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Evaluation antibacterial and antibiofilm activity of the antimicrobial peptide P34 against *Staphylococcus aureus* and *Enterococcus faecalis*

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ABSTRACT

The adhesion ability of bacteria to abiotic surfaces has important implications in food industries, because these organisms can survive for long periods through the biofilm formation. They can be transferred from one place to another in the industry causing contamination of the food processing environment. In this study, the antibacterial and antibiofilm activities of the antimicrobial peptide P34, characterized as a bacteriocin-like substance (BLS P34) were tested against planktonic and sessile cells of *Staphylococcus aureus* and *Enterococcus faecalis* isolated from foods. The BLS P34 showed inhibitory effect against all planktonic cells of *E. faecalis*. The inhibition of biofilm formation and the eradication of pre-formed biofilm were evaluated with the crystal violet assay and with the reduction of 3-bromide [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium. The BLS P34 promoted a reduction of percentage of adhered microbial cells on the surface, not being able to perform the complete elimination of biofilm formation. The metabolic activity of *S. aureus* biofilms decreased considerably between 41-95%. However, *E. faecalis* cells showed up metabolically stimulated. The BLS P34 has the potential antibiofilm for the species *S. aureus*. Studies suggest more detailed approaches to a better understanding of the interactions between the antimicrobial and bacterial cells within the biofilm structure.

Key words: Biofilm, crystal violet, food industry, MTT assay, peptide P34.

INTRODUCTION

After the first report on biofilms by Zobell (1943), concern about them is still great, especially in the food, biomedical and environmental fields (Marques et al. 2007, Laird et al. 2011). Biofilms are multicellular structures formed by the attachment and aggregation of microorganisms followed by coating with a polysaccharide-rich extracellular matrix (Garrett et al. 2008). There are many advantages to an organism forming a biofilm, since these structures are more resistant to adverse environmental conditions compared to planktonic cells. Biofilms are resistance with makes their elimination from food processing environment a big challenge (Simões et al. 2006, Simões and Vieira 2009).

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Biofilms are a big problem in food sectors such as dairy processing, fresh produce, poultry and red meat processing (Chen et al. 2007), and usually are associated with serious hygiene problems causing deterioration and recontamination of the product (Gram et al. 2007). Several reports describe the persistence of some foodborne pathogens affecting the quality and safety of food products. Most outbreaks whose etiologic agents are transmitted by food seems to be associated with biofilms. In recent years, there have been a significant number of reports correlating the persistence of foodborne pathogens on food contact surfaces and the occurrence of biofilms (Simões and Vieira 2009, Yaron and Römling 2015, Bridier et al. 2015). *Enterococcus* spp. and *Staphylococcus* spp. are Gram positive pathogens associated with foodborne outbreaks. These bacteria have virulence factors, such as, aggregation substances, extracellular surface proteins, and ability to form biofilms (Chapman 2003, Fisher and Phillips 2009, Marinho et al. 2013). The *E. faecalis* was responsible for an outbreak with lenezolid resistance (LRE) in a hospital, affecting 13 people between 2004 and 2005 in Tennessee, USA (Kainer et al. 2007). Resistant bacteria may be transferred to humans through the food chain, and colonize the gastrointestinal tracts and/or may be able to transfer resistance genes to the resident microbiota (Fisher and Phillips 2009, Frazzon et al. 2009, Cassenego et al. 2011).

Staphylococcal foodborne disease (SFD) is one of the most common foodborne disease worldwide resulting from the contamination of food by preformed by *Staphylococcus aureus* enterotoxins (Kadariya et al. 2014). This species can produce many different enterotoxins (Schelin et al. 2011), which are stable to heat and have varying degrees of toxicity to the human body (Balaban and Rasooly 2000, Hennekinne et al. 2012). It is one of the most common causes of reported foodborne diseases in the United States (Kadariya et al. 2014). The ability of *S. aureus* to adhere and form biofilms improves their survival and growth in food processing plants, providing an additional physiological advantage as a causative agent of foodborne diseases (Herrera et al. 2007, Hennekinne et al. 2012).

Conventional cleaning and disinfection regimens may also contribute to inefficient biofilm control and its dissemination. Consequently, new control strategies are constantly emerging with the main incidence in the use of biological approaches (Simões et al. 2010). An alternative for the prevention and eradication of biofilm formation, would consider that antimicrobial peptides could be used. The antimicrobial peptide P34 was isolated and characterized as a bacteriocin-like substance (BLS) by Motta et al. (2007a) and the inhibitory activity was detected against *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella* Enteritidis (Motta et al. 2007b, 2008). Furthermore, some studies showed low cytotoxicity in eukaryotic cells and antiviral activity against pathogenic virus of domestic animals (Vaucher et al. 2010, Silva et al. 2014).

In this context, the objective of this study was to investigate the ability of *E. faecalis* and *S. aureus* isolates from food in Southern Brazil to form biofilm, and evaluate the potential antibiofilm activity of the BLS P34 produced by *Bacillus* sp. P34, a strain isolated from Piau-com-pinta (*Leporinus* sp.) a fish from Brazilian Amazon basin.

MATERIALS AND METHODS

BACTERIAL STRAINS

Eleven *S. aureus* strains obtained from fresh and frozen chicken meat samples and eleven *E. faecalis* strains isolated from food samples (potato, carrot, chicken salt, frozen chicken, ricotta and dairy products including colonial cheese) were evaluated. These isolates were previously identified by Martins et al. (2013) and Riboldi et al. (2009).

All isolates were revalued for colony morphology, Gram stain, catalase test, coagulase,

and mannitol fermentation/or bile esculin test. The isolates were maintained in cryotubes containing 10% (v/v) skim milk and 20% (v/v) glycerol, stored at -20ºC. Bacterial cultures were grown in Brain Heart Infusion agar (BHI, Himedia) at 37ºC for 24 h, to obtain pure colonies before analysis.

PRODUCTION AND PARTIAL PURIFICATION OF BLS P34

The production and partial purification was determined in accordance with Motta et al. (2007a). For the production of BLS P34, *Bacillus* sp. P34 was grown in 100 ml BHI medium at 30ºC in a rotary shaker at 180 rpm (Motta and Brandelli 2008). After culturing for 24 h, the cells were harvested by centrifugation at 10,000 g for 15 min at 4ºC. The filtrate was precipitated with ammonium sulfate at 20% (w/v) saturation, and then dissolved in 10 mM phosphate buffer pH 7.0. This solution was purified by gel filtration chromatography using a Sephadex G-100 column. The antimicrobial activity titre was determined by the serial twofold dilution method previously described by Mayr-Harting et al. (1972). Activity was defined as the reciprocal of the last serial dilution giving an inhibition zone and expressed as arbitrary unit (AU) per milliliter. The AU ml^{-1} was determined against *Listeria monocytogenes* ATCC 7644 as indicator strain due susceptibility to BLS P34. The fractions showing antimicrobial activity were pooled, sterilized through a 0.22 µm filter (Millipore, Bedford, USA) and frozen stored (Motta et al. 2007a).

ANTIMICROBIAL ACTIVITY OF BLS P34 AGAINST PLANKTONIC CELLS OF *Staphylococcus aureus* AND *Enterococcus faecalis*

Antimicrobial activity was determined essentially as described by Motta and Brandelli (2002). An aliquot of 20 µl of BLS P34 (1600 AU) was applied on surface of BHI agar plates previously inoculated with a swab submerged in suspensions of *E. faecalis* or *S. aureus* that corresponded to a 0.5 McFarland turbidity standard solution. The strain *L. monocytogenes* ATCC 7644 was used as a susceptible control. Plates were incubated at the optimal growth temperature of the test organisms (37ºC) and inhibitory zones were measured after 24 h.

BIOFILM FORMATION BY *Staphylococcus aureus* AND *Enterococcus faecalis*

Quantitative determination of biofilm was determined by crystal violet assay (CV) in accordance with Stepanović et al. (2007) with some modifications. This methodology was chosen, for being the reference protocol to detection of biofilm formed by isolated food pathogens (Rodrigues et al. 2010, Marinho et al. 2013, Pinto et al. 2015). In summary, three to five colonies were suspended in 4 ml of 0.85% (w/v) NaCl solution and compared with 0.5 McFarland scale (equivalent to 1.5 x 10^8 CFU ml^{-1}). Twenty microliters of this solution was transferred to each well of the microplate. Later, 180 µl of Trypticase Soy Broth (TSB) containing 10 g l^{-1} glucose was added to each well and the microplate was incubated at 37ºC for 24 h. After incubation the plate was washed with 0.85% (w/v) NaCl solution for three times to remove non-adherent cells, 200 µl of 100% methyl alcohol was added for 20 min to fix the adhered cells. The plate was dried at room temperature for 30 min and then 200 µl of 0.5% crystal violet was added for 15 min. After removing the dye solution and washing with sterile distilled water, the attached dye was solubilized with 0.5% (v/v) ethanol and the optical density of the adherent biofilm was determined in a microtiter plate reader (Anthos Zenyth 200) with a filter of 450 nm. All isolates were tested at least eight times in duplicate. *Staphylococcus epidermidis* ATCC 35984 was used as positive control, since this strain is a strong biofilm producer (Pinto et al. 2015). For interpretation of the biofilm results, the isolates were classified as non-producing, weak, moderate and strong-producing, based on the following optic

density (OD) average values: OD (isolate) ≤ OD (control) = non-biofilm-producing; OD (control) ≤ OD (isolate) ≤ 2OD (control) = weak-producing; 2OD (control) ≤ OD (isolate) ≤ 4OD (control) = moderate-producing; 4OD (control) ≤ OD (isolate) = strong-producing.

DETERMINATION OF ANTIBIOFILM INHIBITORY ACTIVITY BY BLS P34

Verification of antibiofilm activity was performed using the CV test and the test of the reduction of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium (MTT) simultaneously according Jadhav et al. (2013) with some modifications. The term "inhibiting" was used to refer to the surface that has been treated with the BLS P34 prior to deposition of biofilm forming microbial cells. The term "eradication" was used to refer to the treatment made of preformed biofilm with the BLS P34.

Inhibition of initial cell attachment

*S*olutions of BLS P34 (equivalent to 1600 AU) were prepared. One hundred microliters of each solution were added to individual wells of 96-well polystyrene microtiter plates for 4 h. Equal volumes of sterile water were added as negative control. Subsequently, wells were washed three times with sterile distilled water and 100 µl of the bacterial cultures (prepared as described above) were then added to the wells. The cultures were added into the wells in quadruplicate and sterile TSB was used as an additional control to confirm the sterility of the medium. The plates were incubated for 24 h at 37ºC. Biofilm formation was assessed using the CV assay and the metabolic activity of the cells incubated with BLS P34 was investigated using the MTT assay.

Eradication of preformed biofilm

Biofilms were allowed to be formed for 24 h prior to addition of BLS P34. Biofilm formation was achieved by transferring 100 µl of bacterial culture (prepared as described above) into the wells of polystyrene microtitre plates in quadruplicate. The microtitre plates were incubated for 24 h at 37ºC to allow cell attachment and initiate biofilm formation. Following incubation, the plate was washed with sterile distilled water to remove non-adhered cells and 100 µl of BLS P34 solution (equivalent to 1600 AU) was added to each well. Equal volumes of sterile distilled water were added as negative control. After the treatment of preformed biofilms, the plates were incubated for 4 h. Following incubation, the biofilms were assessed using the CV assay and the MTT assay.

QUANTIFICATION OF BIOFILM INHIBITION/ ERADICATION POTENTIAL BLS P34

The mean absorbance at 450 nm was used for determining the percentage inhibition of biofilm formation caused by BLS P34 according to the following equation: Percentage inhibition = 100 - [{ A450 nm experimental well with P34 / A450 nm control well without P34} x 100] (Jadhav et al. 2013).

Percentage inhibition = 100 - [{ A_{450} nm experimental well with P34 / A_{450} nm control well without P34} x 100] (Jadhav et al. 2013).

BIOFILM METABOLIC ACTIVITY ASSAY

The evaluation of the metabolic activity of biofilm was determined by the reduction of MTT according to Denizot and Lang (1986) with some modifications. The MTT salt (Sigma-Aldrich, USA) was dissolved in phosphate buffered saline (PBS) to give a final concentration of 5 mg ml^{-1}.

The culture medium or BLS P34 were carefully removed and the wells were washed five times with 0.85% (w/v) NaCl solution and air-dried. One

hundred microliters of MTT solution were pipetted into each well and incubated for 3 h at 37ºC. The insoluble purple formazan (obtained by enzymatic hydrolysis of MTT by the dehydrogenase enzyme found in living cells) was further dissolved in 100 µl of dimethyl sulphoxide (DMSO; Sigma-Aldrich, USA). The absorbance was then measured at 570 nm using a microplate reader (Anthos Zenyth 200).

STATISTICAL ANALYSIS

All experiments were performed at least in triplicates and standard deviation from the mean was calculated. Effect of BLS P34 on biofilm formation was analyzed using One-way Anova by Statistica 10.0 software. Values were considered significantly different each other at $P<0.05$.

RESULTS

ANTIMICROBIAL ACTIVITY AGAINST PLANKTONIC CELLS OF *Staphylococcus aureus* AND *Enterococcus faecalis*

The antimicrobial activity of BLS P34 against *S. aureus* and *E. faecalis* are presented in Table I. Five *S. aureus* strains showed inhibition zones ≥ 10.0 mm, while other strains were not sensitive to BLS P34 (Table I). The BLS P34 was more effective to inhibit *E. faecalis* isolates, since all isolates showed inhibition zones ≥ 12.0 mm. For the indicator strain *L. monocytogenes* ATCC 7644 used as a positive control, an inhibition zone of 13.6 mm was observed (Table I).

BIOFILM FORMATION BY *Staphylococcus aureus* AND *Enterococcus faecalis*

All isolates tested were biofilm producers. Among the eleven isolates of *S. aureus* evaluated, seven (63.3%) were classified as strong biofilm formers and four (36.4%) as moderate formers. Six isolates of *E. faecalis* (54.5%) were showed as strong biofilm formers and five (45.4%) as moderate formers (Table II).

DETERMINATION OF BIOFILM INHIBITORY ACTIVITY OF BLS P34

Inhibition of initial cell adhesion

The BLS P34 caused lower inhibition of initial cell adhesion in *E. faecalis* strains when compared with the *S. aureus* strains (Table III). The highest inhibition of cell adhesion (46.9%) was observed against *S. aureus* F4-1 strain. For other isolates from this group, the inhibition percentage of adhesion ranged from 8.3% to 22.3% (Table III). The *E. faecalis* C-11 showed the highest inhibition percentage of adhesion (31.4%). For the control *S. epidermidis* ATCC 35984 strain, the BLS P34 was able of providing an inhibition of 9.2% (Table III).

Eradication of preformed biofilm

When the BLS P34 was applied over the pre-formed biofilm, *S. aureus* F4-1 strain was the most sensitive (55.7%), similarly to that observed in the previous evaluation. For other isolates of *S. aureus*, the percentage of eradication ranged from 1.1% to 17.6%, excepting for the F2-4 strain that exhibited a negative value (Table III). Only three *E. faecalis* namely C8, E2 and G4, suffered eradication, with results of 2.8%, 6.9% and 7.5% respectively. However, these results were lower when compared to the treatment before the biofilm formation. The other strains showed negative percentage of eradication, which suggests a stimulation originated by the presence of the antimicrobial substance (Table III). The statistical analysis indicates that different conditions of BLS P34 treatment changes the percentage of adhered cells in case of *E. faecalis* group ($P<0.05$). However, for the *S. aureus* group, this difference was not observed. The control *S. epidermidis* ATCC 35984 strain shows 7.5% of eradication.

TABLE I
Antimicrobial activity spectrum of BLS P34 against planktonic cells of *Staphylococcus aureus* and *Enterococcus faecalis* at 37ºC.

Indicator organism	Inhibition zone (mm)	Indicator organism	Inhibition zone (mm)
Control strain			
L. monocytogenes ATCC 7644			13.6
***S. aureus* strains**		***E. faecalis* strains**	
F -2-1	0	A-8 (colonial cheese)	12.0
F -2-3	11.0	C-8 (carrot)	12.2
F -2-4	0	C-11 (potato)	13.5
F -3-3	12.2	E-2 (potato)	12.0
F -4-1	13.0	E-3 (potato)	12.5
F -5-1	11.3	E-4 (potato)	13.0
F -5-2	0	E-7 (chicken salt)	13.3
F -5-3	10.0	G-2 (ricota)	12.3
F -5-4	0	G-4 (frozen chicken)	12.5
F -5-5	0	G-6 (colonial cheese)	12.5
F -8-3	0	G-8 (mozzarela cheese)	12.0

TABLE II
Classification *Staphylococcus aureus* and *Enterococcus faecalis* isolated from food samples according to their ability to form biofilm at 37ºC.

Indicator organism	Classification	Indicator organism	Classification
Control strain			
S. epidermidis ATCC 35984			S
***S. aureus* strains**		***E. faecalis* strains**	
F 2-1	M	A-8	M
F 2-3	S	C-8	S
F 2-4	S	C-11	M
F 3-3	S	E-2	M
F 4-1	S	E-3	S
F 5-1	S	E-4	M
F 5-2	S	E-7	S
F 5-3	S	G-2	S
F 5-4	M	G-4	M
F 5-5	M	G-6	S
F 8-3	M	G-8	S

Isolates (n)	Strong	Moderate	Weak	Non Producer
22	13 (59.09%)	11(50%)	0	0

S: Strog producer; M: Moderate producer. A biofilm was formed for 24 h in control conditions. The biofilm was assayed with the CV method.

TABLE III
Percentage of antibiofilm potential of BLS P34 against *Staphylococcus aureus* and *Enterococcus faecalis* biofilms using the CV assay.

Bacterial group	Treatment		Bacterial group	Treatment	
Staphylococcus aureus	**A**	**B**	***Enterococcus faecalis***	**A**	**B**
F 2-1	22.2	45.7	A-8	2.4	-2.8
F 2-3	15.4	6.9	C-8	22.4	2.8
F 2-4	18.8	-1.8	C-11	31.4	-7.2
F 3-3	19.8	15.1	E-2	23.5	6.9
F 4-1	46.9	55.7	E-3	18.6	-5.8
F 5-1	22.3	5.3	E-4	13.1	-5.0
F 5-2	14.6	9.0	E-7	29.4	-6.0
F 5-3	40.5	9.6	G-2	23.5	-4.6
F 5-4	9.3	8.6	G-4	13.4	7.5
F 5-5	8.8	1.5	G-6	30.0	-7.5
F 8-3	12.2	17.6	G-8	6.6	-1.6
Control strain					
S. epidermidis ATCC 35984				9.2	7.5

Each strain was individually evaluated and the values are expressed as percentage of inhibition of initial cell adhesion (treatment A) or eradication of preformed biofilm (treatment B) evaluated using the CV assay. Negative values indicate stimulation on biofilm formation.

METABOLIC ACTIVITY OF THE BIOFILMS FORMED BY *Staphylococcus aureus* AND *Enterococcus faecalis* TREATED WITH BLS P34

During the initial cell adhesion assay, *S. aureus* isolates showed reduced metabolic activity, with percentages between 41% and 95%, excepting for the isolate F3-3 that exhibited a negative result indicating increased metabolic activity (Table IV). In the assay of eradication of preformed biofilm, the *S. aureus* strains showed metabolic inhibition between 14% and 94%. Only the strain F2-4 exhibited stimulation on metabolic activity in this treatment (-38%).

All *E. faecalis* strains showed negative results in both treatments. Although in the CV assay demonstrated inhibition of some strains, the other microbial cells that were adhered to the wells were metabolically active and stimulated when compared to the control group (Table IV). Only *E. faecalis* strains showed a statistically significant difference when treated with BLS P34 ($P<0.05$). This behavior is probably associated to a higher metabolic stimulation in the treatment for inhibition of cell adhesion. The control *S. epidermidis* ATCC 35984 strain showed metabolism inhibition values of 42% and 51% for the initial cell adhesion and eradication of preformed biofilm tests, respectively.

DISCUSSION

Foodborne diseases cover a wide range of symptons and are triggered by agents that are consumed along with food. Food contamination may occur at any stage of the production process and can result in contamination of the processing environment (Simões et al. 2010, Srey et al. 2013). It has been recognized that biofilms are a frequent source for

TABLE IV
Effect of BLS P34 on metabolic activity of biofilm cells of *Staphylococcus aureus* and *Enterococcus faecalis* at 37ºC.

Bacterial group	Treatment		Bacterial group	Treatment	
Staphylococcus aureus	**A**	**B**	***Enterococcus faecalis***	**A**	**B**
F 2-1	46	59	A-8	-248	-27
F 2-3	95	86	C-8	-143	-23
F 2-4	81	-38	C-11	-222	-2
F 3-3	-97	14	E-2	-155	-17
F 4-1	46	70	E-3	-348	-32
F 5-1	61	71	E-4	-135	-17
F 5-2	66	64	E-7	-388	-29
F 5-3	41	23	G-2	-382	-27
F 5-4	44	88	G-4	-89	-22
F 5-5	93	94	G-6	-135	-25
F 8-3	64	90	G-8	-52	-23
Control strain					
S. epidermidis ATCC 35984				42	51

Each strain was individually evaluated and the values are expressed in percentage of inhibition of metabolic activity. Negative values indicate stimulation of cellular metabolic activity. A: Inhibition of metabolic activity on the initial cell adhesion; B: Inhibition of metabolic activity on the eradication of the preformed biofilm.

infections and almost 80% of persistent bacterial infections in the United States were found to be associated with biofilms (Janssens et al. 2008).

In the present study, all food strains of *S. aureus* and *E. faecalis* were able to form biofilms. In general, *Staphylococcus* sp. isolates have a significant incidence of biofilm formation in the food industry (Ferreira et al. 2014, Pinto et al. 2015). Several mechanisms are involved in the process to establish the biofilm on the surface, like the presence of genes involved in the adhesion process and extracellular polymeric substance (EPS) production (Ferreira et al. 2014). Moreover, the ability of *S. aureus* isolated from industrial surfaces to form biofilm depends on the environmental conditions. The hygienic condition of food handlers is also associated with the propagation of *S. aureus* biofilm producer (Gutiérrez et al. 2012, Souza et al. 2014, Di Ciccio et al. 2015).

Several authors have reported *E. faecalis* strains isolated from food samples with ability to form biofilms (Medeiros et al. 2014, Jahan and Holley 2014, Fernandes et al. 2015). Apparently, the biofilm formation by this bacterial species is influenced by several aspects, such as temperature, exposure to nutrients and sanitizers, and surface characteristics (Marinho et al. 2013, Fernandes et al. 2015). According to Jahan and Holley (2014), their capacity to exchange genetic information by conjugation, contributes to the resistance and dissemination of *E. faecalis* in food.

Planktonic cells are important for the quick proliferation and dissemination of microorganisms to new surfaces, while the sessile cells are considered a chronic factor because of surface adhesion. It is

generally accepted and well documented that cells within a biofilm are more resistant to biocides than their planktonic counterparts (Houdt and Michiels 2010, Simões et al. 2010). The antimicrobial potential of BLS P34 against planktonic cells was explored in this work, and it was observed that the substance was able to inhibit the growth of five *S. aureus* strains. However, all strains of *E. faecalis* were sensitive to the action of this antimicrobial in planktonic condition. Contrary to these results, Motta et al. (2007a) evaluated the same antimicrobial peptide against *S. aureus* and *E. faecalis* and found no inhibitory action. This discrepancy can be associated with the fact that the effectiveness of antimicrobial peptides is often dependable on its concentration and degree of purification, as well as concentration of the indicator strain and culture medium (Lisboa et al. 2006, Motta and Brandelli 2008). Lisboa et al. (2006) evaluated an antimicrobial peptide produced by *Bacillus amyloliquefaciens* at the concentration of 256 AU against a clinical isolate of *E. faecalis* and observed an inhibitory zone of 14 mm. In a study realized by Motta et al. (2008), using the transmission electron microscopy against planktonic cells of *L. monocytogenes*, the authors report that cells treated with BLS P34 showed vesiculation of the protoplasm, pore formation and disintegration of the cells. It is possible that the antimicrobial activity against the isolates of this study has occurred in a similar way, because both are Gram-positive.

In recent years, the use of antimicrobial peptides on surfaces to prevent microbial adhesion proves to be an interesting approach in food processing facilities (Massani et al. 2008, Aminov 2010, Héquet et al. 2011). Bioconservatives molecules such as nisin, pediocin and lauricidin have potential for biofilm control in processing facilities. (Mahdavi et al. 2007, Garcia-Almendarez et al. 2008). Some antimicrobial molecules can make the surface less hydrophobic, and thus, reduce cell adhesion. This attribute suggests its application to control microbial adhesion and helping in the destabilization of biofilms (Gomes and Nitschke 2012).

Although the mechanism of action of BLS P34 against planktonic cells is not fully elucidated, this study provides a new application approach of the substance, providing information about the antibiofilm potential, never studied before. The CV assay showed that BLS P34 was able to eliminate some of the planktonic cells that form the biofilm. Likely these cells by coming in contact with the conditioned surface with the antimicrobial, were reduced in number, influencing the adhesion capacity. Therefore, the conditioning of the surface became less favorable to cell adhesion.

To provide a better understanding on the effect of BLS P34 on biofilms is necessary the correlation between the results of CV technique and the MTT assay. Although the CV assay serves as an indicator of adhesion, it does not reveal the metabolic status of the cells. MTT assay is a tetrazolium salt, which in the presence of metabolically active cells is reduced into a product that can be measured colorimetrically, serving as a respiratory indicator of live cells (Krom et al. 2007). In biofilms, the MTT assay was used as an indicator of attached viable cells, while CV stains both viable and non-viable cells that may be attached (Kouidhi et al. 2010).

The metabolic activity of biofilms was theoretically controlled, by environmental conditions at the surface and expression of specific genes induced by adhesion (O'Toole and Kolter 1998). The low cellular metabolism provides resistance to antimicrobials that act during bacterial growth phase. This ability to survive the antimicrobial treatment at slow growth rates ensures the presence of persistent cells (Lewis 2012). The results of the MTT assay confirmed that the BLS P34 inhibited the metabolic activity of biofilms formed by *S. aureus* strains. The decrease in cell

metabolism occurs because most antimicrobial peptides work by interacting with the bacterial cell surface, followed by disruption of cellular integrity (Nawrocki et al. 2014). In a previous study, the BLS P34 showed a similar effect on the bacterial wall of *L. monocytogenes* ATCC 7644 (Motta et al. 2008).

The results of MTT assay also showed that *E. faecalis* biofilms were metabolically active. These strains were genotypically characterized by Medeiros et al. (2014) that found the presence of gelatinase (*gelE*) and cytolysin (*cyl*A) genes. Such virulence factors and phenotypic characteristics of each strain may be associated with metabolic stimulation. Moreover, it is possible that the cell stimulation has occurred due to the antimicrobial molecule has been inactivated and used as a substrate for the cell. Del Papa et al. (2007) report that the degradation of antimicrobial peptides (AMPs) by proteases is a mechanism of resistance found in many Gram-positive species, including *E. faecalis* and *S. aureus*. AMP-degrading proteases generally have broad substrate specificity, are typically found in mammalian pathogens, and include both metallopeptidases and cysteine proteases. Furthermore, Motta et al. (2007b) evaluate the residual activity of the BLS P34 treated with proteolytic enzymes like trypsin, papain, pronase E and proteinase K, and they found loss of antimicrobial potential. The inefficiency to inhibit cellular metabolism of *E. faecalis* using the BLS P34 confirms that biofilms are more resistant to antimicrobial agents than planktonic cells.

The results of this study demonstrated that the BLS P34 has the potential to prevent and eradicate the biofilm formation by *S. aureus* and *E. faecalis*. However, the substance was more effective to reduce the metabolic activity of bacterial cells in *S. aureus* biofilms. These results also indicate the antimicrobial potential of BLS P34 against planktonic cells of *S. aureus* and *E. faecalis*, which can be useful for many applications and must be better explored.

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