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Bovicin HC5 and nisin reduce *Staphylococcus aureus* adhesion to polystyrene and change the hydrophobicity profile and Gibbs free energy of adhesion

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen often multidrug-resistant that not only causes a variety of human diseases, but also is able to survive on biotic and abiotic surfaces through biofilm communities. The best way to inhibit biofilm establishment is to prevent cell adhesion. In the present study, subinhibitory concentrations of the bacteriocins bovicin HC5 and nisin were tested for their capability to interfere with the adhesion of *S. aureus* to polystyrene. Subinhibitory dosages of the bacteriocins reduced cell adhesion and this occurred probably due to changes in the hydrophobicity of the bacterial cell and polystyrene surfaces. After treatment with bovicin HC5 and nisin, the surfaces became more hydrophilic and the free energy of adhesion ($\Delta G_{adhesion}$) between bacteria and the polystyrene surface was unfavorable. The transcriptional level of selected genes was assessed by RT-qPCR approach, revealing that the bacteriocins affected the expression of some important biofilm associated genes (*icaD*, *fnbA*, and *clfB*) and *rnaIII*, which is involved in the quorum sensing mechanism. The conditioning of food-contact surfaces with bacteriocins can be an innovative and powerful strategy to prevent biofilms in the food industry. The results are relevant for food safety as they indicate that bovicin HC5 and nisin can inhibit bacterial adhesion and consequent biofilm establishment, since cell adhesion precedes biofilm formation.

1. Introduction

Staphylococcus aureus is a common human pathogen responsible for food-borne intoxications worldwide, caused by the ingestion of food containing staphylococcal heat-stable enterotoxins (Le Loir et al., 2003). Because *S. aureus* does not compete well with indigenous microbiota in raw foods, contamination is mainly associated with improper handling of cooked or processed foods, followed by storage under conditions which allow the growth of *S. aureus* and production of the enterotoxin (Argudín et al., 2010). Another pathway leading to cross contamination of foods is the capability of some strains to develop biofilms on food-processing surfaces (Vázquez-Sánchez et al., 2013).

Biofilms allow bacteria to better resist the application of external stress stimuli such as desiccation, UV light, and treatment with antimicrobial and sanitizing agents, due to low metabolic activity of cells, phenotypic variability and low diffusion of antimicrobials (Herrera et al., 2007; Parsek and Fuqua, 2004).

Biofilm formation depends on the characteristics of the surface, the bacterial cell, the growth medium, the optical density and other environmental conditions (Donlan and Costerton, 2002). There are several mechanisms by which microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell–cell interactions and grow as a complex structure (Bryers and Ratner, 2004). Adhesion is the initial step in biofilm formation which is mediated mainly by non-specific long-range attractive Lifshitz–van der Waals forces and electrostatic, acid–base, and hydrophobic interaction forces (Busscher et al., 2010; Chung et al., 2014). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication, and exopolysaccharide production are important for biofilm formation and development (Hori and Matsumoto, 2010; Simões et al., 2010).

Stainless steel is the most frequently used material for food-processing equipments. Polymers are another food-contact surface material widely used in the food industry, mainly for packaging. The most commonly used plastics in packaging industry are based on petrochemical products such as polyethylene terephthalate, polyvinyl chloride, polyethylene, polypropylene, polystyrene, and polyamide (Mahalik and Nambiar, 2010). Surface properties, such as hydrophobicity,

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roughness, and a predisposition to protein adsorption, are recognized as important for attachment of microorganisms to surfaces and the subsequent biofilm development (Araújo et al., 2009; Palmer et al., 2007). The hydrophobicity of the surfaces contributes to the water removal. If two surfaces are hydrophobic, it is easier to eliminate the water layer, because the molecules of surfaces have less attraction to water molecules and higher interaction with themselves (Araújo et al., 2009).

In staphylococcal species, the development of biofilms is mainly facilitated by polysaccharide intercellular adhesin (PIA), a glycan of β-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residue of which 15% is non-*N*-acetylated (Rohde et al., 2010). The polysaccharide is synthesized by enzymes encoded by the *icaADBC* operon. The IcaA, IcaC, and IcaD proteins are located in the membrane fraction; the *icaB* gene is mainly found in the culture supernatant and deacetylates PIA when it is localized on the cell surface (Gerke et al., 1998). Co-expression of *icaA* and *icaD* increases N-acetylglucosaminyltransferase activity and slime production (Arciola et al., 2006; Atshan et al., 2013).

Intercellular signaling, often referred to as quorum sensing, is reported to be involved in biofilm development (Yarwood et al., 2004). *S. aureus* quorum sensing system is encoded by the accessory gene regulator (*agr*) locus, a global regulatory network of virulence factors including biofilm development. The *agr* chromosomal locus has two promoters, P2 and P3, that control the expression of the divergent RNAII and RNAIII transcripts, respectively. RNAII encodes the core components of the *agr* system, including the AgrB (transmembrane endopeptidase), AgrD (precursor of the autoinducing peptide), AgrC (histidine kinase), and AgrA (regulator) proteins. The RNAIII transcript is itself the main effector for the *agr* system and thereby coordinates the upregulation of secreted virulence factors and the downregulation of cell surface proteins involved in the bacterial attachment (Novick and Geisinger, 2008; Pang et al., 2010).

Bacteriocins, ribosomally synthesized peptides produced by bacteria, have been suggested to inhibit the adhesion of pathogens to abiotic surface (Winkelströter et al., 2011). Many of these substances may be useful as antimicrobial agents for practical applications (Fagundes et al., 2011). Nisin, the most well-studied bacteriocin, is a lantibiotic produced by *Lactococcus lactis* subsp. *lactis* used in over 50 countries as a food preservative. Bovicin HC5 is a bacteriocin with a broad spectrum of action produced by *Streptococcus bovis* HC5, isolated from the rumen of cattle (Mantovani et al., 2002). Inhibition of the gram-positive foodborne pathogens *S. aureus*, *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens* by the bacteriocins bovicin HC5 and nisin has been reported (Antolinos et al., 2011; Mantovani and Russell, 2003; Pimentel-Filho et al., 2013; Pimentel-Filho et al., 2014; Solomakos et al., 2008; Udompijitkul et al., 2011). Bacteriocins with anti-staphylococcal activity, such as bovicin HC5, could open new possibilities to prevent adhesion and thus, to control biofilm formation. Highlighting the potential of antimicrobial peptides against biofilms as a current alternative, recent studies have evaluated the effectiveness of bacteriocins such as nisin, lacticin Q, nukacin ISK-1, and enterocin AS-48 against staphylococcal biofilms (Caballero Goméz et al., 2013; Cabo et al., 2009; Davison et al., 2010; Okuda et al., 2013).

Another interesting strategy to control bacterial adhesion and subsequent biofilm formation is to modify the surface by conditioning or incorporating bacteriocins directly on surfaces which are in direct contact with food. Nostro et al. (2010) incorporated different concentrations of nisin in poly-ethylene-co-vinyl acetate (EVA) films, used for food packing, to evaluate their effect on the biofilm-forming ability of *L. monocytogenes*, *S. aureus* and *Staphylococcus epidermidis*. The results revealed the efficacy of EVA/nisin films in reducing biofilm formation on their surfaces with more evident effect for *S. epidermidis* than *L. monocytogenes* and *S. aureus* strains.

In this work we aimed to evaluate the effect of subinhibitory dosages of bovicin HC5 and nisin on the adhesion of *S. aureus* to the polystyrene surface, determine changes on the surface hydrophobicity and free energy of adhesion, and investigate if subinhibitory concentration of the bacteriocins affects the expression rate of genes involved in biofilm formation in the evaluated conditions.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in the present study are shown in Table 1.

The bovicin HC5-producing strain *S. bovis* HC5 was cultivated under anaerobic conditions, at 39 °C overnight, in basal medium containing (per liter) 0.292 g K_2HPO_4, 0.292 g KH_2PO_4, 0.48 g $(NH_4)_2SO_4$, 0.48 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.064 g $CaCl_2 \cdot 2H_2O$, 0.5 g cysteine hydrochloride, 4 g Na_2CO_3, 0.1 g trypticase, and 0.5 g yeast extract. Glucose (4 g/l) was added as a carbon source.

S. aureus strains were grown aerobically with vigorous agitation at 37 °C in synthetic medium (Gertz et al., 1999).

2.2. Bacteriocins

Nisin A from *L. lactis* (2.5% nisin, ≥1000 IU/mg; Sigma-Aldrich, Germany) was resuspended in sterile sodium phosphate buffer (10 mM, pH 7.2) and stored at 7 °C until use.

Extract of bovicin HC5 was prepared as described by Mantovani et al. (2002). Briefly, stationary-phase *S. bovis* HC5 was harvested by centrifugation and the cells were washed in sodium phosphate buffer (10 mM, pH 7.2). The cell pellet was re-suspended in acidic NaCl (100 mM, pH 2.0) for 2 h at room temperature (Mantovani et al., 2002). The suspensions were centrifuged to remove cells and the cell-free supernatant was lyophilized. The lyophilized material was suspended in sterile sodium phosphate buffer (10 mM, pH 2.0). Purification of bovicin HC5 was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a semi-preparative column (Shimadzu C18, Japan; length, 150 mm; inner diameter, 4.6 mm; particle size, 5 μm). The column was equilibrated with buffer A (0.1% trifluoroacetic acid [TFA] in water), and the peptide was eluted using a linear gradient of 35 to 50% buffer B (80% acetonitrile, 0.1% TFA in water) at 22 °C and at a flow rate of 1 ml/min. The absorbance was monitored at 214 and 280 nm, and the eluted fraction corresponding to pure bovicin HC5 was lyophilized (Paiva et al., 2011). Bacteriocin stock solutions were stored at 7 °C in sterile sodium phosphate buffer (10 mM, pH 7.2) until use.

Bovicin HC5 concentration was determined using a ninhydrin assay (Starcher, 2001). Briefly, bovicin HC5 extract was mixed with equal volume of hydrogen chloride (12 N) for acid-hydrolysis at 100 °C for 24 h. Ninhydrin (1 g) was dissolved in 37.5 ml of ethylene glycol and 12.5 ml of 4 N sodium acetate buffer (pH 5.5). Stannous chloride solution (1.25 ml) freshly prepared by dissolving 50 mg of stannous chloride in 500 μl of ethylene glycol was added to the ninhydrin-reagent. The acid-hydrolyzed sample was diluted 100 fold with distilled water. To the tubes containing 200 μl of distilled water, 200 μl of the diluted

Table 1
Strains used in this study.

Strain	Origin	Reference
S. bovis		
HC5	Isolated from bovine rumen	Mantovani et al. (2001)
S. aureus		
COL	Isolated from human, MRSA[a]	Shafer and Iandolo (1979)
RN 6911	RN 6390-isogenic *agr* mutant	Novick et al. (1993)
HG 001	NCTC 8325 derivative, MSSA[b]	Herbert et al. (2010)
D4-106.06	Isolated from bovine mastitis	Poutrel and Lerondelle (1978)

[a] MRSA, methicillin-resistant *S. aureus*.
[b] MSSA, methicillin-sensitive *S. aureus*.

samples and 600 μl of ninhydrin-reagent were added. After incubation at 100 °C for 10 min in the dark, the absorbance was measured at 575 nm. The standard curve was constructed with bovine serum albumin (Sigma-Aldrich, Germany), in a concentration of 0.25–10 μg/μl in distilled water.

2.3. Susceptibility to the bacteriocins and adhesion testing

In order to determine the Minimal Inhibitory Concentration (MIC) of bovicin HC5 and nisin, 200 μl of synthetic medium supplemented with increasing bovicin HC5 and nisin concentrations (from 0.2 to 2.0μM) was transferred to 96-well microtiter plates and inoculated with 5×10^5 cfu/ml of exponentially growing *S. aureus* cells (optical density at 500 nm [OD_{500}], 0.5) previously propagated in the same medium without bacteriocins. The minimal concentration that prevented turbidity of the medium after 18 h incubation at 37 °C was designated as the bovicin HC5 and nisin MIC.

To evaluate the effect of bovicin HC5 and nisin on staphylococcal adhesion, assays were carried out using the same experimental design as previously described for MIC experiments. After 18 h of incubation, the culture supernatant was discarded, and the surface-attached cells were stained with 200 μl of 0.1% (w/v) crystal violet for 30 min. Subsequently, the crystal violet was removed and the plate was washed three times with water. After air drying for 15 min at 40 °C, the attached cells were determined at 590 nm with the microtiter plate reader (Biotek, Germany) by addition of 200 μl of 95% (v/v) ethanol.

MIC and adhesion experiments were conducted in three biological replicates and three technical replicates. Data were expressed as the ratio between the absorbance of violet crystal extract (adhered cells) and the optical density of total cells (planktonic cells) (Viana et al., 2009).

2.4. Contact angle measurement

2.4.1. Surface

Polystyrene coupons (20 mm × 10 mm × 1 mm) were first cleaned by washing with liquid neutral detergent and water, followed by rinsing with distilled water and then immersing in 70% ethyl alcohol for 1 h to remove fat. Subsequently, they were rinsed with distilled water and air dried under UV light. The cleaned and sanitized coupons were preconditioned for 18 h at 37 °C by immerging in synthetic medium without bacteriocins or in synthetic medium with 0.4 μM of bovicin HC5 or nisin.

The contact angles between the surface and the ultra-pure water, formamide (LGC Bio, Brazil) and α-bromonaphthalene (Merck, Germany) were determined using a goniometer (Kruss, Germany). Contact angle measurements of 2.0 μl drop were taken each second for 30 s for all liquids. Experiments were conducted in three biological replicates and three technical replicates.

2.4.2. Microorganism

Contact angles of *S. aureus* COL surface were determined on a layer of vegetative cells (Busscher et al., 1984). First, pre-warmed medium at 37 °C was inoculated with cells from an overnight culture to an initial OD_{500} of 0.1 and monitored by measuring the OD until the culture reached an OD_{500} of 0.5. At that time, the culture was exposed to 0.4 μM of bovicin HC5 or nisin followed by static incubation at 37 °C for 18 h. Growth in medium without bacteriocins was performed as a control.

Later, the suspension was centrifuged at 4000 ×*g* at 4 °C for 10 min and then, washed three times in 0.1 M phosphate-buffered saline (PBS). The pellet was resuspended in the same buffer and then filtered using acetate cellulose membrane (0.45 μm pore size, 47 mm diameter). During the filtration, 30 ml of ultra-pure water was added.

To standardize the moisture content, the membranes were transferred into Petri dishes containing 1% (w/v) agar and 10% (v/v) glycerol. The membranes were cut to determine the contact angle with the three different polarity liquids.

2.4.3. Determination of the total interfacial tension (γ_s^{tot})

The total interfacial tension was determined by the sum of the apolar and polar components of the respective surfaces (Eq. (1)):

$$\gamma_l^{tot}(1+\cos\theta) = 2\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + 2\sqrt{\gamma_s^{-}\gamma_l^{+}} + 2\sqrt{\gamma_s^{+}\gamma_l^{-}} \quad (1)$$

where γ_l^{tot} is the total interfacial tension of the liquid; γ^{LW} is the interfacial tension of the interactions of the Lifshitz–van der Waals forces; γ^{+} is the interfacial tension of the electron acceptor component of the acid–base component; γ^{-} is the interfacial tension of the electron donor component of the acid–base component, θ is the contact angle, and s and l indicate surface and liquid, respectively (Van Oss and Giese, 1995).

The three components of the interfacial tension of the surfaces were determined from the contact angles obtained from three liquids with different polarities, whose interfacial tensions are known, as shown in Table 2.

The interfacial tension is the result of the sum of the two components (γ_s^{LW} and γ_s^{AB}):

$$\gamma_s^{LW} = 11.1(1+\cos\theta_B)^2 \quad (2)$$

$$\gamma_s^{AB} = 2\sqrt{\gamma_s^{+}\gamma_s^{-}} \quad (3)$$

$$\gamma_s^{tot} = \gamma_s^{LW} + \gamma_s^{AB} \quad (4)$$

where γ_s^{LW} is the interfacial tension of the interactions of the Lifshitz–van der Waals forces; θ_B is the contact angle obtained with α-bromonaphthalene; γ_s^{AB} is the polar component of the Lewis acid–base interaction; γ_s^{+} is the interfacial tension of the electron acceptor component of the acid–base component; γ_s^{-} is the interfacial tension of the electron donor component of the acid–base component and γ_s^{tot} is the total interfacial tension of the surface.

2.4.4. Free energy of interaction (ΔG_{sws}^{TOT})

The total free energy of interaction among molecules of the surface (s) immersed in water (w) was determined by the sum of the apolar and polar free energies of interaction, ΔG_{sws}^{LW} and ΔG_{sws}^{AB}, respectively.

$$\Delta G_{sws}^{TOT} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB} \quad (5)$$

$$\Delta G_{sws}^{LW} = -2.\sqrt{\gamma_s^{LW}-\gamma_w^{LW}} \quad (6)$$

$$\Delta G_{sws}^{AB} = -4\left(\sqrt{\gamma_s^{+}\gamma_s^{-}} + \sqrt{\gamma_w^{+}\gamma_w^{-}} - \sqrt{\gamma_s^{+}\gamma_w^{-}} - \sqrt{\gamma_w^{+}\gamma_s^{-}}\right). \quad (7)$$

2.4.5. Determination of the total free energy of adhesion ($\Delta G_{adhesion}$)

Using the values of the components of the interfacial tensions, it is possible to determine the $\Delta G_{adhesion}$ between two surfaces (microbial

Table 2
Components of the interfacial tensions of the substances at 25 °C.

Substances	Interfacial tension (mJ/m²)			
	γ_l^{tot}	γ_l^{LW}	γ_l^{+}	γ_l^{-}
α-Bromonaphthalene	44.4	44.4	0.0	0.0
Water	72.8	21.8	25.5	25.5
Formamide	58.0	39.0	2.28	39.6

cells (b) and polystyrene surface (s)):

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (8)$$

$$\gamma_{bs}^{LW} = \gamma_{b}^{LW} + \gamma_{s}^{LW} - 2\sqrt{\gamma_{b}^{LW}\gamma_{s}^{LW}} \quad (9)$$

$$\gamma_{bs}^{AB} = 2\left(\sqrt{\gamma_{b}^{+}\gamma_{b}^{-}} + \sqrt{\gamma_{s}^{+}\gamma_{s}^{-}} - \sqrt{\gamma_{b}^{+}\gamma_{s}^{-}} - \sqrt{\gamma_{b}^{-}\gamma_{s}^{+}}\right). \quad (10)$$

When free energy is related to the interfacial tension, $\Delta G_{adhesion}$ can then be represented by the following:

$$\Delta G_{adhesion} = \Delta G_{bls}^{LW} + \Delta G_{bls}^{AB} \quad (11)$$

$$\Delta G_{bls}^{LW} + \gamma_{bs}^{LW} - \gamma_{bl}^{LW} - \gamma_{sl}^{LW} \quad (12)$$

$$\Delta G_{bls}^{AB} = \gamma_{bs}^{AB} - \gamma_{bl}^{AB} - \gamma_{sl}^{AB} \quad (13)$$

where γ_{bs} is the interfacial tension between the bacterial surfaces and the adhesion surface; γ_{bl} is the interfacial tension between the bacterial surfaces and the liquid; and γ_{sl} is the interfacial tension between the adhesion surfaces and the liquid.

The $\Delta G_{adhesion}$ values allow for the evaluation of the thermodynamics of the adhesion process: if $\Delta G_{adhesion} < 0$, the process is favorable; if $\Delta G_{adhesion} > 0$, the process is unfavorable.

2.5. Impact of bovicin HC5 and nisin on biofilm-related gene expression in S. aureus

2.5.1. Sample preparation and total RNA extraction

Expression of the genes *icaD*, *fnbA*, and *clfB*, related to adhesion and biofilm formation, and *rnaIII*, gene involved in the quorum sensing mechanism was evaluated after exponentially growing cells of *S. aureus* COL (OD_{500} 0.5) have been exposed to 0.4 μM bovicin HC5 or nisin in synthetic medium for 18 h, statically at 37 °C. Untreated cells were also evaluated after 18 h of growth in synthetic medium.

Bacterial cells were collected by centrifugation for 5 min at 6000 ×*g*. Total RNA was obtained by phenol–chloroform extraction (TRIzol®, Invitrogen, USA) according to the manufacturer's instructions after a 40 min pre-treatment of the cells with 0.8 μg/μl of lysozyme and 2.0×10^{-5} U/μl of lysostaphin in 300 μl of TE buffer. Purified RNA was eluted in 30 μl of Ultrapure™ RNase/DNase-free distilled water (Invitrogen, USA) and stored at −20 °C. The concentration and purity of the RNA were evaluated by spectrometry at 260 nm and 280 nm. The integrity of the RNA was confirmed by 1% (w/v) agarose gel electrophoresis.

2.5.2. cDNA synthesis and RT-qPCR

Aliquots of 2 μg of total RNA were treated with RQ1 RNase-free DNase (Promega, USA) for DNA elimination according to the manufacturer's instructions. cDNA synthesis was performed using Improm-II Reverse Transcription System kit (Promega, USA) following the manufacturer's recommendations. The mixture was incubated at 25 °C for 5 min, 42 °C for 2 h and 70 °C for 15 min.

The relative mRNA levels of biofilm-related genes were analyzed by quantitative real-time polymerase chain reaction (RT-qPCR) using SYBR® Green PCR-Master Mix (Applied Biosystems, USA) in 25 μl reaction volumes containing 1 μl of cDNA template, 1 μl of each primer at 0.5 μM (Table 3), 12.5 μl of SYBR® Green PCR-Master Mix and 9.5 μl of Ultrapure™ RNase/DNase-free distilled water and the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Abundance of each specific mRNA was calculated relatively to the expression of the housekeeping gene DNA gyrase, B subunit (*gyrB*) based on $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The amplifications were performed in 96-well PCR plates using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The fluorescence data for each sample were converted to cycle threshold (Ct) values using the software Bio-Rad CFX Manager 2.0. Experiments were conducted in three biological replicates and three technical replicates. Statistical differences were examined using one-way ANOVA followed by Tukey's test. A *p*-value less than 0.05 was considered to be statistically significant.

Table 3
Sequences of primers used for RT-qPCR.

Gene	Nucleotide sequence of primers (5′–3′)	Reference
icaD	1-GGGTGGATCCTTAGTGTTACAATTTT 2-TGACTTTTTGGTAATTCAAGGTTGTC	Korem et al. (2010)
fnbA	1-CGACACAACCTCAAGACAATAGCGG 2-CGTGGCTTACTTTCTGATGCCGTTC	Ster et al. (2005)
clfB	1-AATGCCATCATTGCACCAAA 2-CACAAAACTGATGCTTTACCAGAAA	Korem et al. (2010)
rnaIII	1-AGTCACCGATTGTTGAAATGATATCT 2-AGGAAGGAGTGATTTCAATGGC	Pang et al. (2010)
gyrB	1-ATCGGTGGCGACTTTGATCTA 2-CCACATCGGCATCAGTCATAA	Korem et al. (2010)

3. Results

3.1. Inhibition of S. aureus by bovicin HC5 and nisin

The MICs of the bacteriocins bovicin HC5 and nisin on four different strains of *S. aureus* were investigated in synthetic medium and the results are shown in Table 4. The MIC value average for bovicin HC5 and nisin was 1.05 μM ± 0.90 and 0.90 μM ± 0.26, respectively.

Subinhibitory dosages of bovicin HC5 and nisin were tested for their capacity to inhibit adhesion of *S. aureus* in polystyrene 96-well microtiter plates after 18 h of incubation using the crystal violet method. Adhesion was estimated by calculating the ratio between the absorbance of violet crystal extract and the optical density of total cells. The presence of the bacteriocins in synthetic medium clearly reduced adhesion of the strains COL and RN 6911 to the polystyrene surface (Fig. 1), and the effect of bovicin HC5 and nisin seemed to be similar for that of COL strain. While only bovicin HC5 was able to reduce adhesion of *S. aureus* HG 001, only nisin at the highest concentration evaluated reduced adhesion of *S. aureus* D4-106.06 to polystyrene.

3.2. Contact angle and total free energy of interaction (ΔG_{sws}^{TOT})

As we observed that subinhibitory dosages of bovicin HC5 and nisin were more effective in adhesion reduction than growth reduction, COL strain was selected to investigate if the presence of bovicin HC5 and nisin in low concentrations could change the hydrophobicity of the bacterial surface and also the polystyrene surface and thus, interfere with the adhesion process.

Table 4
Minimal Inhibitory Concentration of bovicin HC5 and nisin on *S. aureus* strains.

S. aureus strains	MIC of bacteriocins (μM)	
	Bovicin HC5	Nisin
COL	1.2	1.0
RN 6911	1.2	1.2
HG 001	0.8	0.6
D4-106.06	1.0	0.8

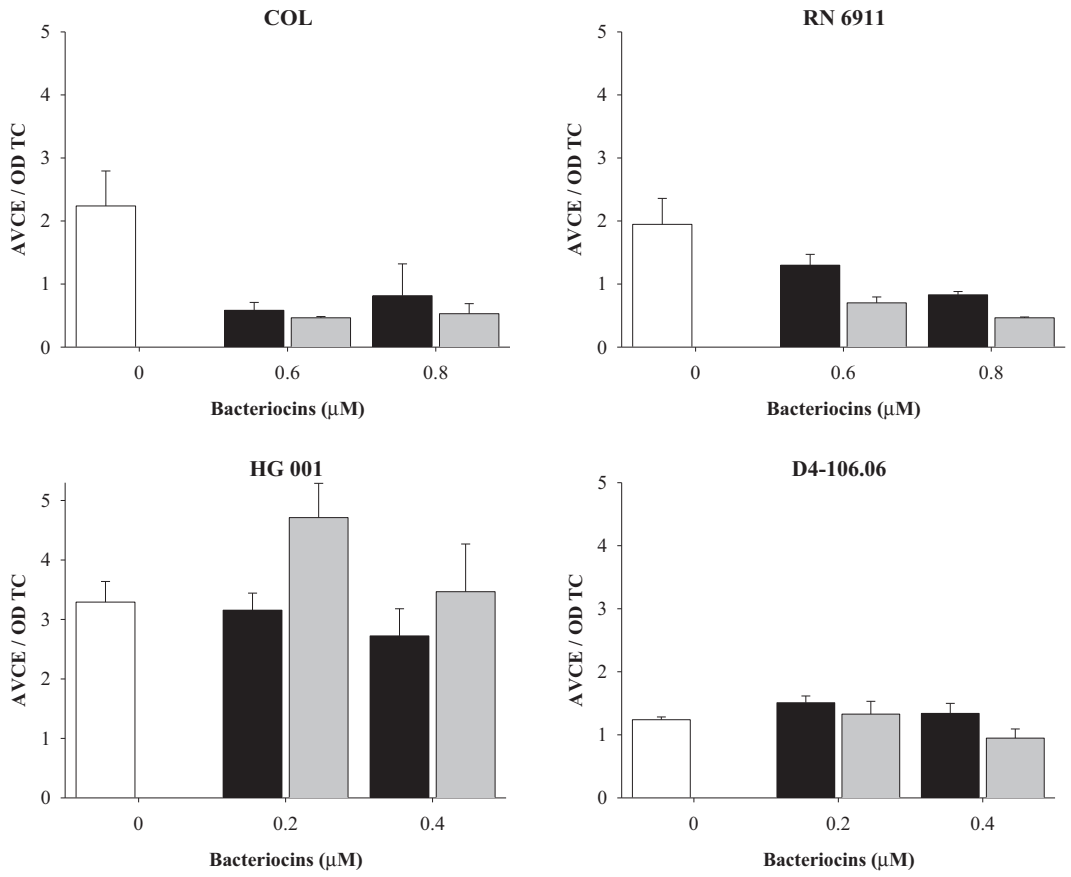

Fig. 1. Ratio between the absorbance of violet crystal extract (AVCE) and the optical density of total cells (OD TC) of *S. aureus* following bovicin HC5 or nisin treatment. *S. aureus* COL, RN 6911, HG 001, and D4-106.06 were cultivated in synthetic medium for 18 h in the presence of different concentrations of bovicin HC5 (black bars) and nisin (gray bars). Untreated control (white bars) is shown.

The measurement of the water contact angle (Θ_W) with the polystyrene surface exposed for 18 h to synthetic medium without bacteriocins was greater than 65°, indicating a hydrophobic profile according to the classification system proposed by Vogler (1998). The presence of the bacteriocins in the synthetic medium drastically changed the hydrophobicity of the polystyrene surface, since Θ_W with the surface was much lower than 65° (Table 5). *S. aureus* COL cellular surface, hydrophilic in the absence of bacteriocins, remained hydrophilic with a small decrease in the contact angle when bacteria grew in the presence of 0.4 μM of bovicin HC5 or nisin.

The ΔG^{TOT}_{sws} values are considered a quantitative criterion for hydrophobicity evaluation. According to the calculations, the polystyrene surface treated with synthetic medium without bacteriocins was classified as hydrophobic ($\Delta G^{TOT}_{sws} < 0$) (Table 5). The presence of bovicin HC5 and nisin decreased the hydrophobicity of the polystyrene surface. The bacterial surface, which is hydrophilic, remained hydrophilic ($\Delta G^{TOT}_{sws} > 0$) even after 18 h of exposure to the bacteriocins (Table 5).

3.2.1. Free energy adhesion ($\Delta G_{adhesion}$)

According to thermodynamic theory, adhesion is considered favorable only if the process results in a decrease in total free energy. Thus, the adhesion process was thermodynamically favorable ($\Delta G_{adhesion} = -9.58$ mJ/m^2) only when the synthetic medium in contact with the polystyrene surface contained no bacteriocins. The free energy of adhesion in the presence of bovicin HC5 and nisin was 23.30 mJ/m^2 and 24.06 mJ/m^2, respectively, with the adhesion process considered thermodynamically unfavorable, confirming the previous results that both bacteriocins decreased the adhesion of *S. aureus* COL.

Table 5
Averages and standard deviation of contact angle measurements with water (Θ_W), formamide (Θ_F) and α-bromonaphthalene (Θ_B) and total free energy of interaction (ΔG^{TOT}_{sws}) of *S. aureus* COL and the polystyrene surface (PS) treated with bovicin HC5 and nisin.

Surface/bacteria	Contact angles (°)[a]			ΔG^{TOT}_{sws} (mJ/m^2)
	Θ_W	Θ_F	Θ_B	
PS + synthetic medium	82.4 ± 4.4	66.2 ± 5.5	22.9 ± 1.4	−63.7
PS + synthetic medium + 0.4 μM bovicin HC5	33.4 ± 7.9	30.2 ± 2.9	32.7 ± 4.8	22.4
PS + synthetic medium + 0.4 μM nisin	40.5 ± 1.3	37.0 ± 0.8	40.9 ± 2.7	18.1
S. aureus + synthetic medium	25.3 ± 2.9	17.7 ± 0.3	45.0 ± 1.5	20.3
S. aureus + synthetic medium + 0.4 μM bovicin HC5	21.9 ± 3.1	16.6 ± 0.7	45.9 ± 1.9	22.8
S. aureus + synthetic medium + 0.4 μM nisin	23.5 ± 1.1	25.3 ± 3.8	45.8 ± 2.3	28.4

[a] Average of three repetitions.

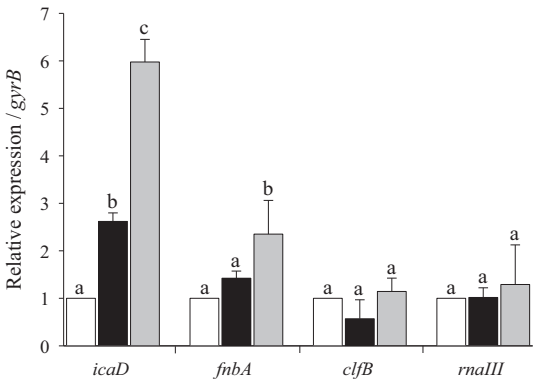

Fig. 2. Comparison of the expression profiles of selected biofilm formation-related genes (*icaD*, *fnbA* and *clfB*) and quorum sensing-related gene (*rnaIII*) in *S. aureus* COL. Cells were grown in synthetic medium and total RNA was extracted from the cells after 18 h of exposure to 0.4 μM of bovicin HC5 (black bars) and nisin (gray bars) and the expression profile was analyzed by RT-qPCR. Untreated control (white bars) is shown. Means with different letters are significantly different ($p < 0.05$).

3.3. Effect of bovicin HC5 and nisin on the expression of biofilm-related genes in S. aureus

The effect of both bacteriocins on the expression of selected genes related to adhesion and biofilm formation (*icaD*, *fnbA* and *clfB*) and also on the *rnaIII*, involved in the quorum sensing mechanism, was investigated.

Low concentrations of bovicin HC5 and nisin increased significantly ($p < 0.05$) the expression of *icaD*, one of the most studied genes related to biofilm formation by staphylococci, by 2.62 and 5.97 fold, respectively, when compared to the control without bacteriocins (Fig. 2). Nisin interfered more than bovicin HC5 on *icaD* expression. A more pronounced effect of nisin was also observed in the fibronectin A gene expression, since only this bacteriocin increased significantly ($p < 0.05$) the expression of *fnbA* by 2.35 fold. The exposure of *S. aureus* COL for 18 h to 0.4 μM of bovicin HC5 or nisin did not cause significant ($p > 0.05$) changes in the transcription profile of *clfB*, clumping factor B, and *rnaIII*, main effector of *agr* quorum sensing system in *S. aureus* (Fig. 2).

4. Discussion

For a long time, *S. aureus* has caused problems in health care. In order to control *S. aureus* growth and biofilm formation various natural substances have been tested. In this work, bovicin HC5 and nisin were tested against different strains of *S. aureus* aiming to determine MIC and also the effects of subinhibitory concentrations on cell adhesion to the polystyrene surface. Bovicin HC5 and nisin were effective against all strains of *S. aureus* tested in synthetic medium and a similar inhibitory effect was observed for both bacteriocins. The minimum concentrations of bovicin HC5 and nisin able to inhibit the growth of all tested strains were 1.4 and 1.2 μM, respectively. The lantibiotics bovicin HC5 and nisin share a similar mode of action using lipid II, an essential precursor in the biosynthesis of cell wall, as docking molecule and forming pores at sensitive membranes leading to cell death due to loss of intracellular compounds (Paiva et al., 2011).

Recently, our group reported that the bacteriocins bovicin HC5 and nisin were effective to inhibit *S. aureus* growth in milk (Pimentel-Filho et al., 2013) and in fresh cheese (Pimentel-Filho et al., 2014). These results reinforce the idea that not only nisin, already authorized as a food additive in many countries, but also bovicin HC5 has potential application as a natural food preservative. Other bacteriocins such as enterocin AS-48 (Ananou et al., 2010; Ananou et al., 2004), epidermicin NI01 (Sandiford and Upton, 2012), lacticin Q (Okuda et al., 2013), and plantaricin ZJ008 (Zhu et al., 2014) have been tested and were effective against *S. aureus*, even those called drug or multidrug-resistant.

Development of technologies to control microbial adhesion and biofilm formation is desired for food industries. Our study revealed that subinhibitory dosages of bovicin HC5 and nisin reduced the adhesion of the pathogen to the polystyrene frequently used in trays, utensils and food packing. Low dosages of nisin as 7.5 μM (25 IU/ml) were able to reduce staphylococcal biofilms on polypropylene coupons and were even more effective against planktonic cell of *S. aureus* (Cabo et al., 2009). Bacteriocin-like substances produced by lactobacilli suppressed biofilm formation on the polystyrene surface by *S. aureus* and *S. epidermidis* and induced ultrastructural changes leading to their destruction (Sadowska et al., 2010). Davison et al. (2010) verified that nisin accessed the interior of biofilm cell clusters of *S. epidermidis* on glass faster than other antimicrobial agents as chlorine and glutaraldehyde resulting in a rapid and uniform loss of green fluorescence, indicating at least changes in the membrane permeabilization, without any removal of biofilm. All these results reinforce the idea that bacteriocins can be a potential strategy to prevent adhesion and to control biofilm formation.

Generally, any surface is vulnerable to biofilm development including plastic, glass, metal, wood, and food products but the surface physiochemical properties play an important role in bacterial adhesion (Ferreira et al., 2010). By water contact angle values, a qualitative criterion, we observed that the cell surface of *S. aureus* COL growing in synthetic medium with or without bovicin HC5 and nisin was hydrophilic. Our result was in agreement with reports which found that the *S. aureus* surface is hydrophilic (Hamadi et al., 2005; Kouidhi et al., 2010). The polystyrene surface conditioned for 18 h with synthetic medium was hydrophobic which is considered favorable for bacterial adhesion. Evaluating the hydrophobicity of the polystyrene surface, Biazar et al. (2011) found a contact angle of 90.1° confirming its hydrophobic character. However, after 18 h of conditioning with synthetic medium added to bovicin HC5 or nisin, the polystyrene surface became hydrophilic ($\Theta_W < 65°$). Our data showed that the decrease in the hydrophobicity on treated surfaces indicates one of the reasons for bacterial attachment decrease. Hydrophobicity is an important propriety and represents the wettability of a surface and, in aqueous medium, adhesion is favored between hydrophobic surfaces, which can enter in closer contact by squeezing the water layer between them (Teixeira et al., 2005). After conditioning with surfactant, the polystyrene surface became more hydrophilic (Zeraik and Nitschke, 2010). It has been shown, that *Salmonella* and *Listeria* preferably adhere in a higher number to hydrophobic surfaces than the hydrophilic ones (Donlan and Costerton, 2002; Sinde and Carballo, 2000).

In order to predict the ability of the pathogen to adhere to those treated or untreated polystyrene surfaces, and further to form biofilms, the free energy of interaction between the micro-organisms and the surface, when immersed in the same synthetic medium condition with or without bacteriocin, was calculated. Only in the medium without bacteriocins the adhesion process was thermodynamically favorable. Since the total free energy of adhesion was positive, in the medium containing bacteriocins, the adhesion process was considered unfavorable. Teixeira et al. (2005) also demonstrated that adhesion of some isolates of *Pseudomonas aeruginosa* and *Staphylococcus sciuri* was thermodynamically favorable to stainless steel and rubber, which were considered hydrophobic and unfavorable to glass and polymethylacrylate surfaces, classified as hydrophilic.

Although many studies have been performed aiming on a better understanding of biofilm formation by *S. aureus*, the knowledge on the effect of bacteriocins on gene expression is still limited. Our work investigated exposure to bovicin HC5 and nisin was also related to an altered expression of four selected genes involved in biofilm formation by *S. aureus* COL. Interestingly, we found *icaD* over-expressed in the presence of both bovicin HC5 and nisin. In staphylococci, production of polysaccharide intercellular adhesin (PIA) by the enzyme products of the

icaADBC operon is the best understood mechanism of biofilm development, making the *ica* genes a potential target for biofilm inhibitors (Oduwole et al., 2010). A similar effect was observed by Nuryastuti et al. (2009) when evaluating subinhibitory concentrations of cinnamon oil on the expression of *icaA* in *S. epidermidis*, another important gene of *icaADBC* operon evaluated in studies involving biofilm formation by staphylococcal species. They found that even reducing biofilm formation on the polystyrene surface, 0.01% of cinnamon oil enhanced *icaA* expression (Nuryastuti et al., 2009). On the other hand, low dosages of povidone–iodine, a complex of polyvinyl pyrrilidine and triiodine ions widely used as an antiseptic in trauma and orthopedic surgery, decreased *icaA* expression and, thus, the biofilm forming capacity of *S. aureus* RN4220 (Oduwole et al., 2010). According to the reports in the literature, the expression of the *ica* genes is highly variable and can be induced by variations in the culture conditions, such as an increase in the concentration of sugars or other substances that induce stress (Cho et al., 2002; Oliveira and de Lourdes Cunha, 2010).

While both bacteriocins did not interfere in the expression of *clfB*, only nisin was able to up-regulate *fnbA*. Differences in the expression of *fnbA* and *clfB* in the presence of antimicrobial agents were also reported. Rasigade et al. (2011) reported that although subinhibitory concentration of rifampicin reduced bacterial adhesion to human fibronectin, the antibiotic did not affect *fnbA*/*B* transcription by five *S. aureus* strains. However, low dosages of oxacillin, moxifloxacin and linezolid led to the development of a hyper-adhesive phenotype in the fibronectin adhesion assay, increasing also *fnbA*/*B* expression (Rasigade et al., 2011). Sublethal dosages of tigecycline were investigated on biofilm formation by 16 methicillin-resistant *S. aureus* isolates and the transcriptome analysis revealed that the antibiotic was able to reduce expression of *icaC*, otherwise upregulation of *fnbA* and *clfB*, which encode adhesins which attach to human proteins, was observed (Smith et al., 2010).

Biofilm formation can be induced by conditions that are potentially toxic for bacterial cells, such as high levels of osmolarity, detergents, urea, ethanol, oxidative stress, and the presence of sub-MICs of some antibiotics (Nuryastuti et al., 2009).

No difference in *rnaIII* expression was observed in the presence of bovicin HC5 or nisin. The *agr* system effector, *rnaIII*, regulates the expression of a large number of target genes, including down-regulating biofilm-related genes as *fnbA* and *clfB* (Xue et al., 2012); it was expected that the bacteriocins would enhance the *rnaIII* transcription and consequently reduce the expression of *fnbA* and *clfB*.

To our knowledge, this is the first study reporting that bacteriocins change the hydrophobicity of polystyrene surfaces. Even expressing *icaD* and *fnbA* in the presence of the bacteriocins, the adhesion of *S. aureus* was reduced since the free energy of adhesion was not favorable, indicating a difficulty for the bacteria to get in close contact with the surface. This is a very interesting finding since to prevent microbial adhesion to food contact surfaces is much more effective than to remove biofilms already established. Thus, bovicin HC5 and nisin appear as a potential alternative to inhibit the initial step of biofilm formation.

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