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Study of the effectiveness of staphylococcins in biopreservation of Minas fresh (Frescal) cheese with a reduced sodium content

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ABSTRACT

Reducing salt content in foods such as cheeses, while limiting the growth of spoilage microorganisms and foodborne pathogens, is a difficult challenge. One method that may prove useful is use of staphylococcins, which are bacteriocins produced by staphylococci.

Therefore, staphylococcin antimicrobial activity against six strains of *S. aureus* isolated from cheese was tested aiming at their industrial application in biopreservation of Minas fresh (Frescal) cheese with reduced sodium content. Three staphylococcins were selected for these tests: Pep 5, aureocin A53 and lysostaphin. All three staphylococcins proved to be bacteriolytic against all six strains of *S. aureus*. The antimicrobial activity of the partially purified staphylococcins was subsequently investigated against strains *S. aureus* Q1 and QJ3 in cheese matrices (6.0 log CFU/g) with different NaCl contents (control, a 25% reduction, and a 50% reduction), kept under refrigeration at 4 °C, for 21 days. Both strains were shown to be of concern for food industry as they carry the SEA, SEB and SEH enterotoxin genes, and are resistant to β-lactam drugs and moderate biofilm formers when grown in TSB. When used singly, Pep5, aureocin A53 and lysostaphin reduced approximately 95%, 99% and 99.99% of the viable cell counts, respectively, irrespective of the sodium content of the cheese matrix. The combined action of aureocin A53 and Pep5 resulted in an additional and significant reduction ($p < 0.05$) of ~1.0 log CFU/g when compared with the reduction caused by the use of either one singly. The combined action of lysostaphin and aureocin A53 or lysostaphin and Pep5 resulted in a reduction similar to or slightly smaller ($p > 0.05$) than that observed when lysostaphin was employed singly. Lysostaphin also proved to reduce the number of the staphylococcal viable cells to a level (~ 2.0 log CFU/g) at which enterotoxin production should not reach a sufficient quantity to cause food poisoning. Therefore, lysostaphin may have a practical application in the food industry to control staphylococcal contamination of Minas fresh cheese with a sodium content reduced up to 50%, providing consumers with more safe options to reduce their intake of sodium.

1. Introduction

Cheese consumption is popular in many countries around the world because it is associated with real health benefits for the consumers and also because of its taste and aroma. Health benefits may include naturally occurring probiotic microorganisms in some types of cheese and reducing rates of type II diabetes (Mozaffarian et al., 2010). Moreover, the various types of cheese are good sources of calcium, phosphorus and protein (Johnson et al., 2009). Among dairy products, cheese also has a decisive contribution to the consumption of sodium in the form of sodium chloride (NaCl) by human populations in their daily lives (Moshfegh et al., 2012). Although it is essential for the body, excess sodium intake has been linked to increase in blood pressure (hypertension), being a risk factor for cardiovascular diseases, and to other health problems (Durack et al., 2008; Wyness et al., 2012). Therefore, the World Health Organization (WHO) recommends NaCl consumption not exceed 5 g/day (WHO, 2003), as reduction of the dietary sodium intake could have significant health benefits.

A technologically viable option for reduction of NaCl content in foods is its substitution by potassium chloride (KCl), since the latter salt

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is chemically similar to the former one and studies reported in the literature have shown promising results in relation to the application of this substitution (Gomes et al., 2011; Grummer et al., 2013; Johnson et al., 2009). However, the substitution of NaCl with other salts or simply a reduction in NaCl concentration in the formulation of the food product can create barriers in cheese processing, affecting the physical-chemical, rheological, functional and organoleptic quality of the product and, especially, the microbial activity. Salt addition plays an important role in food safety of cheese, especially processed cheese, due to microbial growth control (Cruz et al., 2011; Johnson et al., 2009). Therefore, elimination or even reduction of NaCl from processed foods may have serious implications, not only enabling enhanced pathogen growth and survival, but also allowing more accelerated food spoilage and consequently a reduction of the shelf life of foods (Taormina, 2010). Therefore, additional control measures should be employed when manufacturing food products with low sodium content to maximize protection from foodborne pathogens and spoilage bacteria.

Among the major pathogens of milk and its derivatives, *Staphylococcus* is one of great concern. *Staphylococcus aureus* is the most important species of the genus (Andrade et al., 2011), causing food poisoning due to enterotoxin production (Franco and Landgraf, 2002). These enterotoxins possess important properties that enable them to cause intoxication, such as: (i) the ability to cause vomiting and gastroenteritis in primates; (ii) the ability to act as superantigens by activating non-specific T lymphocytes, followed by cytokine release and systemic shock, and (iii) resistance to heat and digestion by pepsin (Hennekinne et al., 2010).

However, the pathogen density in foods should be higher than 5.0 log CFU/g for assumptive enterotoxin production (Bulajic et al., 2017).

In the last decades, technological advances in the food industry coupled with shifts in consumer habits to demand for products with no chemical additives, such as nitrates, benzoates and sorbates, have increased the search for new natural biopreservatives. Among them, bacteriocins have emerged as potential candidates (Bali et al., 2016; Gálvez et al., 2007). Bacteriocins are antimicrobial peptides or proteins ribosomally synthesized by prokaryotes, which exhibit inhibitory activity against other prokaryotes (Heng et al., 2007). Some bacteriocins produced by *Staphylococcus* spp., referred to as staphylococcins, have the ability to inhibit several human and animal pathogens, including the foodborne ones (Bastos et al., 2009; Fagundes et al., 2016a, 2016b).

Bacteriocins may be applied to foods in four different ways: (i) added to foods in their purified or partially purified form, as food additives that act to increase shelf life; (ii) applied from a crude concentrate obtained by culturing the producing culture on food substrates (milk or whey), where this preparation may be used as an additive or ingredient; (iii) through its *in situ* production by bacteriocinogenic adjunct, protective or starter cultures, and (iv) immobilized in food packaging (Gálvez et al., 2007).

Staphylococcins, as most bacteriocins characterized so far, offer several desirable properties that make them suitable for food biopreservation: (i) they are not active on and are nontoxic to eukaryotic cells; (ii) they are generally sensitive to proteases and are expected to have little influence on the gut microbiota; (iii) they are usually pH and heat tolerant; (iv) they have a relatively broad antimicrobial spectrum of activity against many pathogenic and spoilage bacteria, and (v) they generally exhibit a bactericidal mode of action and no cross-resistance with other antimicrobials (Bastos et al., 2009; Fagundes et al., 2016a, 2016b).

There is an increased interest in new natural antimicrobials for food biopreservation and staphylococcins are quite unexplored as food biopreservatives. As bacteriocins may act to inhibit bacterial species which are closely related to the producer ones (Heng et al., 2007), the anti-staphylococcal activity of staphylococcins was investigated in Minas fresh (Frescal) cheese with low sodium content. Minas fresh cheese is one of the most popular dairy products consumed in Brazil (Gomes et al., 2011). Staphylococcins previously described but having different structural characteristics and belonging to different bacteriocin classes were chosen for these studies. The staphylococcins investigated (Pep5, aureocin A53 and lysostaphin) were shown to have potential application in biopreservation of Minas fresh cheese, as their antimicrobial activities were not affected by reduction of NaCl content and its replacement by KCl. Therefore, staphylococcins, especially lysostaphin, may be employed as an additional hurdle to avoid staphylococcal contamination and survival in cheese with a sodium content reduced up to 50%, providing consumers with more safe options to reduce their intake of sodium. Reducing salt content in foods, while maintaining quality and safety, is currently a major challenge for the food industry worldwide.

2. Material and methods

2.1. Bacterial strains and growth conditions

Fifteen bacterial strains were isolated from sausages and different types of cheese sold in Brazilian markets, following the procedures described by the Brazilian legislation (MAPA, 2003). The species identification was performed as described below. *Staphylococcus* spp. strains, described in previous studies and used as bacteriocin producers, are listed in Table 1. These strains, isolated from commercial milk, bovine mastitis or humans, produce staphylococcins which belong to different bacteriocin classes. *Micrococcus luteus* ATCC 4698 was used as indicator in all bacteriocin assays because this strain proved to be highly sensitive to several staphylococcins (Bastos et al., 2009). All strains were grown in either TSB (Difco, Sparks, MD, USA) or BHI (Difco) medium at 37 °C for 18 h. For preparation of either solid or soft-agar media, agar was added at 1.5% (w/v) or 0.7% (w/v), respectively. TSB medium was used to grow strains for DNA isolation, while BHI medium was used in all bacteriocin assays. Bacteria were stored in their appropriate medium with 40% glycerol (w/v) at −20 °C until required.

Table 1
Bacteriocin-producing *Staphylococcus* spp. strains used in this study.

Producer strain	Feature (class/subclass)/plasmid	Reference/source
S. aureus A53	Producer of aureocin A53 (IId)/pRJ9	Netz et al. (2002a)
S. aureus A70	Producer of aureocin A70 (IIe)/pRJ6	Netz et al. (2001)
S. aureus 4185	Producer of aureocin 4185 (ND) and aureocyclicin 4185 (IV)/pRJ101	Ceotto et al. (2010a); Potter et al. (2014)
S. aureus C55	Producer of staphylococcin C55 (Ib)/pET-B	Navaratna et al. (1998)
S. epidermidis Tü3298	Producer of epidermin (Ia)/pTü32	Allgaier et al. (1986)
*S. epidermidis*5	Producer of Pep5 (Ia)/pED503	Sahl and Brandis (1981)
S. hyicus 3682	Producer of hyicin 3682 (Ia)/pRJ109	Fagundes et al. (2017)
S. simulans 3299	Producer of nukacin 3299 (Ib)/pRJ97	Ceotto et al. (2010b)
S. simulans NRRL B-2628	Producer of lysostaphin (III)/pRG5	Recsei et al. (1987)

The bacteriocin classes and subclasses are according to the classification scheme proposed by Bastos et al. (2015). The readers are invited to read this review for a better understanding of the relevant characteristics of each subclass. ND. not determined yet.

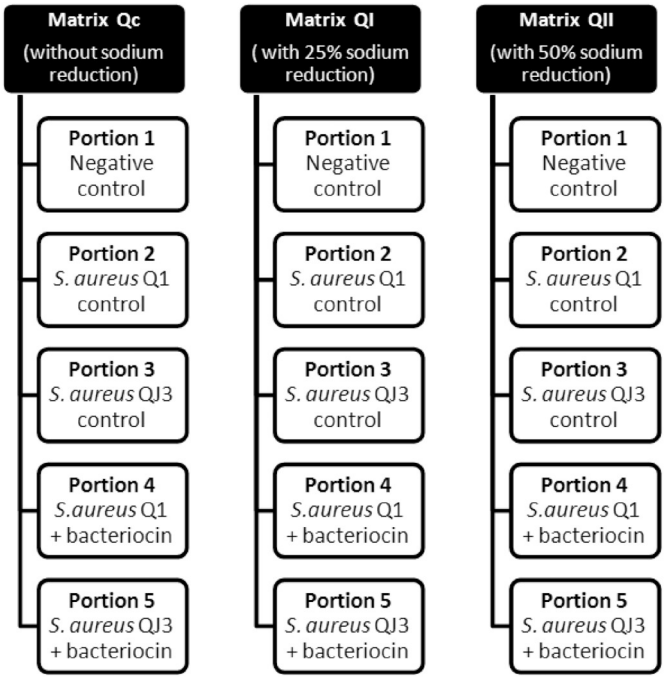

Fig. 1. Schematic flowchart of the experiments performed for each staphylococcin tested. Cheese matrices were divided into 5 portions of 20 g each, which were transferred to sterile vials. *S. aureus* Q1 and QJ3 were inoculated at the final concentration of 6 log CFU/g. After the inoculation of the bacteria, bacteriocins were added: 512 AU/g of aureocin A53 and 256 AU/g of either lysostaphin or Pep5, separately. Control experiments were performed without the addition of bacteriocins.

2.2. Identification of strains isolated from food to the species level

In order to identify the strains isolated from food, aiming to select the *S. aureus* ones for the subsequent tests, they were subjected to Gram staining and conventional biochemical tests as described by Bannerman and Peacock (2007). MALDI-TOF mass spectrometry was then used to confirm the identification of all *S. aureus* strains as described by Ayeni et al. (2015). *Escherichia coli* DH5α was used as a control strain.

2.3. Detection of staphylococcal enterotoxin genes

PCR experiments for detection of the genes coding for the classical staphylococcal enterotoxins (SEA, SEB, SEC, SED and SEE) and the non-classical enterotoxins SEG, SEH, SEI and SE*lJ* in the genome of the *S. aureus* strains were performed as described by Johnson et al. (1991) and Arcuri et al. (2010), respectively. The primers, synthesized by Life Technologies Brasil (São Paulo, Brazil), are described by Fagundes et al. (2016b). The genomic DNA of each strain was isolated as described by Pitcher et al. (1989). The reagents employed in PCR reactions and for DNA isolation were purchased from Promega Biotecnologia (São Paulo, Brazil).

2.4. Antibiotic susceptibility tests

The *S. aureus* strains isolated from food were cultured in Müeller-Hinton agar (Difco). The tests were performed by the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). The following antibiotics (Laborclin; Pinhais, PR, Brazil) were used: amikacin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), penicillin (10 U), rifampicin (5 μg), sulfamethoxazole/trimethoprim (25 μg), tetracycline (30 μg), tobramycin (10 μg) and vancomycin (30 μg). The minimum inhibitory concentration (MIC) for vancomycin (Sigma-Aldrich Brasil Ltda, São Paulo, Brazil) was determined by broth microdilution, using fresh cation-adjusted Müeller-Hinton broth (Difco). *S. aureus* ATCC 25923 and ATCC 29213 were used as controls for the disk diffusion and MIC tests, respectively. All strains were also tested for the presence of the inducible macrolide, lincosamide and streptogramin B (MLS_B) resistance phenotype, as described by CLSI (2015).

2.5. Biofilm formation

The ability of the *S. aureus* strains isolated from food to attach to and to form biofilm on sterile 96-well polystyrene microtiter plates (TPP® 92096; Techno Plastic Products, Trasadingen, Switzerland) was tested as previously described by Potter et al. (2009). Each assay was performed in quadruplicate and repeated three times. The interpretation of the results was done as described by Stepanović et al. (2007), using as negative controls the wells inoculated only with broth.

2.6. Antimicrobial activity of staphylococcins on solid medium

The antimicrobial activity of staphylococcins against all *S. aureus* strains isolated from food was initially tested by the agar-spot assay, essentially as described by Giambiagi-deMarval et al. (1990), using 10 μl (7.0 log CFU) of each producing strain (Table 1) grown as a spot on the surface of a BHI agar plate, and 6.0 log CFU/ml of each target strain in 3 ml of BHI soft agar. After incubation at 37 °C for 24 h, the size of the inhibition zones were given in mm. These experiments were performed in triplicate.

Table 2
Results of the sensitivity tests of the *S. aureus* strains isolated from cheese to staphylococcins.

Bacteriocin(s) produced	Strains of *S. aureus* isolated from cheese					
	Q1	Q2	QRHF1	QRHF2	QRHF3	QJ3
Aureocin A70	–	–	16.0 ± 1.2	16.0 ± 1.2	15.0 ± 0.6	–
Aureocin A53	17.0 ± 0.6	17.0 ± 1.2	17.0 ± 0.6	18.0 ± 0.0	18.0 ± 1.0	18.0 ± 1.0
Aureocin 4185/Aureocyclicin 4185	–	–	–	–	–	–
Pep5	16.0 ± 0.6	17.0 ± 0.6	20.0 ± 0.6	17.0 ± 1.0	17.0 ± 1.2	15.0 ± 0.6
Epidermin	–	–	–	–	–	–
Hyicin 3682	11.0 ± 1.0	17.0 ± 0.6	12.0 ± 1.0	12.0 ± 0.0	12.0 ± 0.6	11.0 ± 0.6
Nukacin 3299	–	–	–	–	–	–
Staphylococcin C55	–	–	–	–	–	–
Lysostaphin	22.0 ± 1.7	22.0 ± 1.2	24.0 ± 1.2	23.0 ± 0.6	23.0 ± 1.0	24.0 ± 1.2

The tests were performed in triplicate and values were expressed as the average size of the inhibition zones ± standard deviation in mm. –, no inhibition was observed.

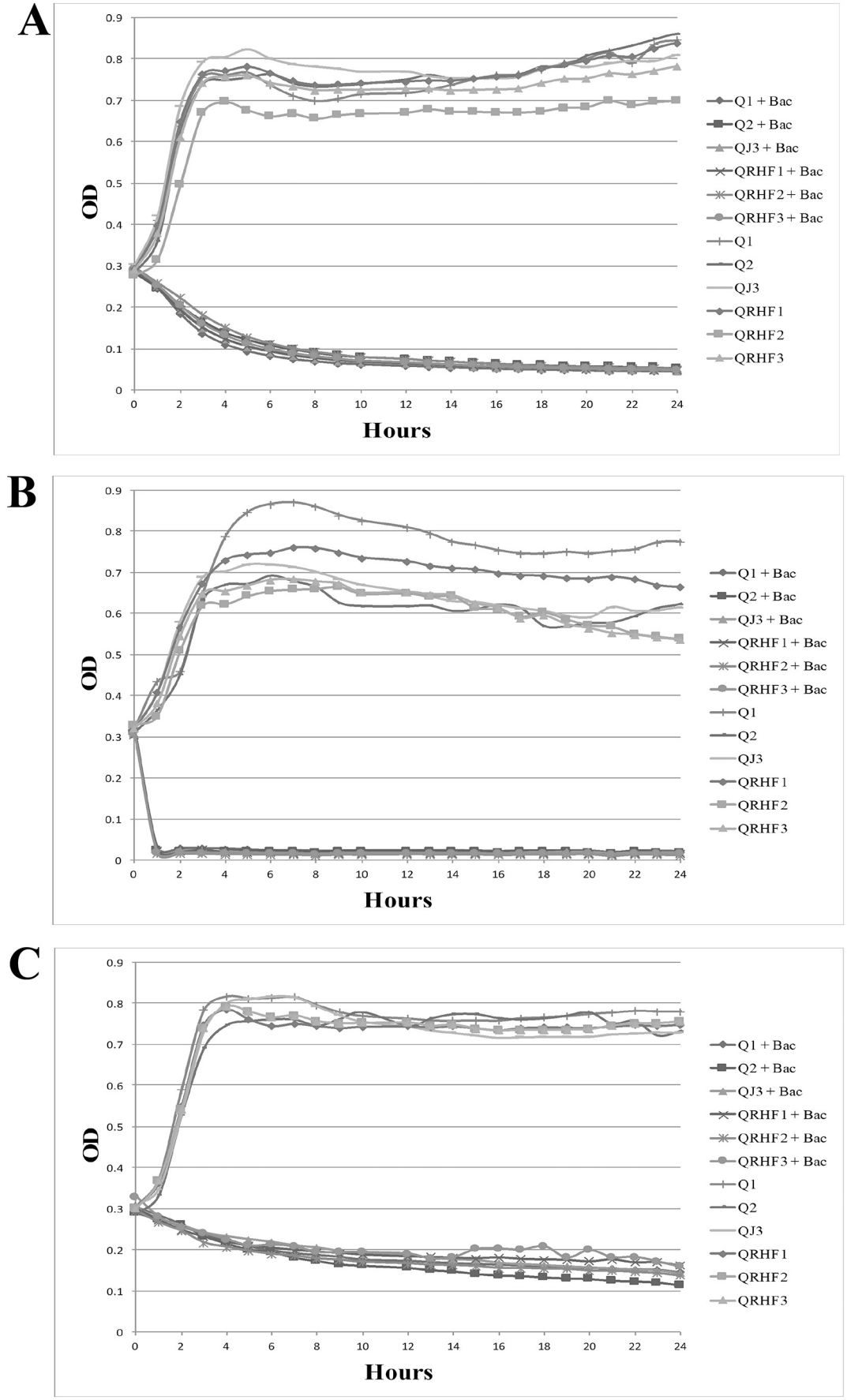

Fig. 2. Activity kinetics of staphylococcins against the six *S. aureus* strains isolated from cheese, in BHI broth, for 24 h at 37 °C, using a culture with an initial OD_{600} of 0.3. The results shown represent the average of three independent experiments. (A) aureocin A53 (4096 AU); (B) lysostaphin (1028 AU); (C) Pep5 (2048 AU).

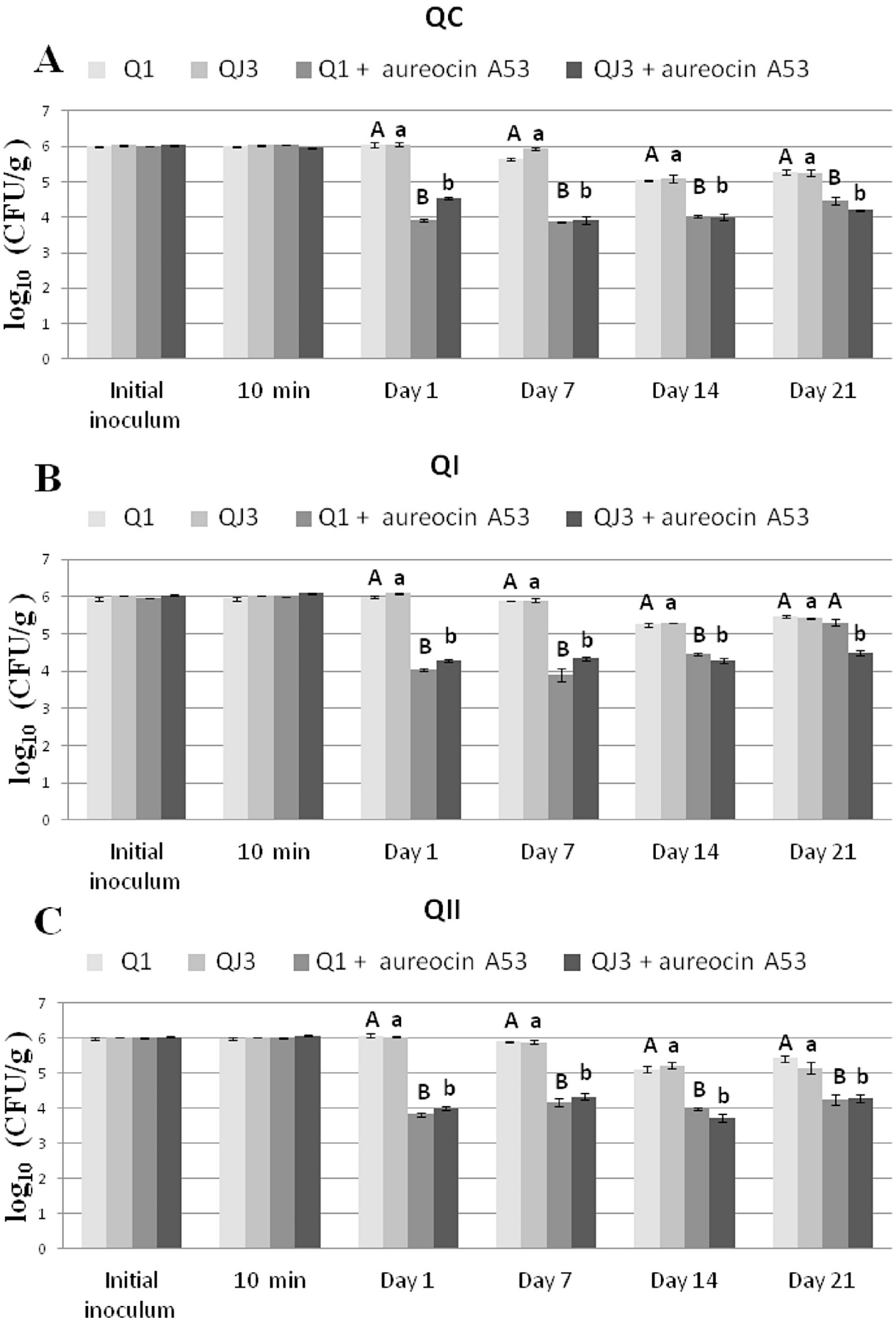

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Fig. 3. Aureocin A53 action (512 AU/g) on *S. aureus* strains Q1 and QJ3 in Minas fresh cheese matrices without sodium reduction (**QC**, A) and with either a 25% reduction (**IQ**, B) or a 50% reduction of the sodium concentration (**QII**, C). All tests were performed in triplicate and the values were expressed as the means of viable cell counts ± standard deviations. The samples were kept under incubation at 4 °C. Uppercase, statistical analyses between cheeses inoculated with strain Q1; lowercase, statistical analyses between cheeses inoculated with strain QJ3. Statistically significant differences were acknowledged when $p < 0.05$.

2.7. Partial purification of staphylococcins

The selected staphylococcins were purified from a culture of the corresponding producer strain, grown in one-liter BHI using optimized conditions for each bacteriocin production as previously described (Nascimento et al., 2004; Recsei et al., 1987; Sahl and Brandis, 1981). The following steps were followed: (i) ammonium sulfate precipitation [65% saturation (w/v; Merck Millipore, Darmstadt, Germany)] and (ii) cation exchange chromatography (SP Sepharose Fast Flow; GE Healthcare, Little Chalfont, United Kingdom), as described by Ceotto et al. (2010b). The staphylococcin preparations were dialysed against ultrapure water and quantified by the microtiter plate assay to determine the arbitrary units per ml (AU/ml), as described by Ceotto et al. (2010b), using 100 µl of two-fold serial bacteriocin dilutions prepared in BHI broth and 100 µl (10^6 cells) of a *M. luteus* ATCC 4698 suspension as the indicator microorganism. AU/ml represented the reciprocal of the highest bacteriocin dilution showing at least a 50% inhibition of the bacterial growth, after incubation at 37 °C for 18 h, when compared with the control with no bacteriocin added, multiplied by 10. The final pH of all staphylococcin preparations was in the range of 5.5–6.0.

2.8. Activity kinetics of staphylococcins against strains of S. aureus isolated from food

The activity kinetics of the partially purified staphylococcins was determined by the microtiter plate assay as described by Ceotto et al. (2010a) using the *S. aureus* strains isolated from food (OD_{600} of ~ 0.3) as the target strains. The amount of each staphylococcin (AU/ml) used in these experiments varied depending on the substance tested and is provided in the results. These experiments were performed in triplicate.

2.9. Minas fresh (Frescal) cheese production

Cheese manufacture followed the methodology described by Gomes et al. (2011) using raw milk (Itaocara, Rio de Janeiro, Brazil) which was pasteurized at 65 °C for 30 min and then cooled to 4 °C. The curd was divided into three equal parts and subjected to dry salting [NaCl, Sigma-Aldrich Brasil Ltda; 0.8% (wt/wt) of the final product], according to the following experiment design. KCl (Sigma-Aldrich Brasil Ltda) was used to replace part of the NaCl at 0, 25 and 50% (wt/wt), while keeping the final concentration of salt consistent [0.8% (wt/wt)] in relation to the total mass of the final product. Therefore, three different combinations were tested: Qc, without reduction in sodium concentration; QI, with a 25% reduction of the sodium concentration; and QII, with a 50% reduction of the sodium concentration. Subsequently, the cheeses were packaged and stored at 4 °C until used. To avoid cheese contamination, good manufacturing practices (GMP) were rigorously followed.

2.10. Evaluation of the inhibition of S. aureus in cheese matrices by the addition of staphylococcins

To test the inhibitory activity of the staphylococcins Pep5, aureocin A53 and lysostaphin in Minas fresh cheese, two strains of *S. aureus* isolated from food, Q1 and QJ3, were used as targets. For each bacteriocin to be tested, three cheese matrices were prepared: Qc, QI, and QII, as described above. Each matrix was divided into five portions (20 g each) which were transferred to sterile vials. The experiments were performed according to the flowchart illustrated in Fig. 1. Each treatment or control included two independent experiments. The strains to be tested were grown on the surface of Casoy agar (Biocen, São Paulo, Brazil) plates for 18 h at 37 °C. Cell suspensions were then prepared in sterile saline solution to the same turbidity of 0.5 of the McFarland Standard (~1.5×10^8 CFU/ml). *S. aureus* Q1 and QJ3 were inoculated into each cheese matrix at the final cell density of 6.0 log CFU/g in order to obtain a deliberately abusive initial count. Such inoculum was used because recent studies reported that such a density of staphylococcal cells has often been found in samples of Minas fresh cheese consumed in Brazil (Amorim et al., 2014; Cardoso et al., 2013; Leite Jr. et al., 2013).

Immediately after the inoculation of the pathogen, a partially-purified preparation of Pep5 (256 AU/g), aureocin A53 (512 AU/g) or lysostaphin (256 AU/g) was added, separately. Combinations of two substances (aureocin A53 and Pep5, aureocin A53 and lysostaphin or Pep5 and lysostaphin) were also tested at the same AU/ml. The matrices were macerated with sterilized spatulas to facilitate homogenization of the bacteria and bacteriocins in the food. Control experiments were performed without the addition of bacteriocins and an equal volume of 0.1% (w/v) peptone water was added instead of the bacteriocin preparation. *S. aureus* cell counts were enumerated by successive dilutions of the samples in saline solution and plating on Baird-Parker agar (Difco), at time zero and after 10 min, 1 day, 7 days, 14 days and 21 days of storage at 4 °C. The plates were incubated for 48 h at 37 °C. The Baird-Parker agar is used to isolate and differentiate coagulase-positive staphylococci from food. Black colonies with a clear halo around them, due to a lipolytic activity, are presumed to be indicative of *S. aureus* (Hoveida et al., 2019).

2.11. Statistical analysis

Data were assessed using on-way analysis of variance (ANOVA) on ranks and Dunn's *post hoc* test at $p < 0.05$, using the Past 3.22 program for Windows (Hammer & Harper, Oslo, Norway).

3. Results and discussion

3.1. Identification of the strains isolated from food to the species level

S. aureus is a major cause of foodborne illnesses. The main goal of the present study was the investigation of the potential use of staphylococcins to either prevent or reduce *S. aureus* contamination in food. Therefore, bacterial strains were isolated from different types of foods and identified to the species level. Six out of the 15 isolates were identified as *S. aureus* by Gram-staining and biochemical tests, and the species identification was further confirmed by MALDI-TOF mass spectrometry (data not shown). They were referred to as *S. aureus* Q1, Q2, QJ3, QRHF1, QRHF2 and QRHF3. All of them were isolated from different types of cheese sold in Brazilian markets. The isolation of these strains aimed at their subsequent use in food matrices to mimic staphylococcal contamination.

3.2. Detection of staphylococcal enterotoxin genes

The majority of the staphylococci-related food poisoning outbreaks have been associated with the classical SE, which can also be produced by staphylococci isolated from samples of bovine milk (Nunes et al., 2016; Seyoum et al., 2016). On the other hand, among the non-classical enterotoxins, SEG, SEH, SEI and SE*l*J were the most prevalent ones found in *S. aureus* isolates from dairy products in the southeast region of Brazil (Arcuri et al., 2010). Therefore, the presence of the genes coding

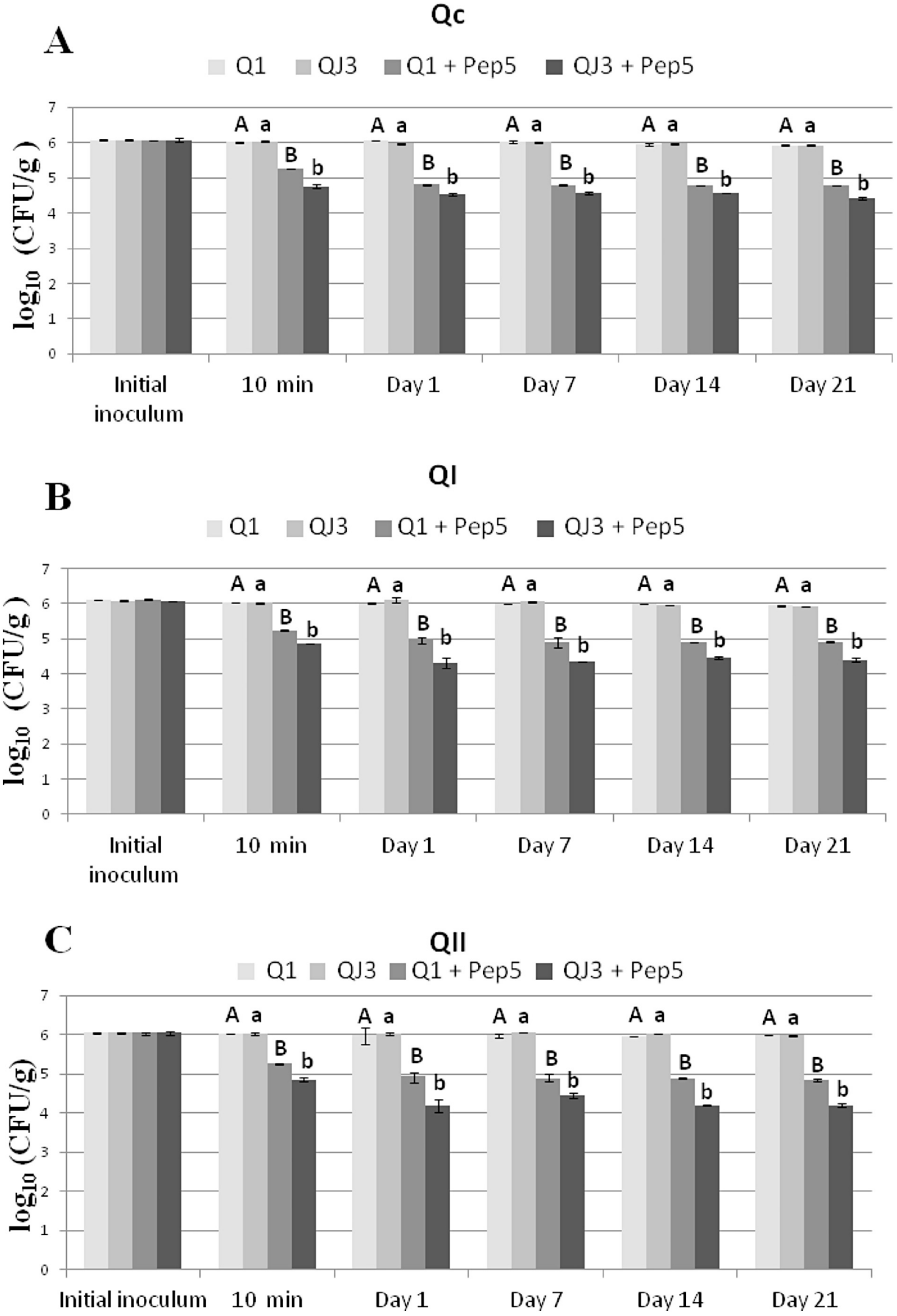

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Fig. 4. Pep5 action (256 AU/g) on *S. aureus* strains Q1 and QJ3 in Minas fresh cheese matrices without sodium reduction (**QC,** A) and with either a 25% reduction (**IQ**, B) or a 50% reduction of the sodium concentration (**QII**, C). All tests were performed in triplicate and the values were expressed as the means of viable cell counts ± standard deviations. The samples were kept under incubation at 4 °C. Uppercase, statistical analyses between cheeses inoculated with strain Q1; lowercase, statistical analyses between cheeses inoculated with strain QJ3. Statistically significant differences were acknowledged when $p < 0.05$.

for the prevalent SE was also investigated in the genome of the six staphylococcal strains by PCR. The strains Q1, Q2 and QJ3 were positive for the presence of the genes encoding SEA, SEB, and SEH, while the strains QRHF1, QRHF2 and QRHF3 were positive only for the presence of the gene coding for SEH (Table S1). Therefore, the classical enterotoxins SEA and SEB should be produced by three strains, while the non-classical SEH seems to be produced by all of them. Ferreira et al. (2016) also reported that the gene encoding SEH was the most frequently detected enterotoxin gene among *S. aureus* isolates from the artisanal cheese samples marketed in Goiânia, Brazil. Therefore, our results agree with the data found in the literature and also show that the *S. aureus* strains isolated from cheese, studied in the present work, possess a toxigenic potential. It is important to highlight that the enterotoxin encoding genes found in all six strains of *S. aureus* code for enterotoxins (SEA, SEB and/or SEH) that have been linked to food poisoning outbreaks (Argudin et al., 2010; Liu et al., 2014).

3.3. Antibiotic resistance profile

To investigate if the staphylococcal strains also carry drug resistance genes, their drug-resistance profile was investigated as well. All six strains were classified as resistant to penicillin and ampicillin (Table S2), both belonging to the β-lactam group of drugs, suggesting probably the occurrence of cross-resistance between both drugs due to the action of a β-lactamase (Drawz and Bonomo, 2010). The sensitivity to cefoxitin corroborates this hypothesis, since it indicates that all strains are not methicillin resistant and probably do not possess the PBP2a protein (CLSI, 2015). The prevalence of β-lactam resistance is often found among *S. aureus* isolates from milk and its derivatives (Xu et al., 2014).

Only strains QRHF1, QRHF2 and QRHF3 (50%) were resistant to tetracycline. A similar percentage of tetracycline resistance (44%) was also found among strains of *S. aureus* isolated from ready-to-eat foods (Yang et al., 2016). These strains also showed inducible resistance to erythromycin (Table S2), an antibiotic that belongs to the MLS_B group of drugs. Therefore, they exhibited the MLS_{Bi} phenotype and can be considered multidrug-resistant strains.

The presence of drug resistance among staphylococci isolated from cheese may reflect the wide use of antibiotics for either preventing or treating bacterial infections in dairy cattle (Gavilán et al., 2015; Li et al., 2015), which, in turn, may select for the presence, in milk, of staphylococcal cells carrying drug resistance. If GMP were not rigorously followed during pasteurization, drug-resistant staphylococcal cells may remain in milk used in cheese manufacture. Alternatively, post-pasteurization contamination by drug-resistant staphylococci during cheese manufacture could also explain the presence of the pathogen in the cheese samples used for bacterial isolation.

3.4. Biofilm formation

Biofilm is an important bacterial virulence factor, leading to a reduction in the effect of sanitizers and to food contamination in industrial plants, resulting in large economic losses to food industry (Kroning et al., 2016). Therefore, biofilm formation by all six strains of *S. aureus* isolated from cheese was investigated as well. All strains were classified as moderate biofilm formers when grown in TSB medium. The ability of these potentially-virulent staphylococcal isolates to produce biofilm increases the risk of their persistence in the food manufacturing environment (Kroning et al., 2016). Conditions that have been shown to increase biofilm formation, such as glucose addition to the medium, ethanol stress and osmotic stress with NaCl, among others (Potter et al., 2009), were not tested in the present study.

3.5. Staphylococcin activity against the S. aureus strains isolated from cheese

As bacteriocins generally inhibit bacterial species which are closely-related to the producer ones (Heng et al., 2007), the antimicrobial activity of staphylococcins was investigated against the staphylococcal strains isolated from cheese. The selected staphylococcins exhibit different biochemical features, belonging to different bacteriocin classes and even subclasses as shown in Table 1.

Initially, in order to detect the activity of staphylococcins against the six *S. aureus* strains, the agar diffusion method was employed. The results are shown in Table 2. The criteria that were established for selection of the staphylococcins to be used in the subsequent experiments were: (i) the ideal bacteriocin should inhibit all target strains and (ii) generate inhibition zones whose average size is ≥15 mm. Then, the staphylococcins that fit both criteria were Pep5 (class I), aureocin A53 (class II) and lysostaphin (class III).

The selected staphylococcins were then partially purified and used in kinetics activity assays. The results of these experiments are shown in Fig. 2. Aureocin A53, lysostaphin and Pep5 led to a marked reduction of the OD_{600} of all bacterial cultures, when compared with the controls, proving to be bactericidal and lytic to the cells tested. The cell lysis caused by these staphylococcins occurs by different mechanisms. Aureocin A53 bacteriolytic activity is due to generalized permeation and destruction of the cellular membrane (Netz et al., 2002b); lysostaphin bacteriolytic activity is due to its specific action on the pentaglycine of the cross-links of the bacterial cell wall (Browder et al., 1965), and Pep5 activity is due to pore formation leading to the efflux of ions and membrane depolarization, and to inhibition of the synthesis of DNA, RNA, proteins and polysaccharides (Sahl and Brandis, 1982).

3.6. Inhibition of S. aureus in cheese matrices with reduced sodium content by the addition of staphylococcins

In order to evaluated the biopreservative potential of the partially purified staphylococcins in Minas fresh cheese matrices with reduced sodium content, each bacteriocin was tested, singly and in combination, against the *S. aureus* strains Q1 and QJ3 isolated from cheese. Both strains were shown to carry the SEA, SEB and SEH enterotoxin genes, to be resistant to β-lactam drugs, and to be moderate biofilm formers when grown in TSB. In these tests, three Minas fresh cheese matrices were prepared: NaCl 100%, NaCl/KCl - 75/25% and NaCl/KCl - 50/50%. The final salt concentration (0.8% of the final product) in each cheese matrix corresponds to the salt concentration generally found in Minas fresh cheese manufactured in Brazil (Gomes et al., 2011). Absence of *S. aureus* cells in milk used in cheese manufacture was confirmed as no CFU was found in the milk samples tested on Baird-Parker agar plates prior to the experiments.

In most experiments, no significant ($p > 0.05$) difference in the cell number of both strains Q1 and QJ3 was observed when they were used as controls (no bacteriocin added) in all three different types of Minas fresh cheese matrices (Figs. 4, 5 and 6). The cell number was kept at 6.0 log CFU/g ($p > 0.05$) up to day 21. The only exceptions seem to be the matrices used in the experiments with aureocin A53 (Fig. 3), in which a drop of < 1.0 log was observed in the number of viable staphylococcal cells after day 7. These results suggest that no staphylococcal growth was observed in all cheese matrices.

When aureocin A53 (512 AU/g) was added to all cheese matrices

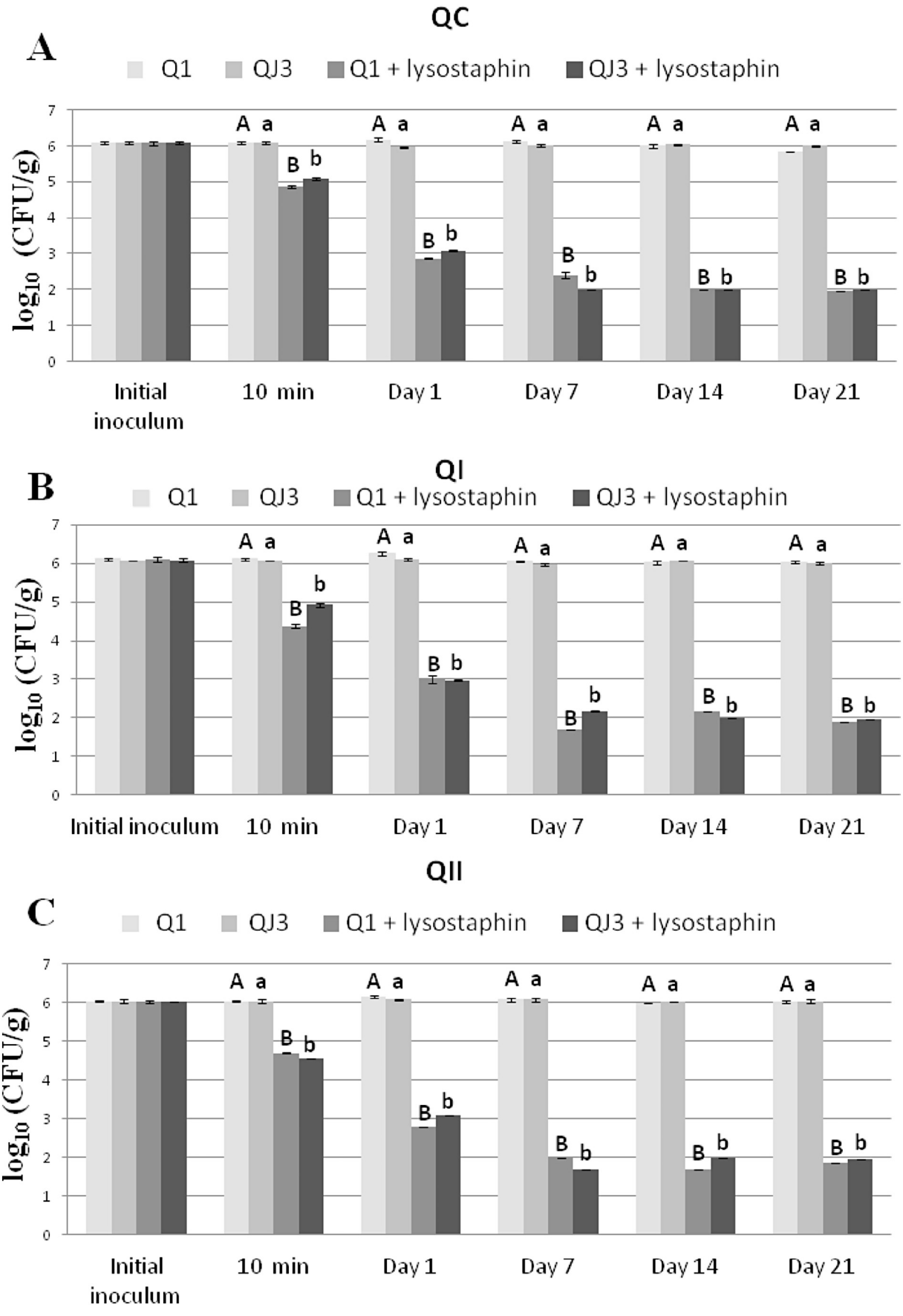

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Fig. 5. Lysostaphin action (256 AU/g) on *S. aureus* strains Q1 and QJ3 in Minas fresh cheese matrices without sodium reduction (**QC**, A) and with either a 25% reduction (**IQ**, B) or a 50% reduction of the sodium concentration (**QII**, C). All tests were performed in triplicate and the values were expressed as the means of viable cell counts ± standard deviations. The samples were kept under incubation at 4 °C. Uppercase, statistical analyses between cheeses inoculated with strain Q1; lowercase, statistical analyses between cheeses inoculated with strain QJ3. Statistically significant differences were acknowledged when $p < 0.05$.

(Fig. 3), there was a significant decline ($p < 0.05$) of 1.5–2.0 log in the number of survivals of *S. aureus* Q1 and QJ3 on the first 24 h of incubation. At the end of the 21st day, a slight but not significant increase ($p > 0.05$) of the bacterial population was detected in both matrices QC (Fig. 3A) and QII (Fig. 3C). In matrix QI (Fig. 3B), a significant increase of 1.0 log ($p < 0.05$) in the number of viable cells of strain Q1 was observed at the end of the experiment. However, staphylococcal recovery may be prevented by increasing the aureocin A53 concentration in the food matrices.

Addition of Pep5 (256 AU/g) to the cheese matrices (Fig. 4) resulted in a significant drop ($p < 0.05$) of 1.0–2.0 log in the number of survivals of both *S. aureus* strains, from the first 10 min of contact until the end of experiment (21 days) and regardless of the matrix sodium content. No recovery of the target strains was observed in these experiments.

The addition of lysostaphin (256 AU/g) to the three different matrices (Fig. 5) gave similar results. After 24 h of incubation, a significant ($p < 0.05$) 3.0-log reduction in the *S. aureus* population was observed, reaching a decrease of ~4.0 log on the 7th day of incubation, which was maintained until day 21, despite the abusive initial *S. aureus* inoculum. The results indicated that lysostaphin, when used singly, proved to be the most effective staphylococcin and that aureocin A53 proved to be the less effective one, when the results are compared at the end of the experiments.

Pinto et al. (2011) also investigated the efficacy of a bacteriocin, nisin, in inhibiting *S. aureus* in Serro cheese, another traditional Brazilian cheese, and the results were similar to those found for Pep5. The authors observed a reduction of 1.2 and 2.0 log in the population of *S. aureus* on the 7th day of ripening for cheese containing 100 IU/ml and 500 IU/ml of the bacteriocin, respectively, compared with the control samples. Pep5 and nisin are lantibiotics, which are linear bacteriocins with a polycyclic structure and post-translationally modified amino acids (Bastos et al., 2009). Both lantibiotics belong to the same subclass, Ia (Bastos et al., 2009). Therefore, it is not surprising that they behaved similarly, resulting in a similar reduction of the initial staphylococcal population in cheese matrices.

Pep5, aureocin A53 and lysostaphin have never been tested before in cheese, and, therefore, the present data are the first description of attempts made to use them for cheese biopreservation. Aureocin A53 is a very atypical class II bacteriocin (linear peptide with no unusual amino acids), whereas lysostaphin belongs to class III (composed of proteins with antimicrobial activity) (Bastos et al., 2009).

According to the Brazilian legislation (ANVISA, 2001), the maximum cell count allowed for *S. aureus* in Minas fresh cheese is 3.0 log CFU/g. This cell density is much lower than that required (> 5 log CFU/g) for the assumptive presence of staphylococcal enterotoxins in food (Bulajic et al., 2017). The lysostaphin preparation used in the present study was able to reduce the abusive initial population of *S. aureus* from 6.0 log CFU/g to < 3.0 log CFU/g in all three cheese matrices on the first 24 h of incubation (Fig. 5). After 21 days of refrigeration, the *S. aureus* population was reduced to ~2.0 log CFU/g. The results showed that lysostaphin alone was capable of reducing pathogen concentration to the safety levels established by ANVISA. No sufficient quantity of SEA, SEB and SEH to cause intoxication should be produced by strains Q1 and QJ3 in the cheese matrices at cell densities of ~2.0 log CFU/g.

To be effective, bacteriocins need to diffuse through the cheese matrix and reach the bacterial cells. However, cheese components may interfere in their diffusion. It has been reported, for example, that nisin can interact with several constituents of food matrices, such as lipids and proteins, reducing its antimicrobial activity (Aly et al., 2012). Among the three staphylococcins tested in this work, lysostaphin seems to be the bacteriocin which is less affected by cheese components.

Since significant differences in the behavior of *S. aureus* strains were not observed in the three cheese matrices tested, matrix QI was chosen for the tests using pairwise combinations of the staphylococcins. The choice of this matrix for these experiments was due to the fact that it exhibited physicochemical and organoleptic characteristics that were shown to be more acceptable to consumers than those of the QII matrix (Gomes et al., 2011). The final bacteriocin concentrations used were the same employed in the previous experiments. The combination of aureocin A53 with Pep5 resulted in an additional and significant decrease ($p < 0.05$) of ~1.0 log in the population of *S. aureus* compared with the use of the two bacteriocins separately (Fig. 6A). The combined action of lysostaphin with either aureocin A53 (Fig. 6B) or Pep5 (Fig. 6C) resulted in a decrease in the *S. aureus* population similar to or slightly smaller ($p > 0.05$) than that caused by the action of lysostaphin alone. Such results suggest that either this bacteriocin acts more rapidly in the target cells, not allowing the action of aureocin A53 and Pep5, or these staphylococcin combinations neither show a synergism nor an additive effect against the *S. aureus* strains employed.

The effectiveness of nisin combined with bovicin HC5 to inhibit the growth of *Listeria monocytogenes* and *S. aureus* in Minas fresh cheese has been tested by Pimentel-Filho et al. (2014). Bacteria (4.0 log CFU/g) were inoculated separately and both bacteriocins were added at 600 AU/g, each. After nine days of storage at 4 °C, the bacteriocins reduced the number of L. *monocytogenes* viable cells to < 1 CFU/25 g, but the *S. aureus* population was reduced by only 1.0 log unit. Lysostaphin, used in the present study, proved to be more effective than nisin and bovicin HC5 in reducing the staphylococcal population in Minas fresh cheese.

4. Conclusions

To avoid or to reduce food contamination by *S. aureus* is one of the most efficient forms to prevent staphylococcal foodborne poisoning. According to Hennekinne et al. (2012), hygiene procedures as proper handling, control of raw materials, cleaning and disinfection of equipment must be adopted. However, these methods are not always sufficient to prevent contamination by *S. aureus* and, once present in the food, others control measures need to be employed to either prevent staphylococcal growth or eliminate the pathogen. The use of bacteriocins is considered a valuable biological alternative. Therefore, three staphylococcins with different biochemical and structural features were tested in this work to control the staphylococcal growth in Minas fresh cheese matrices with different sodium content. The strains chosen for this investigation were isolated from cheese, carry the genes coding for SEA, SEB and SEH, and proved to be resistant to β-lactam drugs and to form biofilm, characteristics that are considered to be quite relevant for food safety. Moreover, a recent study has shown that SEA production by *S. aureus* is not affected by NaCl concentrations up to 10% (Elahi and Fujikawa, 2019). Therefore, staphylococcal enterotoxin production is expected to occur in all cheese matrices tested in the present study if the staphylococcal cell density is kept at > 5 log CFU/g. However, staphylococcal enterotoxin production was not assayed in the present study.

The results of the analyses performed indicated that all three staphylococcins employed (Pep5, aureocin A53 and lysostaphin), especially the latter one, have a rapid activity against *S. aureus* in Minas fresh cheese. Lysostaphin also proved to reduce the number of the

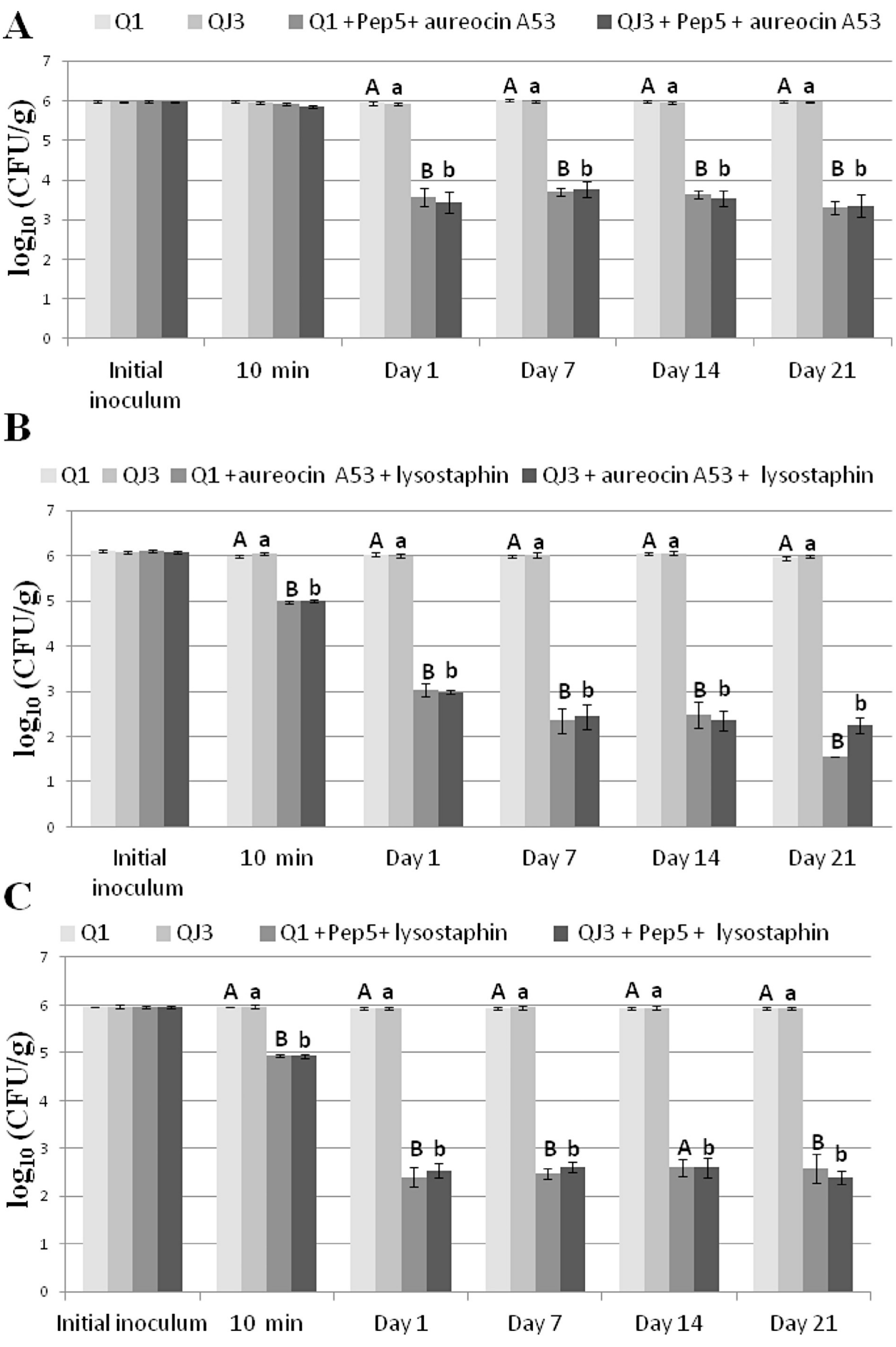

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Fig. 6. Combined action of: aureocin A53 and Pep5 (A); aureocin A53 and lysostaphin (B), and lysostaphin and Pep5 (C) on *S. aureus* strains Q1 and QJ3 in the Minas fresh cheese matrix with a 25% reduction in sodium concentration. Staphylococcin concentrations: aureocin A53 (512 AU/g), Pep5 (256 AU/g), and lysostaphin (256 AU/g). All tests were performed in triplicate and the values were expressed as the means of viable cell counts ± standard deviations. The samples were kept under incubation at 4 °C. Uppercase, statistical analyses between cheeses inoculated with strain Q1; lowercase, statistical analyses between cheeses inoculated with strain QJ3. Statistically significant differences were acknowledged when $p < 0.05$.

staphylococcal cells to a safety level (~2.0 log CFU/g) and, therefore, may have a practical application in the food industry to control staphylococcal contamination of Minas fresh cheese, an ordinary food consumed in Brazil.

This work is the first study to investigate the potential application of staphylococcins in the biopreservation of Minas fresh cheese. The staphylococcin activity was not affected by reduction of sodium content and its replacement by KCl. Therefore, staphylococcins may be used as an additional hurdle to improve safety of cheese with reduced sodium content. Consumption of low-salt foods is recommended by WHO to improve public health, as excess sodium intake has been associated with the development of several health problems, especially hypertension (Durack et al., 2008), which leads to cardiovascular diseases (Grummer et al., 2013). Such health complications can be prevented by decreasing dietary sodium intake (Taormina, 2010). Therefore, consumers, and especially people at a high risk for cardiovascular diseases, may benefit from cheese with low salt content preserved by staphylococcins. Lastly, we cannot forget that the use of bacteriocins as biopreservatives is a complementary tool for GMP in food manufacture and should never be used with the objective to substitute them.

Declaration of Competing Interest

No conflict of interest is declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.05.014.

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