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Presence of *Staphylococcus aureus* and staphylococcal enterotoxin A Production and Inactivation in Brazilian Cheese Bread

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HIGHLIGHTS

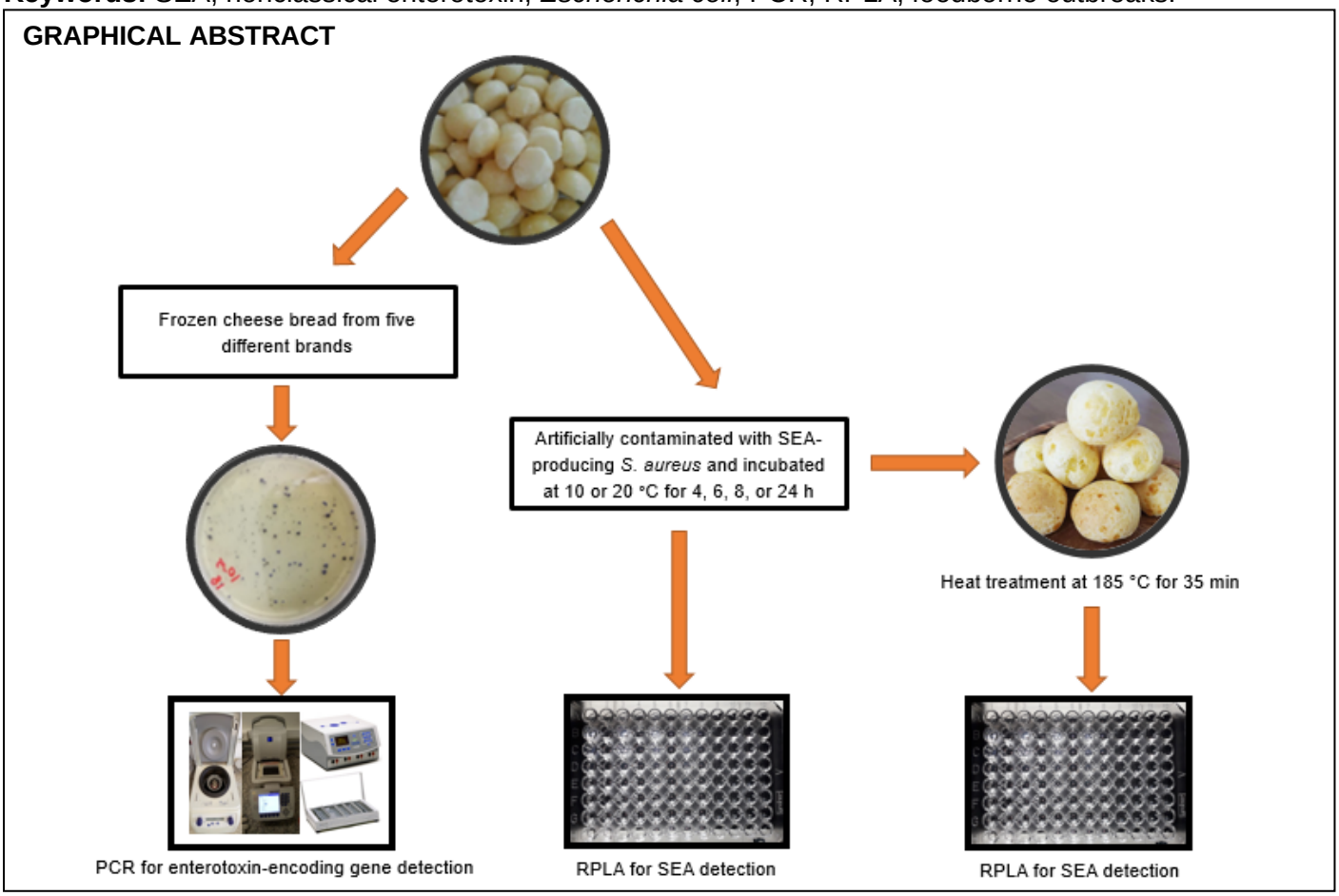
- The study shows the *S. aureus* counts of the 100 frozen cheese bread samples analyzed.
- 27 samples (27.0%) had *S. aureus* counts greater than 5.0 × 10^3 CFU/g.
- None of the 62 *S. aureus* strains isolated carried the genes *sea*, *seb*, *sec*, or *sed*.
- Heat treatment at 180 °C for 35 minutes was not sufficient to inactivate SEA.

Abstract: Ingesting food contaminated by pathogens and/or their toxins can cause foodborne illness. In this sense, this study investigated the occurrence of enterotoxigenic *S. aureus* in frozen cheese bread dough and assessed the production and thermosensitivity of SEA in artificially contaminated cheese dough. *E. coli* counts were determined by MPN. Confirmation of the presence of *S. aureus* was carried out by biochemical and molecular identification. The detection of the genes *sea*, *seb*, *sec*, and *sed* was performed using the PCR. The detection of SEA in artificially contaminated cheese dough, before and after baking at 180 ºC for 35 minutes was performed using the RPLA. All samples contaminated with *E. coli* had NMP < 5.0×10^2 CFU / g. None of the isolated *S. aureus* strains expressed the classical enterotoxin genes *sea*, *seb*, *sec*, and *sed*. However, as there are 23 serologically distinct staphylococcal enterotoxins, we cannot rule out the

possibility that strains expressed other enterotoxin-encoding genes. Laboratory tests showed the presence of SEA in cheese bread dough artificially contaminated with SEA-producing *S. aureus* after 8 h of incubation at 10 or 20 °C. Heat treatment at 180 °C for 35 minutes was not sufficient to inactivate SEA in artificially contaminated cheese bread previously incubated for 24 h at 10 or 20 °C. These results indicate a potential health risk to consumers of cheese bread and underscore the need for ingredient quality control and measures to prevent the multiplication of *S. aureus* during product manufacture and storage.

Keywords: SEA; nonclassical enterotoxin; *Escherichia coli*; PCR; RPLA; foodborne outbreaks.

GRAPHICAL ABSTRACT



INTRODUCTION

Cheese bread is a traditional Brazilian food originated in the state of Minas Gerais and widely consumed throughout the country and abroad. The mandatory ingredients in cheese bread production are cassava flour, milk or water, cheese, vegetable oil or butter, eggs, and salt [1]. Brazilian cheese bread is a perishable product that is susceptible to microbial contamination, mostly as a result of the use of contaminated raw materials or inadequate manufacturing practices during preparation. Pathogen survival and multiplication in cheese bread may lead to foodborne diseases.

One of the most important foodborne pathogens that is capable of contaminating cheese bread dough is enterotoxigenic *Staphylococcus aureus*. The human nasal vestibule has been described as a natural habitat for the bacterium. This implies that hands can be easily contaminated and, in industries where hygiene standards and manufacturing practices are poor, cheese bread may be contaminated during production. The problem is further aggravated by the fact that cheese, one of the basic ingredients of cheese bread, is associated with outbreaks of staphylococcal intoxication [2-4]. *S. aureus* can cause clinical and subclinical mastitis in cattle, contaminating milk and dairy products [5].

S. aureus is the main species associated with staphylococcal intoxication, although enterotoxins can also be produced by other coagulase-positive or -negative species [5]. Staphylococcal intoxication occurs after ingestion of foods containing preformed staphylococcal enterotoxins. *S. aureus* growth and enterotoxin production are influenced by different factors as food composition, temperature (10 °C to 46 °C), water activity (0,86 to 0,99) and pH (4,9 to 9,6) [6,7].

To date, 23 serologically distinct staphylococcal enterotoxins have been described, categorized into emetic (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SER, SES, and SET) and non-emetic (SElJ, SElU, SElV, SElX, and SElW) [8-10]. About 95% of all cases of staphylococcal food poisoning are caused by SEA, SEB, SEC, SED, and SEE [11]. Staphylococcal enterotoxins are heat stable, being resistant to cooking temperatures and times commonly used in domestic and industrial practice [12-14].

Several countries have reported outbreaks of staphylococcal food poisoning from milk and dairy products contaminated with classical staphylococcal enterotoxins [15]. The outbreak with the highest number of cases ever reported (*n* = 13,420) occurred in Japan and was due to different products made from contaminated milk and skimmed milk powder. Asao and coauthors [16] found that fluid milk was contaminated with 0.38 ng/g of SEA and milk powder with 3.7 ng/g of SEA. Ikeda and coauthors [17] reported the presence of SEH, in addition to SEA, in skimmed milk powder samples, indicating that SEH might have been involved in staphylococcal food poisoning.

In Brazil, there were 6,903 reports of foodborne outbreaks between 2009 and 2018, resulting in 672,873 affected individuals, 122,187 treated patients, 16,817 hospitalizations, and 99 deaths. *S. aureus* was implicated as the etiological agent in 9.5% of these cases [18]. Ezequiel Dias Foundation reported in Minas Gerais, between January 2006 and April 2007, 27 foodborne outbreaks resulting in 1019 affected people and 394 patients. These outbreaks were confirmed by a coagulase positive Staphylococcus count greater than 10^5 CFU/g of food and/or by the detection of enterotoxins [19]. In 2018, the European Food Safety Authority and the European Centre for Disease Prevention and Control reported that 3,908 foods produced in different European countries were analyzed and 46 foods (23 cheese samples) were contaminated with staphylococcal enterotoxins [20].

Little information is available on the contamination of frozen cheese bread sold in Brazil. Ferrari, Winkler, and Oliveira [21] assessed the microbiological quality of cheese bread of different brands produced and sold in Londrina, Brazil, and found that all samples had *S. aureus* counts above 5,0 x 10^3 UFC/g, that is the limit defined by Brazilian legislation. Tomich and coauthors [22] also found that 66.7% of cheese bread samples analyzed had coagulase-positive staphylococci counts above 5,0 x 10^3 UFC/g.

This study investigated the occurence of enterotoxigenic *S. aureus* in frozen cheese bread dough sold in Londrina, Paraná, Brazil, and assessed the production of SEA in cheese bread dough and its inactivation by thermal processing.

MATERIAL AND METHODS

Sample Collection Procedures

This study analyzed frozen cheese bread products from five different brands produced in Londrina, Paraná, Brazil. Twenty samples from each brand were purchased between March and July 2018 from five different supermarkets, totaling 100 samples. Products (400 g) with packages in good condition were randomly selected, purchased, and transported to the laboratory in an isothermal container within 1 h of collection. Analyses were performed at the Laboratory of Food Microbiology of the Center for Agricultural Sciences of the State University of Londrina. The brands were coded as A, B, C, D, and E, and the supermarkets as 1, 2, 3, 4, and 5. Table 1 shows the number of samples analyzed by place of purchase and brand.

Table 1. Distribution of frozen cheese bread samples analyzed in the study by place of purchase and brand.

Place of purchase	Number of samples					
	Brand A	Brand B	Brand C	Brand D	Brand E	Total
Supermarket 1	10	-	-	-	5	15
Supermarket 2	-	-	-	-	10	10
Supermarket 3	5	5	-	-	5	15
Supermarket 4	-	10	-	-	-	10
Supermarket 5	5	5	20	20	-	50
Total	20	20	20	20	20	100

Determination of Thermotolerant Coliforms, *Escherichia coli, and S. aureus*

Twenty-five grams of the examined cheese bread samples were weighed aseptically and homogenized with 225 mL of 0.1% sterile buffered peptone water (Acumedia, Acumedia, Lansing, MI, USA) in a homogenizer (Stomacher 400, Lab System, Seward, Norfolk, UK) for 1 min. From this initial dilution (10^{-1}), two serial dilutions (10^{-2} and 10^{-3}) were prepared using tubes containing 9 mL of 0.1% sterile buffered peptone water [23].

Thermotolerant coliform and *E. coli* counts were determined by the most probable number (MPN) method, according to Kornacki, Gurtler, and Stawick [23]. *S. aureus* counts were determined following the method of Bennett, Hait, and Tallent [24]. Catalase, coagulase, and DNAse tests were used for biochemical screening. Coagulase-positive *Staphylococcus* isolates were tested for aerobic and anaerobic mannitol fermentation and aerobic maltose fermentation. The protocol proposed by Martineau and coauthors [25] was used for molecular characterization of biochemically confirmed *S. aureus* isolates, with strain USA 300 used as positive control.

Detection of *sea*, *seb*, *sec*, and *sed* Genes in *S. aureus* Isolates

All *S. aureus* strains isolated from cheese bread samples were subjected to analysis of *sea*, *seb*, *sec*, and *sed* expression. DNA extraction, PCR conditions, and data analysis followed the procedures described by Johnson and coauthors [26] as modified by Cunha and coauthors [27]. The following standard strains of *S. aureus* were used as positive controls: ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), and ATCC 23735 (*sed*). Non-enterotoxigenic *S. aureus* ATCC 25923 was used as negative control [28].

Investigation of the Ability of SEA-Producing *S. aureus* to Express Enterotoxin in Cheese Bread Dough

Preparation and Artificial Contamination of Cheese Bread Dough

A suspension of SEA-producing *S. aureus* (ATCC 13565) in 0.85% saline was adjusted to the turbidity of a 0.5 McFarland standard (8 log colony-forming units, CFU/mL). The drop count method was used to determine the best dilution and inoculum size for contamination of cheese bread dough [29].

All the ingredients used in the preparation of cheese bread dough were analyzed as described in section "Determination of thermotolerant coliforms, *Escherichia coli*, and *S. aureus*" to ensure that they were not contaminated with *S. aureus*. The dough was prepared by mixing 1,000 g of sour cassava flour, 500 g of grated cheese, 350 mL of soybean oil, 250 mL of milk, five whole eggs, and 20 g of salt. Milk and soybean oil were heated to a boil and then poured over the cassava flour. This process, known as scalding, is traditionally used to promote starch gelatinization. The scalded flour was cooled to 25 °C, mixed with eggs, salt, and grated cheese, and kneaded aseptically to avoid contamination.

The dough was divided into six portions of 100 g. Four portions were subdivided into samples of 25 g, rolled into balls (3 cm diameter and 1 cm height), and individually contaminated with 0.25 mL of 10^3 CFU/g of SEA-producing *S. aureus* that was instantly absorbed by the cheese bread dough. The two uncontaminated portions were each subdivided into four portions of 25 g and used as negative controls. Contaminated and uncontaminated samples were individually packaged in sterile bags and incubated at 10 or 20 °C for 4, 6, 8 or 24 h. After incubation, samples were evaluated for SEA production and SEA inactivation, as described below (sections "Evaluation of the ability of enterotoxigenic *S. aureus* to produce SEA in cheese bread dough" and "Evaluation of SEA inactivation by thermal processing"). The experiment was repeated twice.

Evaluation of the Ability of Enterotoxigenic S. aureus to Produce SEA in Cheese Bread Dough

Dough samples (25 g) were analyzed for SEA production. Briefly, samples were homogenized in 50 mL of 0.85% sterile saline solution for toxin extraction. Aliquots of 30 mL were collected and centrifuged at 900 × *g* and 4 °C for 30 min (Eppendorf AG, Hamburg, Germany). Supernatants were filtered through 0.45 and 0.2 µm Millipore membranes, and filtrates were analyzed for SEA using a reversed passive latex agglutination (RPLA) test kit (SET-RPLA, Denka Seiken Co. Ltd., Tokyo, Japan).

RPLA assays were performed in V-bottom 96-well polystyrene microplates (Greiner Bio-One, Americana-SP), according to the manufacturer's instructions. Plates were incubated at room temperature for 20 to 24 h, and the results were read according to the manufacturer's interpretation criteria.

Evaluation of SEA Inactivation by Thermal Processing

Contaminated dough samples (25 g) were baked at 180 °C for 35 min. Baked samples were analyzed by RPLA (as described in section "Evaluation of the ability of enterotoxigenic *S. aureus* to produce SEA in cheese bread dough") to assess whether staphylococcal enterotoxins were degraded by thermal processing. Baked cheese bread was also analyzed for the presence of *S. aureus* following the procedures described in section "Determination of thermotolerant coliforms, *Escherichia coli*, and *S. aureus*".

Statistical Analysis

Data were subjected to analysis of variance and Tukey's test at $P < 0.05$ using R version 3.6.0 (Boston, MA, USA).

RESULTS AND DISCUSSION

Microbiological Evaluation of Frozen Cheese Bread

Brazilian legislation does not define standards of identity for cheese bread or specific limits for enterotoxigenic *S. aureus*. According to the Brazilian Health Regulatory Agency (ANVISA), cheese bread cannot contain more than 5.0×10^3 CFU/g of coagulase-positive staphylococci [30,31]. Table 2 shows the *S. aureus* counts of the 100 cheese bread samples analyzed in the study. Sixty samples (60%) had less than 1.0×10^2 CFU/g. Of the 40 samples (40%) found to be contaminated with *S. aureus*, 27 (27%) had counts greater than 5.0×10^3 CFU/g.

Table 2. *Staphylococcus aureus* counts (CFU/g) in frozen cheese bread by brand.

Count range	Brand A *n* (%)	Brand B *n* (%)	Brand C *n* (%)	Brand D *n* (%)	Brand E *n* (%)
$<1.0 \times 10^{2a}$	13 (65)	14 (70)	3 (15)	13 (65)	17 (85)
1.0×10^2 to 1.0×10^{3b}	0 (0)	0 (0)	1 (5)	0 (0)	0 (0)
1.0×10^3 to 5.0×10^{3c}	2 (10)	2 (10)	3 (15)	3 (15)	2 (15)
$>5.0 \times 10^{3d}$	5 (25)	4 (20)	13 (65)	4 (20)	1 (5)

Note: n, number of samples; CFU, colony-forming units.

[a] Limit of detection.

[b] Range between the limit of detection and the acceptable level, as defined by Brazilian legislation.

[c] Range between the acceptable and the intermediary acceptable level, as defined by Brazilian legislation.

[d] Values above the maximum limit defined by Brazilian legislation.

Thermotolerant coliform and *E. coli* counts were determined as additional parameters to assess the hygienic quality of cheese bread. Twenty-five samples (25.0%) were found to be contaminated with thermotolerant coliforms and 17 (17.0%) with *E. coli*. However, microbial counts were lower than 5.0×10^2 MPN/g, the upper limit established for cheese bread by Brazilian Resolution no. 331 and Normative Instruction no. 60 [30,31].

None of the 62 *S. aureus* strains isolated from the 40 contaminated cheese bread samples carried the genes *sea*, *seb*, *sec*, or *sed*, responsible for the production of SEA, SEB, SEC, and SED, respectively. Other studies have detected staphylococcal enterotoxin genes in *S. aureus* strains isolated from contaminated dairy products [3,32-39].

Contamination of cheese bread by *S. aureus* and the presence of preformed enterotoxins in cheese bread dough can result from the use of contaminated ingredients or from contamination with *S. aureus* and production of staphylococcal enterotoxins during manufacture and storage. Babic´ and coauthors [**Erro! Fonte de referência não encontrada.**] observed that milk storage at temperatures below 8 °C during production and distribution significantly decreases the risk of *S. aureus* multiplication and enterotoxin production.

Two outbreaks of staphylococcal intoxication have been reported by do Carmo and coauthors [3] due to the ingestion of cheese and raw milk contaminated with counts of *S. aureus* ranging from 2.4×10^3 CFU / g to 2.0×10^8 CFU / g. SEA and SEB enterotoxins were detected in raw milk samples and SEA, SEB and SEC in cheese samples. Senger and Bizani [39] evaluated 60 samples of Minas cheese and 31.67% had *S. aureus* counts greater than 5×10^2 CFU / g and the presence of SEA, SEB, SEC, SED and SEE. Castro and coauthors [38] observed the presence of *S. aureus* that carried the *sea* and *sec* genes in samples of raw milk, artisanal Minas cheese and cheese producers.

Between 2014 and 2017, Ciupescu and coauthors [**Erro! Fonte de referência não encontrada.**] evaluated three outbreaks of staphylococcal intoxication from three different types of cheese contaminated with 1.2×10^6 to 5.3×10^8 CFU/g of coagulase-positive staphylococci. The strains expressed *sed* and other enterotoxin-encoding genes (*seg*, *seh*, *sei*, *sej*, and *ser*), revealing the diverse enterotoxigenic profile of isolates and the expression of nonclassical enterotoxin genes. Ercoli and coauthors [**Erro! Fonte de referência não encontrada.**], in a study on foodborne *S. aureus* outbreaks, found that cases were associated with the consumption of whipped cream contaminated with *S. aureus* strains expressing *sea* and also *seg*, *seh*, and *sei*. Other studies reported the presence of only nonclassical staphylococcal enterotoxins in foods associated with outbreaks of staphylococcal intoxication [4,**Erro! Fonte de referência não encontrada.**]. It is important to bear in mind that these studies were limited by the lack of standardized immunological assays for detection of nonclassical enterotoxins.

In the present study, we analyzed the expression of four classical staphylococcal enterotoxin genes. However, the isolated strains might have carried genes that were not assessed, such as genes encoding SEE or one of the 18 other staphylococcal enterotoxins. Mello and coauthors [**Erro! Fonte de referência não encontrada.**] isolated *S. aureus* strains from cows with subclinical mastitis in Brazil and found that strains expressed not only classical enterotoxin genes (*sea*, *seb*, *sec*, and *see*) but also *seg*, *seh*, *sei*, and *ser*.

An outbreak of staphylococcal food poisoning in Minas Gerais, Brazil, with 4,000 cases and 16 deaths, occurred because of contamination of food by handlers and inadequate storage at room temperature for 24 h [45]. Regarding the foodborne outbreak that occurred after the 2017 central Italy earthquake, research showed that enterotoxigenic *S. aureus* strains isolated from pasta salad were human-derived [46]. Continued hygiene education of food handlers is, therefore, essential to reduce the risk of staphylococcal intoxication.

Potential of *S. aureus* to Produce SEA in Cheese Bread Dough

Cheese bread dough samples were artificially contaminated with SEA-producing *S. aureus* ATCC 13565 and incubated at 10 or 20 °C for 4, 6, 8, or 24 h. After 8 h of incubation, all samples incubated at 20 °C contained at least 10^5 CFU/g of *S. aureus*. The highest *S. aureus* count obtained after 24 h of incubation was 2.6×10^6 CFU/g. Significant interaction effects between incubation time and temperature were observed. At both temperatures, *S. aureus* counts increased with increasing incubation time. For all incubation times, the highest counts were observed in samples incubated at 20 °C.

In samples incubated at 10 °C, *S. aureus* counts were significantly higher after 24 h of incubation, not differing ($P > 0.05$) between 4, 6, and 8 h (Table 3). In samples incubated at 20 °C, the highest counts were observed after 24 h of incubation, followed by 8 h. Counts did not differ between samples incubated for 4 and 6 h (Table 3).

Table 3. Mean counts (CFU/g) of SEA-producing *Staphylococcus aureus* and presence of SEA, as detected by reversed passive latex agglutination**, in cheese bread dough artificially contaminated with SEA-producing *Staphylococcus aureus* and incubated at different temperatures for different periods of time.

Incubation time	Incubation temperature			
	Dough before baking		Baked cheese bread	
	10 °C	20 °C	10 °C	20 °C
4 h	$2.20 \times 10^{4\ aA}$ SEA -	$5.30 \times 10^{4\ aA}$ SEA -	* SEA -	* SEA -
6 h	$2.85 \times 10^{4\ aA}$ SEA -	$6.90 \times 10^{4\ aA}$ SEA -	* SEA -	* SEA -
8 h	$9.25 \times 10^{4\ aA}$ SEA +	$2.90 \times 10^{5\ bB}$ SEA +	* SEA -	* SEA -
24 h	$2.00 \times 10^{6\ bA}$ SEA +	$2.40 \times 10^{6\ cA}$ SEA +	* SEA +	* SEA +

Note: Means in a column followed by different lowercase letters and means in a row followed by different uppercase letters differ significantly by Tukey's test ($P < 0.05$).
- SEA not detected.
+ SEA detected.
**S. aureus* was not detected in baked samples.
**The limit of detection of the RPLA method is 1.0 ng/mL.

SEA detection was performed by RPLA and results are presented in Table 3. SEA was detected in samples incubated for 8 and 24 h at 10 and 20 °C. *S. aureus* multiplication and production of staphylococcal enterotoxins are influenced by intrinsic factors of the food matrix and extrinsic factors, such as food production, storage, and handling conditions. There is no definite relationship established between *S. aureus* count and enterotoxin production. In this study, enterotoxin production occurred in samples contaminated with 4.4×10^4 CFU/g or more of *S. aureus*. Tatini and coauthors [47] detected SEA in milk with counts from 10^4 CFU / g of *S. aureus*, while Resta and Oliveira [48] and Santana and coauthors [49] reported that food samples with more than 10^5 CFU/g of *S. aureus* were positive for enterotoxins.

SEA inactivation was assessed after baking the artificially contaminated dough samples for 35 min at 180 °C. Heat treatment was sufficient to inactivate SEA in samples that had been incubated for 8 h at 10 or 20 °C but not in samples incubated for 24 h at either temperature, as shown in Table 3. Thermal inactivation of staphylococcal enterotoxins depends, among other factors, on the initial enterotoxin levels in the food matrix.

Necidová and coauthors [50] evaluated the thermal stability of SEA, SEB, and SEC in milk previously contaminated with 10^4 to 10^5 CFU/g of enterotoxigenic *S. aureus* and incubated at 37 °C for 24 h. After heat treatment at 72, 85, or 92 °C for 15 s, all samples were negative for *S. aureus*, but SEA, SEB, and SEC were detected in 87.5% (35/40), 52.5% (21/40), and 45.0% (18/40) of samples, respectively. Nevertheless, heat treatment significantly reduced enterotoxin levels, and SEB was detected at the lowest concentrations. Tibana and coauthors [51] analyzed the thermal stability of SEA, SEB, and SEC in a buffered system containing 100 ng/mL of enterotoxins. SEC had the highest thermal resistance, followed by SEA and SEB. The authors found that domestic cooking temperatures and times were not sufficient to completely inactivate enterotoxins. In another study, Necidová and coauthors [13] assessed the thermal stability of SEA, SEB, and SEC in fluid milk contaminated with 38 different strains of enterotoxigenic *S. aureus*, autoclaved at 100, 110, or 121 °C for 3 min. Heat treatment reduced enterotoxin levels, but the prevalence was 36.8% (14/38), 34.2% (13/38), and 31.6% (12/38) in samples autoclaved at 100, 110, and 121 °C, respectively. SEA was detected at the highest level and with the highest frequency. Skimmed milk powder associated with the 2000 staphylococcal food intoxication in Japan had been processed at 130 °C for 2-4 s. Although *S. aureus* cells were destroyed, SEA, which was likely produced during the storage of raw milk, retained its biological and immunological properties [16].

S. aureus multiplication and enterotoxin production are influenced by different factors inherent to food processing. To reduce staphylococcal food poisoning, food industries and handlers must enforce good hygiene practices to avoid enterotoxigenic *S. aureus* contamination and ensure adequate storage and processing conditions to prevent microbial multiplication.

CONCLUSION

None of the *S. aureus* strains isolated from contaminated cheese bread samples expressed the classical enterotoxin genes evaluated in this study. However, as there are 23 serologically distinct staphylococcal enterotoxins, we cannot rule out the possibility that strains expressed other enterotoxin-encoding genes. The presence of *S. aureus* above the limit defined by Brazilian legislation in several samples indicates a potential danger to consumer health.

Heat treatment at 180 °C for 35 min was not sufficient to inactivate SEA in artificially contaminated cheese bread previously incubated for 24 h at 10 or 20 °C. Thermal deactivation depends, among other factors, on initial SEA levels.

These results highlight the importance of controlling the microbiological quality of raw materials used for cheese bread dough production and reinforcing hygiene measures to prevent *S. aureus* multiplication during manufacture and storage of the product. Our data may guide food surveillance programs and the assessment of good manufacturing practices and hazard analysis and critical control points in cheese bread industries.

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Conflicts of Interest: The authors declare no conflict of interest.

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