Viability of *Staphylococcus aureus* and expression of its toxins (SEC and TSST-1) in cheeses using *Lactobacillus rhamnosus* D1 or *Weissella paramesenteroides* GIR16L4 or both as starter cultures

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ABSTRACT

Staphylococcus aureus is one of the main causative agents of food poisoning. This bacterium is an important component of cheese microbiota and plays an important role in foodborne diseases. Another important component of the microbiota is the lactic acid bacterium, which actively participates in processes that define the physicochemical, sensorial, and microbiological features of cheese. Of the various microbiological interactions in cheese, the interaction between lactic acid bacteria and *Staph. aureus* is most relevant. To this end, we evaluated the viability of *Staph. aureus* strains and the expression of their enterotoxins in cheeses produced experimentally, using *Weissella paramesenteroides* GIR16L4 or *Lactobacillus rhamnosus* D1 or both as starter cultures. Over 7 d, we observed that the presence of lactic acid bacteria did not impair *Staph. aureus* growth. However, via qPCR we observed a change in the gene expression of staphylococcal enterotoxins, suggesting that molecular communication exists between *Staph. aureus* strains and lactic acid bacteria in cheese. **Key words:** cheese, staphylococcal enterotoxin, gene expression, lactic acid bacterium

INTRODUCTION

The global food market is constantly challenged to improve technology, production rate, and safety from microbiological contamination. Foods are susceptible to contamination by diverse microorganisms, leading to the development of foodborne diseases in both animals and humans. These diseases could be caused by

the microorganisms themselves or by their metabolites (Stamford et al., 2006). From 1998 to 2015, 19,119 foodborne outbreaks were recorded in the United States (an average of approximately 1,000 cases per year), with 14,681 hospitalizations and 337 deaths (CDC, 2017). In Brazil, from 2007 to 2016, 6,848 foodborne outbreaks were recorded, with 17,517 hospitalizations and 111 deaths. In 2017, 133 outbreaks and 2,014 affected people were recorded in Brazil (Brazil, Ministério da Saúde, Secretaria de Vigilância em Saúde, 2017). The main microbiological agents involved in those outbreaks were *Escherichia coli* (7.3%), *Salmonella* spp. (7.3%), and *Staphylococcus aureus* (5.7%; Brazil, Ministério da Saúde, Secretaria de Vigilância em Saúde, 2016).

Among all etiological agents associated with foodborne outbreaks, *Staph. aureus* has been reported to be one of the most common pathogenic microorganisms (Le Loir et al., 2003; Stamford et al., 2006). These gram-positive bacteria are the causative agents for bovine mastitis and are responsible for contaminating milk and cheese. *Staphylococcus aureus* can produce toxic shock proteins, such as toxic shock syndrome toxin 1 (TSST-1), and thermostable enterotoxins, such as staphylococcal enterotoxins, which cause food poisoning commonly characterized by nausea, vomiting, abdominal cramps, and, in some cases, diarrhea (Benkerroum, 2017). To date, 24 staphylococcal enterotoxins have been described, of which SEA, SEB, SEC (1, 2, and 3), SED, and SEE are the most well studied (Benkerroum, 2017).

Cheese contains diverse microorganisms; their interactions determine whether the cheese is safe for consumption as well as the physicochemical and sensorial characteristics of the cheese. These microorganisms include lactic acid bacteria (LAB), which synthesize metabolites that can inhibit the growth of pathogens such as *Staph. aureus*, *Salmonella* spp., *E. coli*, and *Listeria monocytogenes* (Penido et al., 2018; Sant'Anna et al., 2019). The metabolites include organic acids

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(lactic, propionic, and acetic acids); bacteriocins such as nisin, lactacinin, pediocin, and plantaricin; and hydrogen peroxide (Ross et al., 2000; Dabiza and El-Deib, 2007; Le Marc et al., 2009; Al-Delaimy and Hamamdeh, 2013; Hor and Liong, 2014).

Although several studies have reported the antagonistic activity of LAB strains on pathogenic strains of *Staph. aureus*, these studies have been limited to in vitro approaches. To date, the inhibitory effect of LAB strains on *Staph. aureus* growth and enterotoxin gene expression has not been evaluated in cheese. This study evaluated *Staph. aureus* colonization and *sec* and *tsst1* gene expression in experimentally produced cheese, using different LAB strains as starter cultures.

MATERIALS AND METHODS

Bacterial Samples

In this study, we used 2 *Staph. aureus* strains: FRI361 (from the Food Research Institute, University of Wisconsin Madison; Otero et al., 1990), a staphylococcal enterotoxin C (SEC) producer (*sec*⁺); and N315, a methicillin-resistant strain (Kuroda et al., 2001) and an SEC and toxic shock syndrome toxin 1 producer (*sec*⁺, *tsst1*⁺). Two LAB strains, *Lactobacillus rhamnosus* D1 (isolated from Brazilian Minas artisanal cheese) and *Weissella paramesenteroides* GIR16L4 (isolated from raw milk) were used. The LAB strains have been identified to species level by Sandes et al. (2014) and were used in this study due to their inhibitory effect on other *Staph. aureus* strains (Costa et al., 2013; Silva, 2016; Acurcio et al., 2017). The *Staph. aureus* and LAB strains were cultured aerobically in brain heart infusion broth (BHI; Difco, Detroit, MI) and in de Man, Rogosa, and Sharpe broth (MRS; Merck, Darmstadt, Germany), respectively.

In Vitro Approaches

Susceptibility of *Staph. aureus* Strains to Lactic Acid. To assess the acid susceptibility of *Staph. aureus* strains, 1 mL of stationary-phase cultures of *Staph. aureus* FRI361 and N315 were inoculated in 100 mL of BHI, supplemented with 0.1, 0.2, or 0.3% (vol/vol) lactic acid. The cultures were incubated at 37°C for 18 h. At 0, 4, 8, and 18 h, 100-μL aliquots were collected, diluted in peptone saline, and plated on Baird Parker agar (Difco). Plates were incubated at 37°C for 48 h, and viable colonies were counted.

Susceptibility of *Staph. aureus* to LAB Strains. This assay was performed as previously described (Sandes et al., 2017). Briefly, 5 μL of stationary-phase

cultures of *L. rhamnosus* D1 and *W. paramesenteroides* GIR16L4 were spotted onto MRS agar and incubated under aerobic conditions for 48 h at 37°C. Thereafter, the cells were killed by exposing them to chloroform vapor and UV light for 30 min. The *Staph. aureus* FRI361 and N315 strains were cultured in BHI medium until they reached stationary phase, inoculated into BHI soft agar (0.75% wt/vol agar), and then overlaid on the surface of MRS plates containing spots of dead LAB strains. After incubating at 37°C for 24 h, the inhibitory activity of LAB was determined by measuring the growth inhibition zone, using a digital pachymeter (Mitutoyo Sul Americana Ltd., São Paulo, Brazil).

In-Cheese Approaches

Cheesemaking Process. Before cheese production, skim milk powder (Itambé, Belo Horizonte, Brazil) was evaluated for presence of inhibitory substances (Neal and Calbert, 1955), colony-forming units of *Staph. aureus* (Lancette and Tatini, 2001) and LAB (MacFaddin, 1980), presence of *Salmonella* spp. (Andrews et al., 2001), and most probable number of coliforms (Kornacki and Johnson, 2001). The presence of staphylococcal enterotoxin genes in skim milk was evaluated via PCR, using 10 pmol each of *sec* and *tsst-1* primer (Table 1), 25 ng of DNA, and master mix from Phoeutria (Belo Horizonte, Brazil) containing 5 U/μL of *Taq* polymerase, under the following conditions: 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min.

To produce cheese, a solution of 15 g of skim milk powder was diluted in 100 mL of sterile distilled water, heated to 40°C, and incubated at 37°C for 20 min. The last step was performed to completely dissolve solid particles in water. This concentration of milk (15% wt/vol) was chosen because it produced clots with the highest stability during cheese production. Thereafter, milk was supplemented with rennet and 50% (wt/vol) CaCl₂ solution, and the starter cultures were inoculated as shown in Table 2. After 40 min of coagulation, the solid phase (curd) was placed in a sterile cheesecloth, and the curd was mixed uniformly with 1 g of NaCl. The cheese was stored at 7°C for 7 d in a biochemical oxygen demand refrigerator, and small samples were collected on the first and seventh day after production.

Due to loss of bacteria during cheese processing, we plated the cheese samples. For this, 1 g cheese sample was homogenized, serially diluted, and plated in MRS or Baird-Parker medium. Sample collection for physicochemical, microbiological, and molecular assays was performed only when bacterial populations reached 10⁸ cfu/g, this point being considered d 0.

Table 1. Targets and primers used in this study

RNA target ¹	Primer sequence (5'-3')		Amplicon size (bp)	Primer (μmol/L)
	Forward	Reverse		
TSST-1	TTCACATATTTGTAAAGTGTGACGCCACT	TACTAATGAATTTTTTATCGTAAGCCCTT	180	2.0
SEC	AATAAAGCGTTGATTCTAAAGTGTGAA	ATCAAAATCGGATTAAACATTATCCATTG	80	1.5
16S	CNACGCGAAGAACCTTAC	CGACAGCCATGCANCACT	98	0.3
Coa	CCGCTTCAACTTCAGCCTAC	TTAGGTGCTACAGGGGCAAT	204	0.1
FemA	AAAAAGCACATAACAGCG	GATAAAGAAGAAACCAAGCAG	132	0.1

¹TSST-1 = toxic shock syndrome toxin 1; SEC = staphylococcal enterotoxin C; Coa = coagulase-positive staphylococci.

Physicochemical and Microbiological Analyses.

The different cheeses produced were physicochemically analyzed for (1) moisture content, by the gravimetric method (IDF, 2004a); (2) nitrogenous compounds, by the Kjeldahl method (IDF, 2004b); (3) titratable acidity (Merck, 1993); and (4) pH, using an indicator strip. For microbiological analyses, 10 g of cheese was weighed and homogenized using a stomacher (MK1204, Boitton, Porto Alegre, Brazil) in 90 mL of 0.1% NaCl (wt/vol) solution, corresponding to 10^{-1} dilution. Thereafter, samples were serially diluted to 10^{-6} and plated by the spread-plate method on Baird-Parker (Lancette and Tatini, 2001) or MRS agar (MacFaddin, 1980) to perform counts for *Staph. aureus* and LAB strains, respectively. For these assays, samples were collected on the first and seventh days of storage. The physicochemical and microbiological analyses were performed in triplicate.

RNA Isolation. The RNA assay was performed as previously described (Ulve et al., 2008; Sandes et al., 2017). Briefly, total RNA was isolated from 3 g of cheese stored for 1 and 7 d. The quality and quantity of RNA were evaluated using agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), measuring absorbance ratios at 280/260 and 260/230 nm. Samples were treated with turbo DNase (Ambion, Austin, TX) for cDNA synthesis, which was performed using 1 μg of RNA and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Expression Analysis of *sec* and *tsst1*. Quantitative reverse transcription (qRT)-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene-specific primers for *sec* and *tsst1*, as described by Klotz et al. (2003) and Jarraud et al. (2002). The housekeeping gene 16S was used for normalization (Huse et al., 2008). Experimental approach was optimized by adjusting primer concentration for optimal specificity and efficiency (Table 1). Amplification was performed using the following protocol: initial step, 95°C for 30 s, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The dissociation curve was obtained as follows: 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Expression levels in cheese inoculated only with FRI361 (*sec*⁺) or with N315 (*tsst1*⁺) strains were used for calibration. Results have been shown graphically as fold-change in gene expression, using mean and standard deviation of the amount of gene expression, according to Hellemans et al. (2007).

Contaminant Analysis. Molecular analyses were performed in the control cheese (without inoculum) to verify eventual cross-contamination between cheeses, by using specific primers for coagulase-positive staphy-

Table 2. Combinations of *Staphylococcus aureus* and lactic acid bacteria strains used for cheesemaking

Cheese	Inoculum (10^8 cfu) ¹			
	FRI361	N315	<i>Weissella paramesenteroides</i>	<i>Lactobacillus rhamnosus</i>
Control ²	—	—	—	—
FRI361 <i>sec</i> ⁺	Yes	—	—	—
FRI361 <i>sec</i> ⁺ vs. <i>W. paramesenteroides</i>	Yes	—	Yes	—
FRI361 <i>sec</i> ⁺ vs. <i>L. rhamnosus</i>	Yes	—	—	Yes
FRI361 <i>sec</i> ⁺ vs. <i>W. paramesenteroides</i> / <i>L. rhamnosus</i>	Yes	—	Yes	Yes
N315 <i>sec</i> ⁺ <i>tsstI</i> ⁺	—	Yes	—	—
N315 <i>sec</i> ⁺ <i>tsstI</i> ⁺ vs. <i>W. paramesenteroides</i>	—	Yes	Yes	—
N315 <i>sec</i> ⁺ <i>tsstI</i> ⁺ vs. <i>L. rhamnosus</i>	—	Yes	—	Yes
N315 <i>sec</i> ⁺ <i>tsstI</i> ⁺ vs. <i>W. paramesenteroides</i> / <i>L. rhamnosus</i>	—	Yes	Yes	Yes

¹*Staphylococcus aureus* and lactic acid bacteria were cultured in brain heart infusion broth and de Man, Rogosa, and Sharpe broth, respectively, until stationary phase was reached. Thereafter, bacterial samples were serially diluted, and the inoculum concentration was adjusted to 10^8 cfu. Experimental cheeses were inoculated with *Staphylococcus aureus* strains FRI361, a staphylococcal enterotoxin C producer (SEC; *sec*⁺), and N315, a methicillin-resistant strain and an SEC and toxic shock syndrome toxin 1 producer (*sec*⁺ *tsstI*⁺).

²No inoculum was used.

lococci (Coa) and *Staph. aureus* (FemA; Table 1). Cheese samples inoculated with FRI361 or with N315 were used as positive controls, as these were treated only with *Staph. aureus* during production. The cDNAs were amplified by PCR using 0.2 μ L of each primer and 2.5 ng of cDNA, under the following conditions: 94°C for 5 min, 35 cycles of 94°C for 2 min, 56°C for 2 min (Coa) or 57°C for 2 min (FemA), 68°C for 1 min, and final extension 72°C for 7 min. Furthermore, contamination was analyzed via qPCR for *sec* and *tsstI*, to ascertain whether the toxins studied were present, as they could be expressed only by coagulase-negative staphylococci. The conditions used in the reaction have been described previously.

Statistical Analysis

All assays were performed in triplicate with 3 independent repetitions. A total of 9 different cheeses were produced in triplicate (Table 1) and stored for 1 d. Another batch of 9 different cheeses was produced under the same conditions and stored for 7 d. Data were analyzed in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). The Shapiro-Wilk test was used to determine data distribution. Parameterized samples were analyzed through one-way ANOVA to evaluate differences between groups inside a period, or through 2-way ANOVA to evaluate differences between groups in different periods. Dunnett's correction was employed as post hoc multiple comparison test, considering a level of significance higher than 95% ($P < 0.05$). Non-parameterized samples were also submitted to ANOVA and Duncan's tests. Student-Newman-Keuls and Friedman tests were used for microbiological and physicochemical assays, respectively.

RESULTS AND DISCUSSION

Skim Milk Powder Standard

Staphylococcus aureus, thermotolerant coliforms, *Salmonella* spp., and LAB strains were not detected in the skim milk powder.

Staph. aureus FRI361 and N315 are Resistant to Lactic Acid

Staphylococcus aureus strains FRI361 and N315 were able to multiply in the presence of 0.1, 0.2, and 0.3% lactic acid (Figure 1), suggesting that these strains were acid tolerant. These results contradict other studies, which showed an inhibitory effect of lactic acid produced by LAB strains on *Staph. aureus* growth (Alomar et al., 2008; Charlier et al., 2008; Al-Delaimy and Hamamdeh, 2013; Hor and Liong, 2014; Nouaille et al., 2014). Therefore, we speculated that the inhibition of *Staph. aureus* strains by LAB may be due to the action of other inhibitory substances, such as bacteriocins or hydrogen peroxide (Sandes et al., 2017).

Physicochemical Analyses

Control cheeses showed 72.47 and 71.16% moisture and 16.29 and 15.12% total protein at d 1 and 7 after processing, respectively. These values are within the standards established by Brazilian legislation for cheeses with very high moisture content (Brazil, Ministério da Agricultura, Pecuária e Abastecimento, Secretaria de Defesa Agropecuária, 1996). Mean results for titratable acidity and pH of cheeses treated with different conditions are shown in Tables 3 and 4.

Table 3. Means of titratable acidity and pH in cheese prepared using different lactic acid bacteria strains and *Staphylococcus aureus* FRI361, a staphylococcal enterotoxin C producer (*sec*⁺), stored at 7°C for 1 and 7 d

Cheese	Titratable acidity (%)		pH	
	Day 1	Day 7	Day 1	Day 7
Control	0.034 ^{A,a}	0.029 ^{B,a}	7.0 ^{A,a}	7.0 ^{A,a}
FRI361 <i>sec</i> ⁺	0.021 ^{BC,a}	0.035 ^{B,b}	7.0 ^{A,a}	7.0 ^{A,a}
FRI361 <i>sec</i> ⁺ vs. <i>Weissella paramesenteroides</i>	0.021 ^{C,a}	0.052 ^{AB,b}	6.5 ^{B,a}	6.0 ^{B,a}
FRI361 <i>sec</i> ⁺ vs. <i>Lactobacillus rhamnosus</i>	0.022 ^{B,ca}	0.042 ^{B,a}	7.0 ^{A,a}	6.0 ^{B,b}
FRI361 <i>sec</i> ⁺ vs. <i>W. paramesenteroides</i> / <i>L. rhamnosus</i>	0.028 ^{B,a}	0.095 ^{A,b}	5.7 ^{C,a}	5.5 ^{C,a}

^{A-C}Different uppercase superscripts in columns indicate that comparison between groups was statistically different ($P < 0.05$).

^{a,b}Different lowercase superscripts in rows indicate that comparison between groups was statistically different ($P < 0.05$).

Over 7 d of storage we observed an increase in titratable acidity in all inoculated cheeses (Tables 3 and 4), probably due to acid produced during microbial metabolism. No statistical differences were observed in the pH of the cheeses inoculated with *Staph. aureus* alone (Tables 3 and 4). In contrast, the use of LAB as starter culture reduced the pH over the time of storage, especially for cheeses made with *Staph. aureus* FRI361 and *L. rhamnosus* D1; *Staph. aureus* N315, *W. paramesenteroides* GIR16L4, or *L. rhamnosus* D1; and *Staph. aureus* N315, *W. paramesenteroides* GIR16L4, and *L. rhamnosus* D1 ($P < 0.05$).

Microbiological Analyses

As stated earlier, microorganisms were not detected in milk powder, attesting to its quality and safety as a raw material for cheesemaking. Although the cheeses were produced under sterile conditions, we observed growth of LAB and staphylococci in control samples, which were produced without bacterial inoculation (Tables 5 and 6). This can be explained by possible contamination after processing or during storage, or both. However, LAB counts in these cheeses were statistically lower than LAB counts observed in other cheeses ($P < 0.05$). Regarding staphylococci growth, a similar observation was made: control cheese showed lower counts of

Staph. aureus compared with those subjected to other treatments ($P < 0.05$; Tables 5 and 6). This result was expected, as control cheeses were not inoculated with *Staph. aureus*.

To evaluate whether the contamination could have interfered with subsequent analyses, we performed PCR for coagulase-positive *Staph. aureus*, using CoA and FemA primers; no amplicon was detected, indicating that the control cheese was not contaminated with coagulase-positive *Staph. aureus* (data not shown). Moreover, when we performed qPCR for *sec* and *tsst1*, no difference in relative gene expression was observed between d 1 (without contamination) and d 7 (with contamination), indicating that contamination of the control cheese did not change the relative gene expression of the toxins (Figure 2).

Regarding storage time, no difference in *Staph. aureus* count was observed between the different cheeses except in the control cheese and in the cheese inoculated with *Staph. aureus* N315, *W. paramesenteroides*, and *L. rhamnosus* (Table 6). Although the pH of the cheeses decreased with storage time, it was not sufficient to decrease *Staph. aureus* growth (Tables 3–6). Moreover, in the present study, the growth of *Staph. aureus* FRI361 and N315 were not affected by LAB, demonstrating that these strains were resistant to environmental and microbiological changes. This result could be a warn-

Table 4. Means of titratable acidity and pH in cheese prepared using different lactic acid bacteria strains and *Staphylococcus aureus* N315, a methicillin-resistant strain that is a staphylococcal enterotoxin C producer (*sec*⁺) and a toxic shock syndrome toxin 1 producer (*tsst1*⁺), stored at 7°C for 1 and 7 d

Cheese	Titratable acidity (%)		pH	
	Day 1	Day 7	Day 1	Day 7
Control	0.034 ^{A,a}	0.029 ^{B,a}	7.0 ^{A,a}	7.0 ^{A,a}
N315 <i>sec</i> ⁺ <i>tsst1</i> ⁺	0.017 ^{CD,a}	0.030 ^{B,b}	7.0 ^{A,a}	7.0 ^{A,a}
N315 <i>sec</i> ⁺ <i>tsst1</i> ⁺ vs. <i>Weissella paramesenteroides</i>	0.020 ^{C,a}	0.049 ^{A,b}	6.6 ^{A,a}	5.8 ^{C,b}
N315 <i>sec</i> ⁺ <i>tsst1</i> ⁺ vs. <i>Lactobacillus rhamnosus</i>	0.014 ^{D,a}	0.038 ^{B,b}	7.0 ^{A,a}	6.5 ^{B,b}
N315 <i>sec</i> ⁺ <i>tsst1</i> ⁺ vs. <i>W. paramesenteroides</i> / <i>L. rhamnosus</i>	0.027 ^{B,a}	0.057 ^{AB,b}	6.0 ^{B,a}	5.5 ^{C,b}

^{A-D}Different uppercase superscripts in columns indicate that comparison between groups was statistically different ($P < 0.05$).

^{a,b}Different lowercase superscripts in rows indicate that comparison between groups was statistically different ($P < 0.05$).

Figure 1. *Staphylococcus aureus* growth (strains FRI361 and N315) in 0.1, 0.2, and 0.3% lactic acid.

ing, as *Staph. aureus* counts found in this study can be considered high enough to trigger toxin production that could result in staphylococcal poisoning (Jay et al., 2005).

Toxin Expression

When we compared the relative expression of toxins in cheese inoculated with *Staph. aureus* N315 (Figure 3a and b), we observed reduced *tsst1* and *sec* expression from d 1 to the d 7 ($P < 0.05$), suggesting high initial toxin levels followed by a decrease in toxin levels, which may be explained by a physiological adaptation process, wherein the synthesis of virulence factors was reduced. We also observed that when *Staph. aureus* N315 was co-inoculated with *W. paramesenteroides* or *L. rhamnosus* or both, initial expression levels of *tsst1* and *sec* were reduced, compared with cheese inoculated with N315 alone, and remained at low levels during 7 d of storage. These results lead us to speculate that expression of these genes could be regulated by the same

quorum-sensing mechanism; however, this hypothesis needs to be tested further.

When we analyzed *sec* expression in cheeses inoculated with *Staph. aureus* FRI361 (Figure 3c), we observed that co-inoculation with *W. paramesenteroides* or *L. rhamnosus* or both triggered higher expression of toxins, which was reduced through d 7 of incubation. On d 1 of storage, the cheeses inoculated with *Staph. aureus* FRI361 behaved in an opposite manner to the cheeses inoculated with *Staph. aureus* N315, where LAB co-inoculation induced *sec* expression, indicating that FRI361 might be regulated by a different quorum-sensing mechanism than N315.

Previously, similar results have been described. Even et al. (2009) evaluated gene expression of virulence factors in *Staph. aureus* cocultured with *Lactococcus lactis* and observed that several regulatory virulence factors genes, such as *agr* and *sarA*, were underexpressed. Duquenne et al. (2010) co-inoculated *Staph. aureus*, *L. lactis*, and *Lactococcus cremoris* in cheeses and reported decreased expression of *sea* and *sed* over

Table 5. Average count of lactic acid bacteria and *Staphylococcus aureus* FRI361, a staphylococcal enterotoxin C producer (*sec*⁺; log cfu/g) in cheese stored at 7°C for 1 and 7 d¹

Cheese	Lactic acid bacteria		<i>Staphylococcus aureus</i> FRI361 <i>sec</i> ⁺	
	Day 1	Day 7	Day 1	Day 7
Control	ND ^{A,a}	6.03 ^{A,b}	ND ^{A,a}	5.73 ^{A,b}
FRI361 <i>sec</i> ⁺	ND ^{A,a}	ND ^{C,a}	7.08 ^{B,a}	7.61 ^{B,a}
FRI361 <i>sec</i> ⁺ vs. <i>Weissella paramesenteroides</i>	6.87 ^{B,a}	7.85 ^{B,b}	7.32 ^{B,a}	7.87 ^{B,a}
FRI361 <i>sec</i> ⁺ vs. <i>Lactobacillus rhamnosus</i>	6.84 ^{B,a}	7.71 ^{B,b}	7.22 ^{B,a}	7.82 ^{B,a}
FRI361 <i>sec</i> ⁺ vs. <i>W. paramesenteroides</i> / <i>L. rhamnosus</i>	7.31 ^{B,a}	7.86 ^{B,a}	8.03 ^{C,a}	7.76 ^{B,a}

^{A-C}Different uppercase superscripts in columns indicate that comparison between groups was statistically different ($P < 0.05$).

^{a,b}Different lowercase superscripts in rows indicate that comparison between groups was statistically different ($P < 0.05$).

¹ND = not detected.

Table 6. Average count of lactic acid bacteria and *Staphylococcus aureus* N315, a methicillin-resistant strain that is a staphylococcal enterotoxin C producer (*sec*⁺) and a toxic shock syndrome toxin 1 producer (*tsst*⁺; log cfu/g) in cheese stored at 7°C for 1 and 7 d¹

Cheese	Lactic acid bacteria		<i>Staphylococcus aureus</i> N315 <i>sec</i> ⁺ <i>tsst</i> ⁺	
	Day 1	Day 7	Day 1	Day 7
Control	ND ^{A,a}	6.03 ^{A,b}	ND ^{A,a}	5.73 ^{A,b}
N315 <i>sec</i> ⁺ <i>tsst</i> ⁺	ND ^{A,a}	ND ^{C,a}	7.07 ^{B,a}	7.58 ^{B,a}
N315 <i>sec</i> ⁺ <i>tsst</i> ⁺ vs. <i>Weissella paramesenteroides</i>	7.40 ^{B,a}	7.63 ^{B,a}	7.49 ^{B,a}	7.72 ^{B,a}
N315 <i>sec</i> ⁺ <i>tsst</i> ⁺ vs. <i>Lactobacillus rhamnosus</i>	6.99 ^{B,a}	7.71 ^{B,b}	7.53 ^{B,a}	7.46 ^{B,a}
N315 <i>sec</i> ⁺ <i>tsst</i> ⁺ vs. <i>W. paramesenteroides</i> / <i>L. rhamnosus</i>	7.28 ^{B,a}	7.87 ^{B,b}	8.18 ^{B,a}	7.61 ^{B,b}

^{A-C}Different uppercase superscripts in columns indicate that comparison between groups was statistically different ($P < 0.05$).

^{a,b}Different lowercase superscripts in rows indicate that comparison between groups was statistically different ($P < 0.05$).

¹ND = not detected.

72 h. Nouaille et al. (2014) investigated the interaction between *L. lactis* ssp. *lactis* and *Staph. aureus*, suggesting the capacity of LAB to modulate *agr* expression in *Staph. aureus*.

CONCLUSIONS

The results obtained in the present study show that the *W. paramesenteroides* GIR16L4 and *L. rhamnosus* D1 strains were not able to impair the growth of *Staph. aureus* FRI361 and N315 strains in cheeses produced experimentally. However, LAB may be able to change the expression of staphylococcal toxins in a strain-dependent manner. These results must be fur-

ther analyzed to determine the mechanisms of quorum sensing through which LAB modulates expression of staphylococcal toxins.

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Figure 2. Fold changes in gene expression of (a) toxic shock syndrome toxin 1 (*tsst*) and (b) staphylococcal enterotoxin C (*sec*) in control cheeses (without inoculation) compared with cheeses inoculated with *Staphylococcus aureus* N315 (*sec*⁺ *tsst*⁺) or *Staph. aureus* FRI361 (*sec*⁺). Different uppercase letters indicate that comparison between the same groups on d 1 and d 7 was statistically different ($P < 0.05$). Different lowercase letters indicate that comparison between groups on the same day was statistically different ($P < 0.05$).

Figure 3. Level of gene expression of toxic shock syndrome toxin 1 (*tsstI*) and staphylococcal enterotoxin C (*sec*) in cheeses produced with *Weissella paramesenteroides* or *Lactobacillus rhamnosus*, or both, as starter cultures, inoculated with *Staphylococcus aureus* N315 (*sec*⁺ *tsstI*⁺) or *Staph. aureus* FRI361 (*sec*⁺). Different uppercase letters indicate that comparison between the same groups on d 1 and d 7 was statistically different ($P < 0.05$). Different lowercase letters indicate that comparison between groups on the same day was statistically different ($P < 0.05$).

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REFERENCES

- Acurcio, L. B., S. H. C. Sandes, R. W. Bastos, F. M. Sant'anna, S. H. S. P. Pedroso, D. C. Reis, Á. C. Nunes, G. D. Cassali, M. R. Souza, and J. R. Nicoli. 2017. Milk fermented by *Lactobacillus* species from Brazilian artisanal cheese protect germ-free-mice against *Salmonella* Typhimurium infection. *Benef. Microbes* 8:579–588. <https://doi.org/10.3920/BM2016.0163>.
- Al-Delaimy, K. S., and Y. M. Hamamdeh. 2013. Inhibition of *Staphylococcus aureus* by lactic acid bacteria and/or *Bifidobacterium lactis* during milk fermentation and storage. *J. Microbiol. Biotechnol. Food Sci.* 2:2406.
- Alomar, J., P. Loubiere, C. Delbes, S. Nouaille, and M.-C. Montel. 2008. Effect of *Lactococcus garvieae*, *Lactococcus lactis* and *Enterococcus faecalis* on the behavior of *Staphylococcus aureus* in microfiltered milk. *Food Microbiol.* 25:502–508. <https://doi.org/10.1016/j.fm.2008.01.005>.
- Andrews, W. H., R. S. Flowers, J. Silliker, and J. S. Bailey. 2001. *Salmonella*. Pages 357–376 in *Compendium of Methods for the Microbiological Examination of Foods*, 4th ed. F. P. Downes and K. Ito, ed. Am. Public Health Assoc, Washington, DC.
- Benkerroum, N. 2017. Staphylococcal enterotoxins and enterotoxin-like toxins with special reference to dairy products: An overview. *Crit. Rev. Food Sci. Nutr.* 58:1943–1970. <https://doi.org/10.1080/10408398.2017.1289149>.
- Brazil, Ministério da Agricultura, Pecuária e Abastecimento, Secretaria de Defesa Agropecuária. 1996. Portaria n. 147, de 07 de março de 1996. Regulamentos Técnicos de Identidade e Qualidade dos Produtos Lácteos. Diário Oficial da União.
- Brazil, Ministério da Saúde, Secretaria de Vigilância em Saúde. 2016. Surtos de Doenças Transmitidas por Alimentos no Brasil. Accessed May 13, 2017. <http://portal.arquivos.saude.gov.br/images/pdf/2016/dezembro/09/Apresentacao-Surtos-DTA-2016.pdf>.
- Brazil, Ministério da Saúde, Secretaria de Vigilância em Saúde. 2017. Surtos de Doenças Transmitidas por Alimentos no Brasil. Accessed Jul. 4, 2017. <http://portal.arquivos.saude.gov.br/images/pdf/2017/maio/29/Apresentacao-Surtos-DTA-2017.pdf>.
- CDC (Centers for Disease Control and Prevention). Foodborne Outbreak Online Database. 2017. Accessed Apr. 27, 2017. <https://www.cdc.gov/foodborneoutbreaks/Default.aspx/>.
- Charlier, C., S. Even, M. Gautier, and Y. Le Loir. 2008. Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactococcus lactis* in milk. *Int. Dairy J.* 18:197–203. <https://doi.org/10.1016/j.idairyj.2007.03.015>.
- Costa, H., M. Souza, L. Acúrcio, A. Cunha, M. Resende, and A. Nunes. 2013. Potencial probiótico in vitro de bactérias ácido-láticas isoladas de queijo-de-minas artesanal da Serra da Canastra, MG. *Arq. Bras. Med. Vet. Zootec.* 65:1858–1866. <https://doi.org/10.1590/S0102-09352013000600038>.
- Dabiza, N., and K. El-Deib. 2007. Biochemical evaluation and microbial quality of Ras cheese supplemented with probiotic strains. *Pol. J. Food Nutr. Sci.* 57:295–300.
- Duquenne, M., I. Fleuret, M. Aigle, C. Darrigo, E. Borezée-Durant, S. Derzelle, M. Bouix, V. Deperrois-Lafarge, and A. Delacroix-Buchet. 2010. Tool for quantification of staphylococcal enterotoxin gene expression in cheese. *Appl. Environ. Microbiol.* 76:1367–1374. <https://doi.org/10.1128/AEM.01736-09>.
- Even, S., C. Charlier, S. Nouaille, N. L. Ben Zakour, M. Cretenet, F. J. Cousin, M. Gautier, M. Coccain-Bousquet, P. Loubière, and Y. Le Loir. 2009. *Staphylococcus aureus* virulence expression is impaired by *Lactococcus lactis* in mixed cultures. *Appl. Environ. Microbiol.* 75:4459–4472. <https://doi.org/10.1128/AEM.02388-08>.
- Hellemans, J., G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele. 2007. qBase relative quantification framework and soft-

- ware for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19. <https://doi.org/10.1186/gb-2007-8-2-r19>.
- Hor, Y. Y., and M. T. Liong. 2014. Use of extracellular extracts of lactic acid bacteria and bifidobacteria for the inhibition of dermatological pathogen *Staphylococcus aureus*. *Zhonghua Pifuke Yixue Zazhi* 32:141–147. <https://doi.org/10.1016/j.dsi.2014.03.001>.
- Huse, S. M., L. Dethlefsen, J. A. Huber, D. M. Welch, D. A. Relman, and M. L. Sogin. 2008. Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLoS Genet.* 4:e1000255. <https://doi.org/10.1371/journal.pgen.1000255>.
- IDF (International Dairy Federation). 2004a. Cheese and processed cheese: Determination of total solids content. IDF Standard 004. Int. Dairy Fed., Brussels, Belgium.
- IDF (International Dairy Federation). 2004b. Milk: Determination of nitrogen content. IDF Standard 020–3. Int. Dairy Fed., Brussels, Belgium.
- Jarraud, S., C. Mougé, J. Thioulouse, G. Lina, H. Meunier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect. Immun.* 70:631–641. <https://doi.org/10.1128/IAI.70.2.631-641.2002>.
- Jay, J. M., M. J. Loessner, and D. A. Golden. 2005. *Modern Food Microbiology*. 6th ed. Van Nostrand Reinhold Publishing Co., New York, NY.
- Klotz, M., S. Oppé, K. Heeg, and S. Zimmermann. 2003. Detection of *Staphylococcus aureus* enterotoxins A to D by real-time fluorescence PCR assay. *J. Clin. Microbiol.* 41:4683–4687. <https://doi.org/10.1128/JCM.41.10.4683-4687.2003>.
- Kornacki, J. L., and J. L. Johnson. 2001. Enterobacteriaceae, coliforms and *Escherichia coli* as quality and safety indicators. Pages 69–80 in *Compendium of Methods for the Microbiological Examination of Foods*. 4th ed. F. P. Downes and K. Ito, ed. American Public Health Association, Washington, DC.
- Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357:1225–1240. [https://doi.org/10.1016/S0140-6736\(00\)04403-2](https://doi.org/10.1016/S0140-6736(00)04403-2).
- Lancette, G. A., and S. R. Tatini. 2001. *Staphylococcus aureus*. Pages 387–400 in *Compendium of Methods for the Microbiological Examination of Foods*. 4th ed. F. P. Downes and K. Ito, ed. American Public Health Association, Washington, DC.
- Le Loir, Y., F. Baron, and M. Gautier. 2003. *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.* 2:63–76.
- Le Marc, Y., L. Valík, and A. Medvedová. 2009. Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. *Int. J. Food Microbiol.* 129:306–311. <https://doi.org/10.1016/j.ijfoodmicro.2008.12.015>.
- MacFaddin, J. F. 1980. *Biochemical Tests for Identification of Medical Bacteria*. 2nd ed. Williams and Wilkins, Baltimore, MD.
- Merck. 1993. *Reactivos, Diagnostica, Productos Químicos* 1992/93. Merck, Darmstadt, Germany.
- Neal, C., and H. Calbert. 1955. The use of 2, 3, 5-triphenyltetrazolium chloride as a test for antibiotic substances in milk. *J. Dairy Sci.* 38:629–633. [https://doi.org/10.3168/jds.S0022-0302\(55\)95015-3](https://doi.org/10.3168/jds.S0022-0302(55)95015-3).
- Nouaille, S., L. Rault, S. Jeanson, P. Loubière, Y. Le Loir, and S. Even. 2014. Contribution of *Lactococcus lactis* reducing properties to the downregulation of a major virulence regulator in *Staphylococcus aureus*, the agr system. *Appl. Environ. Microbiol.* 80:7028–7035. <https://doi.org/10.1128/AEM.02287-14>.
- Otero, A., M. L. Garcia, M. C. Garcia, B. Moreno, and M. S. Bergdoll. 1990. Production of staphylococcal enterotoxins C₁ and C₂ and thermonuclease throughout the growth cycle. *Appl. Environ. Microbiol.* 56:555–559.
- Penido, F. C. L., F. B. Piló, S. H. C. Sandes, Á. C. Nunes, G. Colen, E. S. Oliveira, C. A. Rosa, and I. C. A. Lacerda. 2018. Selection of starter cultures for the production of sour cassava starch in a pilot-scale fermentation process. *Braz. J. Microbiol.* 49:823–831. <https://doi.org/10.1016/j.bjm.2018.02.001>.
- Ross, R. P., C. Stanton, C. Hill, G. F. Fitzgerald, and A. Coffey. 2000. Novel cultures for cheese improvement. *Trends Food Sci. Technol.* 11:96–104. [https://doi.org/10.1016/S0924-2244\(00\)00057-1](https://doi.org/10.1016/S0924-2244(00)00057-1).
- Sandes, S., L. Alvim, B. Silva, L. Acurcio, C. Santos, M. Campos, C. Santos, J. Nicoli, E. Neumann, and Á. Nunes. 2017. Selection of new lactic acid bacteria strains bearing probiotic features from mucosal microbiota of healthy calves: Looking for immunobiotics through in vitro and in vivo approaches for immunoprophylaxis applications. *Microbiol. Res.* 200:1–13. <https://doi.org/10.1016/j.micres.2017.03.008>.
- Sandes, S. H., L. B. Alvim, B. C. Silva, D. F. Zanirati, L. R. Jung, J. R. Nicoli, E. Neumann, and Á. C. Nunes. 2014. *Lactobacillus* species identification by amplified ribosomal 16S–23S rRNA restriction fragment length polymorphism analysis. *Benef. Microbes* 5:471–481. <https://doi.org/10.3920/BM2013.0092>.
- Sant'Anna, F. M., S. U. Wetzels, S. H. S. Cicco, R. C. Figueiredo, G. A. Sales, N. C. Figueiredo, C. A. Nunes, S. Schmitz-Esser, E. Mann, M. Wagner, and M. R. Souza. 2019. Microbial shifts in Minas artisanal cheeses from the Serra do Salitre region of Minas Gerais, Brazil throughout ripening time. *Food Microbiol.* 82:349–362. <https://doi.org/10.1016/j.fm.2019.02.016>.
- Silva, R. S. Caracterização da microbiota da glândula mamária bovina saudável e com mastite subclínica e seleção de bactérias potencialmente probióticas isoladas desse ecossistema. 2016. PhD thesis, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil.
- Stamford, T. L. M., C. G. M. Silva, R. A. Mota, and A. Cunha Neto. 2006. Enterotoxigenicidade de *Staphylococcus* spp. isolados de leite in natura. *Food Sci. Technol. (Campinas)* 26:41–45. <https://doi.org/10.1590/S0101-20612006000100007>.
- Ulve, V. M., C. Monnet, F. Valence, J. Fauquant, H. Falentin, and S. Lortal. 2008. RNA extraction from cheese for analysis of in situ gene expression of *Lactococcus lactis*. *J. Appl. Microbiol.* 105:1327–1333. <https://doi.org/10.1111/j.1365-2672.2008.03869.x>.