragments were separated by PFGE in 1% (w/v) agarose gels in a CHEF-DRIII system (Bio-Rad, Hercules, USA) with pulse times increasing from 2 to 35 s for 21 h at 13 °C, with a voltage gradient of 6 V cm^{-1} and 120° angle (Santos, Teixeira, Fonseca, & Gontijo Filho, 1999). Lambda-ladder PFGE marker of 50–1000 kb (New England Biolabs) was used as a molecular weight marker. Analysis of the restriction profiles was performed by visual inspection of the photographic register of the ethidium bromide stained gels. Strains were considered to be closely related if they differed by no more than three bands according to van Belkum et al. (2007).

2.9. Crude hyicin 4244 preparation

The cell-free supernatant was obtained from a 1 l culture of *Staphylococcus hyicus* 4244 grown at 37 °C for 24 h in BHI, under shaking (180 rpm), and centrifuged at 10,000*g* for 15 min at 4 °C. Ammonium sulfate [40% (w/v); Vetec, Rio de Janeiro, Brazil] was added to the supernatant, which was kept under shaking for 4 h at 4 °C. The antimicrobial substance was precipitated from the supernatant by centrifugation at 10,000*g* for 25 min at 4 °C, dissolved in 50 ml phosphate buffer (5 mM, pH 6.5), dialyzed against the same buffer through a 2000 Da cutoff membrane and treated at 65 °C for 15 min. Antimicrobial activity (AU ml^{-1}) was determined by the agar-well diffusion assay as described by Coelho et al. (2007).

2.10. Activity of hyicin 4244 against L. monocytogenes and S. aureus in skimmed milk and in TSB broth

To assess the efficacy of the crude hyicin 4244 preparation in reducing viable cell counts of *S. aureus* and *L. monocytogenes* in food, the activity of this BLIS against both food pathogens was tested in skimmed milk. In this experiment, the strains *S. aureus* A70 Bac^- and *L. monocytogenes* ATCC 19117 were used. The milk was inoculated with approximately 10^7 cfu ml^{-1} of each bacterium (previously grown in TSB broth at 37 °C for 18 h). The two milk samples were divided into two portions: in one, 1000 AU ml^{-1} of the crude BLIS were added and, in the other, equal volume of phosphate buffer (5 mM, pH 6.5) was added, as a control without antimicrobial activity. All samples were incubated at 37 °C. At the desired intervals (0, 2, 4, 6, 8 and 24 h), culture aliquots were serially diluted in a sterile saline solution and plated in triplicate on TSB medium. Plates were incubated at 37 °C for 48 h and the average number of colonies was used to calculate the number of viable cells in cfu ml^{-1}. Three independent trials were carried out as above for statistical analysis. The same experiment was also performed in TSB broth.

2.11. Statistical analysis

All experiments of growth inhibition by hyicin 4244 were carried out in triplicate, and the means and standard deviations were calculated with the Excel program (Microsoft Corp., USA). Statistical analyses were performed using the Student's *t*-test of the statistical program R version 2.15.0 (Lucent Technologies, USA), considering $p < 0.05$.

3. Results

3.1. Detection of the AMS$^+$ strains

Forty-seven *Staphylococcus* spp. strains isolated from milk samples of animals with bovine mastitis from 33 different Brazilian dairy herds were tested for AMS production. Fourteen strains (29.8%) from 11 different herds exhibited antagonistic activity against *C. fimi* NCTC 7547. During subsequent tests of AMS production on solid medium, the strains 643, 4189, 3276, and 3398 proved to be inconstant producers, i.e., the detection of the AMS was observed in some experiments, but not in others. Therefore, these four strains were not investigated further. The strains 318, 770, 3699, 4369, and 5475 exhibited inhibition zones between 11 and 18 mm, while the remaining five strains (4059, 4231, 4244, 5409, and 5580) generated inhibition zones larger than 20 mm.

3.2. Effect of proteolytic enzymes and sodium hydroxide on AMS activity

All ten AMS tested were resistant to 0.2 N NaOH (Table 2), ruling out the possibility that the inhibition zones exhibited against the target strains were due to acids from the producer-strain metabolism. Except for the AMS produced by the strains 3699 and 4244, the remaining AMS were sensitive to at least one proteolytic enzyme tested, suggesting their proteinaceous nature, a characteristic of BLIS. Therefore, from hereafter, the AMS will be considered as BLIS.

3.3. Plasmid profiles

The plasmid profiles of the BLIS$^+$ staphylococcal strains are also shown in Table 2. The strains 4244, 5475, and 5580 did not present any plasmidic DNA. Seven strains showed at least two plasmid forms. The strains 4059 and 4231 exhibited identical profiles (Fig. 1), showing plasmid forms with sizes similar to those of pRJ6, the bacteriocinogenic staphylococcal plasmid that encodes aureocin A70. The remaining strains exhibited plasmid profiles which were distinct from each other and that included large and/or small forms.

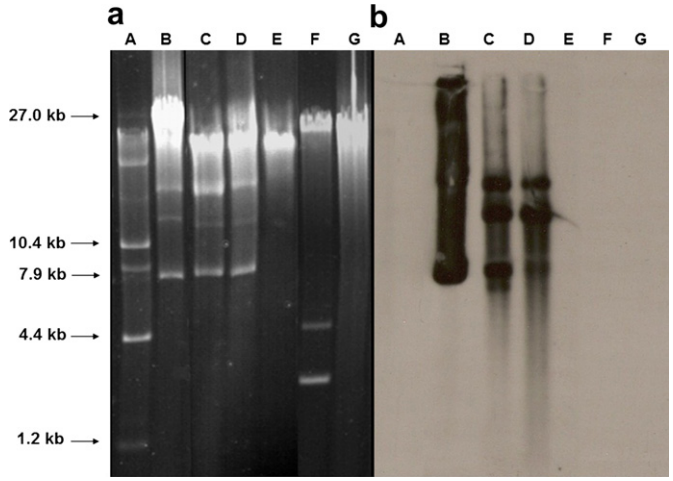

Fig. 1. Plasmid profile of the BLIS-producing strains (a) and Southern blot hybridization using the operon *aurABCD*, amplified by PCR, as probe (b). The lanes represent plasmidic DNA isolated from the following strains: (A) *S. aureus* MB196 (used as a negative control and as a plasmid molecular size marker); the positions of molecular size markers (in kb) are indicated on the left; (B) *S. aureus* A70 (positive control); (C) 4059; (D) 4231; (E) 4244; (F) 5409, and (G) 5580.

3.4. Spectrum of activity of the BLIS produced by the staphylococcal strains

In order to determine the antimicrobial spectrum of the BLIS under study, 30 strains, including LAB, food-borne pathogens and bacterial strains isolated from different foods, were used as targets (Table 3). The strains 318, 770, 3699, and 4369 did not inhibit any of these micro-organisms, but only *C. fimi* NCTC 7547, while the strain 5475 exhibited antimicrobial activity only against *M. luteus* ATCC 4698.

Except for the two enterococcal, one listerial and two staphylococcal strains, BLIS 4059 and 4231 inhibited all the target strains used. BLIS 5409 was able to inhibit strains of different genera, but did not show activity against any staphylococcal strain. The BLIS produced by the strain 5580 exhibited activity against most (63.3%)

Table 2
Characteristics of the BLIS-producing staphylococcal strains isolated in this study.

BLIS-producing strains	Effect of proteolytic enzymes (1 mg ml^{-1})[b]				NaOH (0.2 N)[b]	Plasmid profile (size in kb)
	Protease XXIII	Pronase	Trypsin	Proteinase K		
318	S	S	S	S	R	4.5; 2.8
770	R	S	R	R	R	18.5; 13.0; 8.0; 6.1; 4.4; 3.0
3699	R	R	R	R	R	>20.0 (2 forms); 15.0; 14.0; 11.5; 8.0; 5.1; 4.4; 3.1
4059	R	R	S	S	R	14.0; 12.0; 8.3
4231	S	S	S	S	R	14.2; 12.0; 8.3
4244	R	R	R	R	R	–
4369	S	S	S	R	R	7.1; 3.9; 2.5
5409	R	S	R	S	R	5.4; 3.4
5475	R	S	S	S	R	–
5580	R	S	R	R	R	–
A70[a]	S	S	S	S	R	7.9 (SC form of pRJ6)

–, Absence of plasmid forms.
SC, supercoiled.
[a] Strain A70 was included as a control.
[b] S, sensitivity to proteolytic enzyme; R, resistance to either proteolytic enzyme or NaOH.

Table 3
Spectrum of activity of the BLIS produced by strains of *Staphylococcus* spp.

Target strains	BLIS-producing strains				
	4059	4231	4244	5409	5580
L. casei ATCC 398	++	++	+++	++	±
L. sakei DSM 20017	++	++	+++	++	±
L. lactis subsp. *cremoris*	++	++	+++	++	++
L. lactis NZ 9000	++	++	+++	++	++
L. lactis 2084	++	++	+++	++	++
M. luteus ATCC 4698	++	++	+++	±	++
E. faecium E89	−	−	+++	−	++
E. faecalis 2758	−	−	++	−	−
L. monocytogenes 11/LM	++	++	+++	−	−
L. monocytogenes L1/L2a	++	++	++	++	−
L. monocytogenes 7898	++	++	++	±	±
L. innocua 397	++	++	+++	++	−
L. innocua ATCC 33090	++	++	+++	−	−
L. monocytogenes L_7	++	++	+++	±	±
L. monocytogenes L_9	++	++	++	±	±
L. monocytogenes L_{12}	++	++	++	±	±
L. monocytogenes L_{29}	++	++	+++	±	±
L. monocytogenes L_{30}	++	++	+++	±	±
L. monocytogenes L_{32}	++	++	+++	±	±
Staphylococcus spp. $QRFH_2$	±	++	+++	−	±
Staphylococcus spp. QM_2	±	±	++	−	−
Staphylococcus spp. $5S_9$	±	±	+++	−	−
Staphylococcus spp. $6H_1b$	±	±	++	−	++
Staphylococcus spp. $13H_1$	±	±	++	−	−
Staphylococcus spp. $13S_2$	−	−	++	−	±
Staphylococcus spp. Sal_{12}	±	±	++	−	−
Staphylococcus spp. Sal_{17}	−	−	+++	−	−
Staphylococcus spp. LI_1	++	++	++	−	±
Staphylococcus spp. LI_2	±	±	+++	−	±
Staphylococcus spp. L_1N_4	±	±	++	−	−

(+) inhibition zones between 12 and 20 mm; (++) inhibition zones between 21 and 29 mm; (+++) inhibition zones ≥ 30 mm; (−) no inhibition. Only the producer strains that inhibited more than one of the target strains are shown in this table.

bacterial targets, while strain 4244 exhibited the broadest spectrum of activity, inhibiting all 30 targets tested.

Based on the results shown above, only the strains 4059, 4231, 4244, 5409, and 5580 were selected for further analyses. According to API and additional identification tests, strains 4059 and 4231 were identified as *S. aureus*, 4244 and 5409 as *S. hyicus*, and 5580 as *S. epidermidis*. Since the BLIS produced by strain 4244 exhibited a broader potential biotechnological application, the identification of strain 4244 as *S. hyicus* was confirmed by 16S rDNA sequencing.

3.5. Cross-immunity against bacteriocins produced by S. aureus, S. epidermidis and S. simulans

Bacteriocin-producing bacteria normally possess a mechanism of immunity to protect themselves from their own products, and such self-immunity is often mediated by the expression of a cognate immunity system. Generally, strains that produce either identical or similar bacteriocins exhibit cross-immunity (Jack et al., 1995). Therefore, the inhibitory activity of the $BLIS^+$ strains investigated in the present study was tested against staphylococcal strains which produce known staphylococcins and vice-versa. The results of these cross-immunity tests are shown in Table 4.

Strains 4059 and 4231 were able to inhibit the growth of *S. aureus* A70 Bac^- (cured of the plasmid pRJ6) but not of strain A70, the aureocin A70 producer. Furthermore, strain A70 did not inhibit the strains 4059 and 4231, suggesting the presence of cross-immunity among the AMS produced by these three strains and, therefore, the relatedness among them.

The producer strain of epidermin, aureocin A53, and Pep5 were able to inhibit all five *Staphylococcus* spp. associated with bovine mastitis, suggesting that none of the BLIS from this study is identical to these three staphylococcins. The BLIS produced by strain 4244 inhibited all nine staphylococcin-producing strains, suggesting that this BLIS is distinct from these nine staphylococcins described in the literature.

Table 4
Immunity/resistance to bacteriocins among BLIS-producing staphylococcal strains.

Target strains	BLIS-producing strains				
	4059	4231	4244	5409	5580
S. aureus A53	++	++	++	+	+
S. aureus A70	−	−	+++	−	+
S. aureus A70 Bac^-	++	++	+++	−	+
S. aureus C55	+	+	++	−	−
S. epidermidis 5	+	+	++	−	−
S. epidermidis 5 Bac^-	+	++	++	−	−
S. epidermidis Tü3298	−	−	+	−	−
S. epidermidis BN280	−	−	+	−	−
S. epidermidis K7	−	−	+++	−	−
S. simulans 3299	++	++	++	−	−
S. aureus COL1881	−	−	+++	−	−
Producer strains (bacteriocin)	**Target strains**				
	4059	4231	4244	5409	5580
S. aureus A53 (aureocin A53)	+	+	+	+	−
S. aureus A70 (aureocin A70)	−	−	−	−	−
S. aureus C55 (staphylococcin C55)	−	−	−	−	−
S. epidermidis 5 (Pep5)	++	+	++	+	−
S. epidermidis Tü3298 (epidermin)	+++	+++	+++	+++	+++
S. epidermidis BN280 (epicidin 280)	−	−	−	−	−
S. epidermidis K7 (epilancin K7)	−	−	−	−	−
S. simulans 3299 (nukacin 3299)	−	−	++	++	−
S. aureus COL1881 (Bsa)	−	−	−	−	+++

(+) inhibition zones between 12 and 20 mm; (++) inhibition zones between 21 and 29 mm; (+++) inhibition zones ≥ 30 mm; (−) no inhibition.

The strain 5409 inhibited the producer strain *S. aureus* A53 and was sensitive to the bacteriocins aureocin A53, Pep5, epidermin, and nukacin 3299, suggesting that BLIS 5409 is different from these four staphylococcins. Additionally, the strain 5580 was inhibited by epidermin and Bsa, and was able to inhibit the producer strains of aureocins A53 and A70, suggesting that BLIS 5580 was not similar to these four bacteriocins.

3.6. Detection of staphylococcin structural genes by PCR and DNA/DNA hybridizations

The five BLIS-producer strains were then tested for the presence of the structural genes involved in production of aureocin A70, aureocin A53, staphylococcin C55, Pep5, epidermin, epicidin 280, epilancin K7, and nukacin 3299 by both PCR and DNA/DNA hybridizations, and for the presence of the Bsa structural gene only by PCR.

By using specific primers for detection of the operon *aurABCD* (coding for the structural genes of aureocin A70), a ~500 bp fragment was amplified in the strains 4059 and 4231 (Fig. 2). In hybridization assays using the operon *aurABCD* as probe, signals were detected in both strains on plasmids with ~8.0 kb, a size similar to that of pRJ6 (Fig. 1).

The remaining three strains which did not present the *aurABCD* operon were tested for the presence of the structural genes *aucA*, *sacαA*/*sacβA*, *pepA*, *eciA*, *elkA*, *epiA*, *nukA*, and *bsaA*2 but none of these genes could be detected on the 4244 and 5580 genomes (data not shown). These results suggest that the strains 4244 and 5580 produce BLIS which are distinct from nine staphylococcins already described in the literature. However, the presence of the structural genes of epidermin and Bsa was detected in strain 5409 through amplification of fragments with approximately 430 and 150 bp, the size expected for the amplicons corresponding to the *epiA* and *bsaA*2 genes, respectively. However, the observed DNA bands for strain 5409 were weaker than those observed for the positive controls (data not shown).

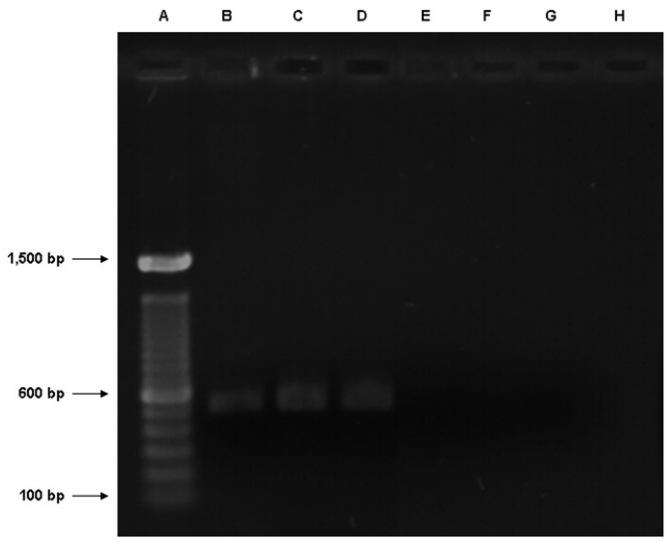

Fig. 2. DNA banding patterns obtained after PCR amplification with specific primers for detection of the operon *aurABCD*. (A) 100-bp DNA ladder (Invitrogen); the positions of molecular size markers (in kb) are indicated on the left; (B) *S. aureus* A70 (positive control); (C) 4059; (D) 4231; (E) 4244; (F) 5409; (G) 5580, and (H) *S. aureus* A53 (negative control).

3.7. PFGE of the BLIS-producer strains

Genomic DNA from the BLIS$^+$ strains was typed by PFGE after digestion with *Sma*I to determine strain similarity. According to the results shown in Fig. 3, strains 4059 (lane B) and 4231 (lane C), isolated from milk of cows of the same herd, exhibited identical PFGE profiles showing that they are the same isolate. However, comparison of the banding patterns of these two strains with the banding pattern of *S. aureus* A70 (the producing strain of aureocin A70) showed that the latter strain exhibited a quite distinct profile (lane A). The other three strains exhibited clearly different profiles, even both *S. hyicus* strains (lanes D and E), which prove to be distinct isolates of this staphylococcal species. The *S. hyicus* strains were isolated from different dairy herds.

3.8. Activity of hyicin 4244 against L. monocytogenes and S. aureus in skimmed milk and in TSB broth

In order to test the potential application of hyicin 4244 as a food biopreservative, 1000 AU ml^{-1} of a crude preparation of this BLIS were added to skimmed milk samples previously inoculated with approximately 7 log cfu ml^{-1} of either *L. monocytogenes* ATCC 19117 or *S. aureus* A70 Bac$^-$. Additionally, this experiment was also performed in TSB broth.

L. monocytogenes grew in skimmed milk from an initial count of 7–8 log cfu ml^{-1} after 24 h at 37 °C (Fig. 4B). However, in the milk added of 1000 AU ml^{-1} of hyicin 4244 the bacteria counts were lower than the counts detected in the growth control ($p < 0.05$), remaining the same as the initial inoculum. This result suggests an inhibitory action of hyicin 4244 in this food matrix. Similar results were found in TSB broth contaminated with *L. monocytogenes* (Fig. 4A).

In TSB broth, *S. aureus* viable counts increased from an initial value of 7–9 log cfu ml^{-1} during 8 h at 37 °C, followed by a slight decrease after 24 h of incubation (Fig. 4C). The addition of hyicin 4244 to TSB resulted in significant reduction ($p < 0.05$) in *S. aureus* counts compared with those of the control. It was observed that the bacterial growth remained below 7 log cfu ml^{-1} during cultivation for 24 h in TSB. Similar results were obtained in skimmed milk inoculated with 7 log cfu ml^{-1} of *S. aureus* (Fig. 4D). However, after 24 h of incubation, there was a slight increase in *S. aureus* counts, possibly due to loss of hyicin 4244 efficacy brought about by such factors as binding of the BLIS to the food matrix and insufficient AMS concentration.

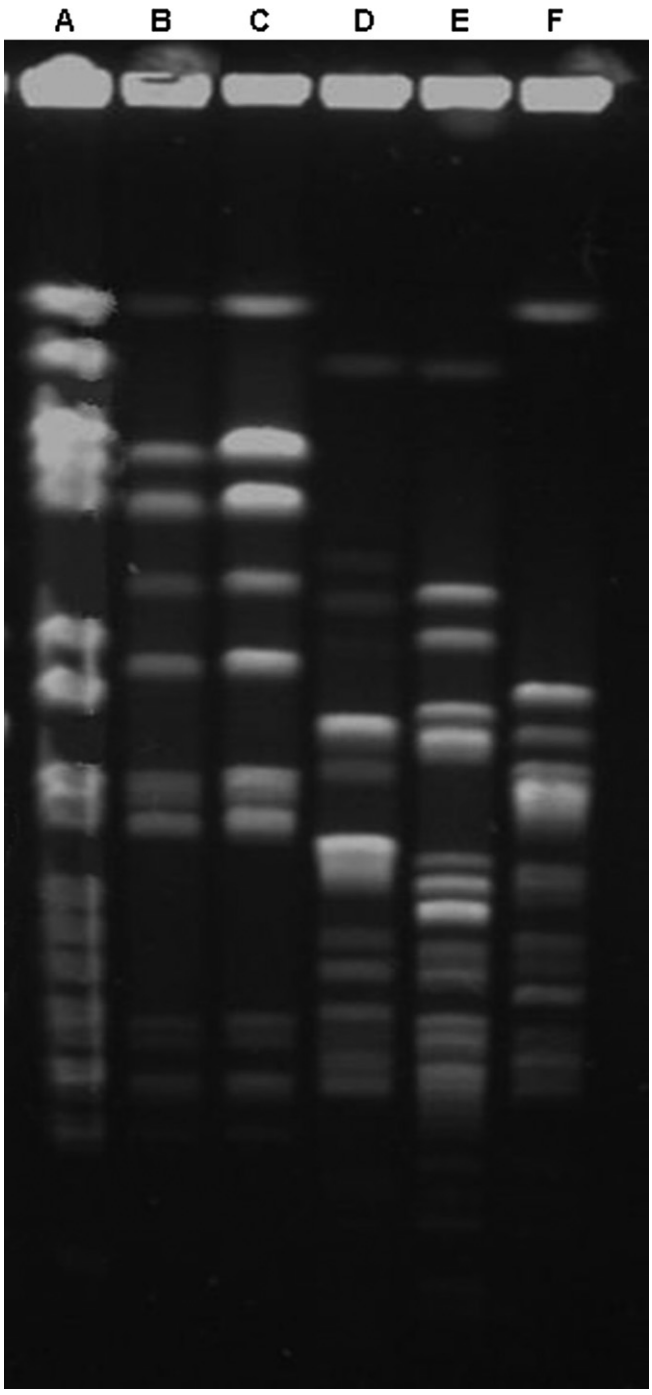

Fig. 3. DNA banding patterns obtained by PFGE of genomic DNA digested with *Sma*I. (A) *S. aureus* A70; (B) 4059; (C) 4231; (D) 4244; (E) 5409, and (F) 5580.

4. Discussion

Bacteriocins have been effectively characterized and tested in food systems to control pathogenic and spoilage micro-organisms, being studied as natural additives in the food industry (Bastos & Ceotto, 2010; Gálvez, López, Abriouel, Valdivia, & Omar, 2008).

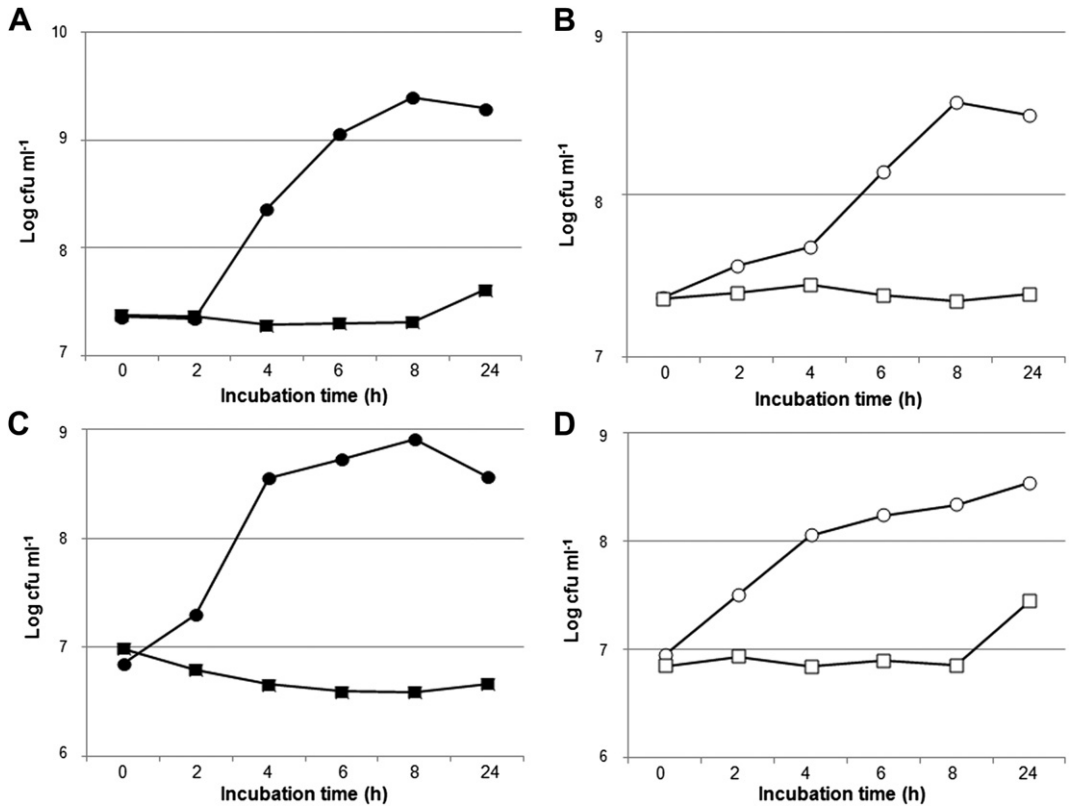

Fig. 4. *L. monocytogenes* and *S. aureus* growth kinetics either in the presence (squares) or in the absence (circles) of 1000 AU ml^{-1} of hyicin 4244. The curves show viable cell counts (cfu/ml). A and B, *L. monocytogenes* growth kinetics in TSB medium and skimmed milk, respectively. C and D, *S. aureus* growth kinetics in TSB medium and skimmed milk, respectively.

Although many bacteriocins have been purified and characterized to date, the only commercially available bacteriocins are nisin, and, to a lesser extent, pediocin PA-1 (Cotter, Hill, & Ross, 2005). Because nisin is shown to be ineffective in some food matrices (e.g. meat), and considering the potential emergence of nisin-resistant bacterial populations, it seems attractive to explore the use of other bacteriocins to prevent microbial growth in food products (Deegan, Cotter, Hill, & Ross, 2006).

Previous studies from our group have described the isolation of bacteriocin-producing staphylococcal strains of different sources in Brazil (Bastos et al., 2009; Ceotto et al., 2009). In the present study, amongst 47 *Staphylococcus* spp. strains isolated from milk from mastitic cattle, 14 strains (29.8%) were shown to produce AMS, but only five (10.6%) exhibited a high antagonistic activity against *C. fimi*.

The main criterion established to characterize AMS as bacteriocins is the presence of a biologically active proteinaceous compound (Cascales et al., 2007; Jack et al., 1995). The AMS produced by most strains tested in the present study were sensitive to at least one proteolytic enzyme suggesting that they might be BLIS. The exceptions were BLIS 3699 and BLIS 4244. However, bacteriocins resistant to proteolytic digestion have already been reported in the literature, such as aureocin A53 (Netz et al., 2002).

Among the five staphylococcal strains that exhibited the broadest antimicrobial spectra of activity, strains 4244 and 5580 carry no plasmidic DNA, suggesting that the genes coding for their corresponding BLIS are located on the bacterial chromosome. Two plasmid forms were observed in strain 5409. However, its bacteriocin genetic determinants are probably chromosomally encoded since the plasmid forms found in this strain (5.4 and 3.4 kb) may represent the OC and SC forms of the same plasmid, which is too small to carry all genes generally required for bacteriocin expression (Bierbaum & Sahl, 2009; Nissen-Meyer, Rogne, Oppegård, Haugen, & Kristiansen, 2009).

The *S. aureus* strains 4059 and 4231 showed three plasmid forms with sizes similar to those of pRJ6, a 7.9-kb plasmid which encodes aureocin A70 (Coelho, Ceotto, Madureira, Nes, & Bastos, 2009). Cross-immunity tests against *S. aureus* A70 and its derivative A70 Bac^{-} and detection of the *aurABCD* operon by PCR and DNA/DNA hybridization confirmed that strains 4059 and 4231 produce a BLIS either similar or identical to aureocin A70, whose genetic determinants are present on their single plasmid. Nevertheless, the PFGE profiles of both strains proved to be identical to each other but distinct from the banding pattern of *S. aureus* A70, confirming that the genes involved in aureocin A70 production can be found in genetically-unrelated strains of *S. aureus*, as already proposed by Ceotto et al. (2012).

Giving that BLIS 4059 was shown to be resistant to protease XXIII and pronase digestion, while aureocin A70 was sensitive, it can be assumed that BLIS 4059 is a more resistant variant of aureocin A70, which can give to the former an advantage when considering its application in food with high enzymatic activity, such as meats (Gálvez et al., 2007).

In relation to strains 4244 and 5580, the immunity/resistance, PCR and DNA/DNA hybridization experiments showed that they produce BLIS which seem to be different from nine staphylococcins described in the literature: aureocins A70 and A53, staphylococcin C55, Pep5, epidermin, epicidin 280, epilancin K7, nukacin 3299/ISK-1, and Bsa.

The BLIS-producing strains 4244 and 5409 were identified as *S. hyicus* and 5580 as *S. epidermidis*. So far, there are five staphylococcins produced by *S. epidermidis* described in the literature:

epidermin and its variants, Pep 5, epicidin 280, epilancin K7, and epidermicin NI01 (Bastos et al., 2009; Sandiford & Upton, 2012). Like epilancin K7, BLIS 5580 seems also to be chromosomally encoded. However, as already mentioned above, our data suggest that BLIS 5580 is different from epilancin K7.

Strain 5409 showed sensitivity to epidermin. However, weak bands were observed for this strain during amplication of both *epiA* and *bsaA2* genes. These results suggest that strain 5409 may produce an epidermin-like bacteriocin. Although similar bacteriocins generally exhibit cross-immunity, Daly et al. (2010) observed that strain 26, a Bsa (an epidermin-like bacteriocin) producer, was also inhibited by epidermin.

Hyicin 4244, the BLIS produced by *S. hyicus* 4244, showed the broadest spectrum of activity, inhibiting all bacterial strains used as targets, including important food-borne pathogens such as *L. monocytogenes* and *S. aureus* isolated from different types of food. Furthermore, hyicin 4244 was able to inhibit the growth of *S. aureus* and *L. monocytogenes* in TSB medium and in skimmed milk during incubation for 24 h at 37 °C. Taken together, these results suggest that this BLIS possesses potential application in food preservation.

Although nisin is already employed as a food preservative in more than 50 countries, it exhibits a low stability in either neutral or alkaline pH which limits its use (Bastos & Ceotto, 2010). Moreover, its continuous use may lead to emergence of resistant mutants, as already reported in the literature (Cotter et al., 2005). Therefore, the search for new bacteriocins, such as hyicin 4244, that compensate for the nisin instability but with a broad spectrum of activity, may play a beneficial role in the control of undesirable microbial populations.

Recently, a bacteriocin produced by *S. hyicus* (hyicin 3682), and related to both epidermin and Bsa, has been described (Fagundes et al., 2011). However, hyicin 4244 seems to be distinct from hyicin 3682 due to its resistance to proteolytic enzymes and to the fact that the producer strains of each hyicin were able to inhibit each other (data not shown). Moreover, hyicin 4244 seems to be different from both Bsa and epidermin.

Due to hyicin 4244 broad spectrum of activity, its purification and detailed chemical and genetic characterization are currently in progress. Moreover, the investigation of its kinetics of activity will be also of great importance to further application of this new staphylococcin as a biopreservative in a food matrix, either alone or combined with other preservation techniques. In a near future, hyicin 4244 gene cluster could also be expressed in nonpathogenic food-grade CoNS species, such as *Staphylococcus xylosus* and *Staphylococcus carnosus*, which play important roles in food technology, maximizing food protection against undesirable microorganisms.

Acknowledgments

A.F.S.D. is recipient of a scholarship from CNPq/Brazil. This study was supported by grants from CNPq, CAPES, and PRONEX to M.C.F.B. We would like to thank Dr. Janaína dos Santos Nascimento (IFRJ) and Patricia Carlin Fagundes for kindly providing the strains of *Staphylococcus* spp. and *Listeria* spp. isolated from food.

References

Allgaier, H., Jung, G., Werner, R. G., Schneider, U., & Zähener, H. (1986). Epidermin: sequencing of a heterodet tetracyclic 21-peptide amide antibiotic. *European Journal of Biochemistry, 160*, 9–22.

Augustin, J., Rosenstein, R., Wieland, B., Schneider, U., Schnell, N., Engelke, G., et al. (1992). Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. *European Journal of Biochemistry, 204*, 1149–1154.

Bannerman, T. L., & Peacock, S. J. (2007). *Staphylococcus, Micrococcus*, and other catalase-positive cocci. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, & R. H. Yolken (Eds.), *Manual of clinical microbiology* (9th ed.) (pp. 390–411). Washington, DC: ASM Press.

Bastos, M. C. F., & Ceotto, H. (2010). Bacterial antimicrobial peptides and food preservation. In M. Ray, & M. Chikindas (Eds.), *Antimicrobial peptides and food quality and safety* (pp. 62–76). United Kingdom: CAB International.

Bastos, M. C. F., Ceotto, H., Coelho, M. L. V., & Nascimento, J. S. (2009). Staphylococcal antimicrobial peptides: relevant properties and potential biotechnological applications. *Current Pharmaceutical Biotechnology, 10*, 38–61.

van Belkum, A., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K., et al. (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clinical Microbiology and Infectious Diseases, 13*, 1–46.

Bierbaum, G., & Sahl, H.-G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. *Current Pharmaceutical Biotechnology, 10*, 2–18.

Bradley, A. J. (2002). Bovine mastitis: an evolving disease. *Veterinary Journal, 164*, 116–128.

Brito, M. A. V. P., & Lange, C. C. (2005). Resíduos de antibióticos no leite. Juiz de Fora: Embrapa Gado de Leite. *Comunicado Técnico, 44*, 1–44.

Brouillette, E., & Malouin, F. (2005). The pathogenesis and control of *Staphylococcus aureus*-induced mastitis: study models in the mouse. *Microbes Infection, 7*, 560–568.

Cascales, E., Buchanan, S. K., Duché, D., Kleanthous, C., Lloubès, R., Postle, K., et al. (2007). Colicin biology. *Microbiology and Molecular Biology Reviews, 71*, 158–229.

Ceotto, H., Dias, R. C. S., Nascimento, J., Brito, M. A. P., Giambiagi-deMarval, M., & Bastos, M. C. F. (2012). Aureocin A70 production is disseminated amongst genetically unrelated *Staphylococcus aureus* involved in bovine mastitis. *Letters in Applied Microbiology, 54*(5), 455–461.

Ceotto, H., Holo, H., Costa, K. F. S., Nascimento, J. N., Salehian, Z., Nes, I. F., et al. (2010). Nukacin 3299, a lantibiotic produced by *Staphylococcus simulans* 3299 identical to nukacin ISK-1. *Veterinary Microbiology, 146*, 124–131.

Ceotto, H., Nascimento, J. S., Brito, M. A. V. P., & Bastos, M. C. F. (2009). Bacteriocin production by *Staphylococcus aureus* involved in bovine mastitis in Brazil. *Research in Microbiology, 160*, 592–599.

Coelho, M. L. V., Ceotto, H., Madureira, D. J., Nes, I. F., & Bastos, M. C. F. (2009). Mobilization functions of the bacteriocinogenic plasmid pRJ6 of *Staphylococcus aureus*. *The Journal of Microbiology, 47*, 327–336.

Coelho, M. L. V., Nascimento, J. S., Fagundes, P. C., Madureira, D. J., Oliveira, S. S., Brito, M. A. V. P., et al. (2007). Activity of staphylococcal bacteriocins against *Staphylococcus aureus* and *Streptococcus agalactiae* involved in bovine mastitis. *Research in Microbiology, 158*, 625–630.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology, 3*, 777–788.

Daly, K. M., Upton, M., Sandiford, S. K., Draper, L. A., Wescombe, P. A., Jack, R. W., et al. (2010). Production of the Bsa lantibiotic by community-acquired *Staphylococcus aureus* strains. *Journal of Bacteriology, 192*, 1131–1142.

Deegan, L. H., Cotter, P. D., Hill, C., & Ross, P. (2006). Bacteriocins: biological tools for biopreservation and shelf-life extension. *International Dairy Journal, 16*, 1058–1071.

Ersfeld-Dressen, H., Sahl, H.-G., & Brandis, H. (1984). Plasmid involvement in production of and immunity to the staphylococcin-like peptide Pep 5. *Journal of General Microbiology, 130*, 3029–3035.

Fagundes, P. C., Ceotto, H., Potter, A., Brito, M. A. V. P., Brede, D., Nes, I. F., et al. (2011). Hyicin 3682, a bioactive peptide produced by *Staphylococcus hyicus* 3682 with potential applications for food preservation. *Research in Microbiology, 162*(10), 1052–1059.

Gálvez, A., Abriouel, H., López, R. L., & Omar, N. B. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology, 120*, 51–70.

Gálvez, A., López, R. L., Abriouel, H., Valdivia, E., & Omar, N. B. (2008). Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. *Critical Reviews in Biotechnology, 28*, 125–152.

Giambiagi-deMarvaL, M., Mafra, M. A., Penido, E. G. C., & Bastos, M. C. F. (1990). Distinct groups of plasmids correlated with bacteriocin production in Staphylococcus aureus. *Journal of General Microbiology, 136*, 1591–1599.

Heidrich, C., Pag, U., Josten, M., Metzger, J., Jack, R. W., Bierbaum, G., et al. (1998). Isolation, characterization, and heterologous expression of the novel lantibiotic epicidin 280 and analysis of its biosynthetic gene cluster. *Applied and Environmental Microbiology, 64*, 3140–3146.

Heng, N. C. K., Wescombe, P. A., Burton, J. P., Jack, R. W., & Tagg, J. R. (2007). The diversity of bacteriocins in Gram-positive bacteria. In M. A. Riley, & M. A. Chavan (Eds.), *Bacteriocins: Ecology and evolution* (pp. 45–92). New York: Springer.

Huijps, K., Lam, T. J. G. M., & Hogeveen, H. (2008). Costs of mastitis: facts and perception. *Journal of Dairy Research, 75*, 113–120.

Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of gram-positive bacteria. *Microbiological Reviews, 59*, 171–200.

van de Kamp, M., van den Hooven, H. W., Konings, R. N., Bierbaum, G., Sahl, H.-G., Kuipers, O. P., et al. (1995). Elucidation of the primary structure of the lantibiotic epilancin K7 from *Staphylococcus epidermidis* K7. Cloning and characterisation of the epilancin-K7-encoding gene and NMR analysis of mature epilancin K7. *European Journal of Biochemistry, 230*, 587–600.

Klostermann, K., Crispie, F., Flynn, J., Meaney, W. J., Ross, P. R., & Hill, C. (2010). Efficacy of a teat dip containing the bacteriocin lacticin 3147 to eliminate gram-positive pathogens associated with bovine mastitis. *Journal of Dairy Research, 77*, 231–238.

Kumar, A. P. (2009). Evaluation of PCR test for detecting major pathogens of bubaline mastitis directly from mastitic milk samples of buffaloes. *Tropical Animal Health and Production, 41*, 1643–1651.

Langer, A. J., Ayers, T., Grass, J., Lynch, M., Angulo, F. J., & Mahon, B. E. (2012). Nonpasteurized dairy droducts, disease outbreaks, and state laws – United States,

1993–2006. Available at. *Emerging Infectious Diseases, 18*(3), 385–389 <www.cdc.gov/Features/RawMilk/>.
LeJeune, J. T., & Rajala-Schultz, P. J. (2009). Unpasteurized milk: a continued public health threat. *Clinical Infectious Diseases, 48*, 93–100.
Nascimento, J. S., Santos, K. R. N., Gentilini, E., Sordelli, D., & Bastos, M. C. F. (2002). Phenotypic and genetic characterization of bacteriocin-producer strains of *Staphylococcoccus aureus* involved in bovine mastitis. *Veterinary Microbiology, 85*, 133–144.
Navaratna, M. A., Sahl, H.-G., & Tagg, J. R. (1998). Two-component anti-*Staphylococcus aureus* lantibiotic activity produced by *Staphylococcus aureus* C55. *Applied and Environmental Microbiology, 64*, 4803–4808.
Netz, D. J. A., Pohl, R., Beck-Sickinger, A. G., Selmer, T., Pierik, A. J., Bastos, M. C. F., et al. (2002). Biochemical characterisation and genetic analysis of aureocin A53, a new, atypical bacteriocin from *Staphylococcus aureus*. *Journal of Molecular Biology, 319*, 745–756.
Netz, D. J. A., Sahl, H.-G., Marcolino, R., Nascimento, J. S., Oliveira, S. S., Soares, M. B., et al. (2001). Molecular characterisation of aureocin A70, a multipeptide bacteriocin isolated from *Staphylococcus aureus*. *Journal of Molecular Biology, 311*, 939–949.
Nissen-Meyer, J., Rogne, P., Oppegård, C., Haugen, H. S., & Kristiansen, P. E. (2009). Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by gram-positive bacteria. *Current Pharmaceutical Biotechnology, 10*, 19–37.
Olde Riekerink, R. G., Barkema, H. W., Kelton, D. F., & Scholl, D. T. (2008). Incidence rate of clinical mastitis on Canadian dairy farms. *Journal of Dairy Science, 91*, 1366–1377.
Piccinini, R., Borromeo, V., & Zecconi, A. (2010). Relationship between *S. aureus* gene pattern and dairy herd mastitis prevalence. *Veterinary Microbiology, 145*, 100–105.
Potter, A., Ceotto, H., Giambiagi-deMarval, M., Santos, K. R. N., Nes, I. F., & Bastos, M. C. F. (2009). The gene *bap*, involved in biofilm production, is present in *Staphylococcus* spp. strains from nosocomial infections. *The Journal of Microbiology, 47*, 319–326.
Pyörälä, S., & Taponen, S. (2009). Coagulase-negative staphylococci-emerging mastitis pathogens. *Veterinary Microbiology, 134*, 3–8.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.) New York, Cold Spring Habor: Cold Spring Harbor Laboratory.
Sandiford, S., & Upton, M. (2012). Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by *Staphylococcus epidermidis* that displays potent activity against staphylococci. *Antimicrobial Agents Chemotherapy, 56*(3), 1539–1547.
Santos, K. R. N., Teixeira, L. M., Fonseca, L. S., & Gontijo Filho, P. P. (1999). DNA typing of methicillin-resistant *Staphylococcus aureus*: isolates and factors associated with their nosocomial acquisition in two Brazilian university affiliated hospitals. *Journal of Medical Microbiology, 48*, 17–23.
Sawant, A., Sordillo, L. M., & Jayarao, B. M. (2005). A survey on antibiotic usage in dairy herds in Pennsylvania. *Journal of Dairy Science, 88*, 2991–2999.
Schukken, Y. H., González, R. N., Tikofsky, L. L., Schulte, H. F., Santisteban, C. G., Welcome, F. L., et al. (2009). CNS mastitis: nothing to worry about? *Veterinary Microbiology, 134*, 9–14.
Simojoki, H., Hyvönen, P., Plumed Ferrer, C., Taponen, S., & Pyörälä, S. (2012). Is the biofilm formation and slime producing ability of coagulase-negative staphylococci associated with the persistence and severity of intramammary infection? *Veterinary Microbiology, 158*, 344–352.
Viguier, C., Arora, S., Gilmartin, N., & O'Kennedy, K. R. (2009). Mastitis detection: current trends and future perspectives. *Trends Biotechnology, 27*, 486–493.