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Analysis of enterotoxin production by *Staphylococcus aureus* in Minas Frescal Cheese under different intrinsic and extrinsic factors

Nathália Cristina Cirone Silva
ncirone@unicamp.br
http://orcid.org/0000-0002-2839-1416
Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brasil.

Erika Carolina Romão Bonsaglia
erikabonsaglia@gmail.com
http://orcid.org/0000-0002-3229-3405
Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo, Brasil.

Paulo Eduardo Budri
paulobudri@rcsi.ie
http://orcid.org/0000-0003-4595-3473
Royal College of Surgeons in Ireland (RCSI), Dublin, Irlanda.

Anderson Clayton da Silva Abreu
a190671@dac.unicamp.br
http://orcid.org/0000-0003-4595-3473
Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brasil.

Carlos Tadeu dos Santos Dias
ctsdias@usp.br
http://orcid.org/0000-0003-1015-1761
Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Piracicaba, São Paulo, Brasil.

Ary Fernandes Junior
ary.fernandes@unesp.br
http://orcid.org/0000-0002-2744-2824
Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo, Brasil.

Vera Lúcia Mores Rall
vera.rall@unesp.br
http://orcid.org/0000-0003-4280-5619
Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo, Brasil.

ABSTRACT

Staphylococcus aureus is a foodborne associated pathogen, mainly in dairy products due to the enterotoxin production. This study analyzed the production of classical toxins in cheese by *S. aureus*, in different temperatures, atmospheres, and levels of contamination. Minas Frescal Cheese were classified according to their microbiota. The cheese samples were classified as low or high level of contamination according to the concentration of that bacteria. An aliquot of 10^5 CFU of each strain (producing enterotoxin A, B, C, or D) in BHI was inoculated into two types of commercial cheeses presenting low and high contamination. Therefore, the samples were incubated at 8 °C, 15 °C, and 35 °C for 24, 48 and 72 hours in aerobic and anaerobic condition. The detection and quantification of enterotoxins were tested against toxin A, B, C and D using reverse passive latex agglutination. There was no enterotoxin production at 8 °C in all treatment, consequently, no statistically significant difference was observed. At 15 °C, the SEA production was significantly higher ($p<0.05$) in BHI broth than in cheese with low contamination in all three times evaluated. Regarding SEB, in aerobic condition at 15 °C after 72 hours, the BHI broth showed significantly higher production than the low contaminated cheese. *S. aureus* produced a different range of enterotoxin according to the substrate. In BHI, the production was higher than in cheese, suggesting that food substrates more appropriate to analyze the real enterotoxin production. Besides, the microbiota presents can interfere in its production.

KEYWORDS: temperature; staphylococcal enterotoxin; microbiota; atmosphere.

INTRODUCTION

Staphylococcus aureus is one of the most common pathogens associated with foodborne disease (AYDIN; SUDAGIDAN; MURATOGLU, 2011; CRAGO et al., 2012), frequently involved in food poisoning outbreaks due to contaminated dairy products (JOHLER et al., 2015; SILVA; RODRIGUES; SILVA, 2020).

S. aureus enterotoxins (SE) SEA, SEB, SEC, SED and SEE are named as classical enterotoxins (CÂNDIDO et al., 2020) and their production is a substantial concern in public health and food industry. Several studies detected the presence of those enterotoxins in food from animal origin, especially dairy products (ABREU et al., 2021; CÂNDIDO et al., 2020; CARFORA et al., 2015), causing a diverse range of gastrointestinal symptoms including nausea, violent vomiting, abdominal cramping, with or without diarrhea (SILVA; RODRIGUES; SILVA, 2020).

Enterotoxin production is affected by different factors, such bacteria counts of 10^5–10^6 CFU/g, (ANGELIDIS et al., 2020; WU; SU, 2014), foods with pH of 5–9.6, water activity (aw) of 0.86–0.99 stored at temperatures of 10–46 °C (ANGELIDIS et al., 2020) and presence of competitive microbiota, especially lactic acid bacteria (LAB) (ALJASIR; D'AMICO, 2020).

Minas Frescal Cheese is one of the most consumed cheeses in Brazil, due to the easy manufacturing process, high yield, low production costs (ROCHA et al., 2020). Its characteristics are that of a Brazilian soft white cheese traditionally produced by enzymatic coagulation of pasteurized milk with rennet and / or other coagulating enzymes, with or without the aid of lactic bacteria, Minas Frescal Cheese can be classified as highly perishable even under refrigeration due to its high moisture content (BRASIL, 2017; ROCHA et al., 2020).

Overall, the production of *S. aureus* enterotoxin needs to be well comprehended in different substrates, such as commercial culture media and dairy products. Although both substrates have been studied in the past years (PIMENTA-MARTINS et al., 2012; ROSENGREN; LINDBLAD; LINDQVIST, 2013), enterotoxin production in food substrates should be further investigated in different conditions, including nutrients source, number of viable contaminant cells and temperature to clarify the risks for public health. In Brazil, the presence of this microorganism in dairy products is a concern once *S. aureus* is the third most identified pathogen causing foodborne disease (SVS, 2019).

Studies of enterotoxin production in food matrices are important because the nutrients source are different when compared with laboratory culture media, thus the production could be variable. Therefore, the aim of this study was to investigate the ability of *Staphylococcus aureus* to produce classic toxins in Minas Frescal Cheese at different temperatures, atmospheres, and level of contamination.

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of 25 samples of different brands of Minas Frescal Cheese, containing an inspection seal, were obtained from different supermarkets in Botucatu, SP, Brazil. Dairy temperatures at the time of collection were measured using a digital

infrared thermometer with laser sighting (Incoterm), the samples were stored and transported in thermal boxes until the time of analysis, which was done immediately after purchase. The 25 samples were analyzed, using 25 grams of each cheese.

MICROBIOLOGICAL QUALITY OF CHEESE SAMPLES

The Minas Frescal Cheese samples were classified according to the colony forming units (CFU/g) of mesophilic, psychotropic bacteria and the most probable number (MPN) of thermo-tolerant coliforms / g. Samples of cheese classified as with low microbiota showed up to 10^2 CFU/g of mesophilic and psychotropic bacteria and no thermo-tolerant coliforms. On the other hand, samples with more than 10^3 CFU/g and more than 10^3 MPN were classified as a highly contaminated. *Staphylococcus* spp. should be absent in all samples of cheese, the parameters were defined from the results obtained from the counts of microorganisms (table 1). All culture media, except when specified, were Oxoid (Oxoid, Basingstoke, UK).

For microbiological analysis, 25 grams of each cheese were weighed and homogenized with 225 mL of saline solution, in Stomacher for 1 minute. From this dilution (10^{-1}), analyzes of the counting of mesophilic, psychotropic and coliform microorganisms were performed, as described below.

DETERMINATION OF THE MOST PROBABLE NUMBER (MPN) OF THERMOTOLERANT COLIFORMS

Determination of the MPN of thermo-tolerant coliforms was carried out according to the American Public Health Association (KORNACKI; GURTLER; STAWICK, 2015) in a three-tube series. Dilution were performed until 10^{-3}, and 1 mL of the dilutions were inoculated in the tubes containing *E. coli* broth (EB). The MPN of thermo-tolerant coliforms was calculated based on their ability to produce gas in tubes at 45 °C/24-48h.

Staphylococcus spp. ENUMERATION

From the 10^{-1} dilution of Minas Frescal Cheese, dilutions were made up to 10^{-4}, in tubes containing 9 mL of saline, 0.1 mL of serial dilutions were added on Baird Parker agar supplemented with 5% egg yolk and tellurite emulsion, spread on the surface with the aid of a Drigaski loop, incubated at 35 ° C / 48 h. The characteristic colonies were subjected to phenotypic tests for confirmation, Gram stain, catalase and coagulase (BENNETT; HAIT; TALLENT, 2015).

MESOPHILIC BACTERIA

From the 10^{-1} dilution of Minas Frescal Cheese, dilutions were made up to 10^{-4}, in tubes containing 9 mL of saline, The Pour-Plate method was used for mesophilic bacterial enumeration. Aliquots (1.0 mL) of each dilution were mixed with 20 mL of plate count agar (PCA) into Petri dishes. CFUs were counted after incubation at 35 °C/48h. In order to calculate the final concentration, the number of colony forming unit (CFU) was multiplied by the inverse of the dilution on the respective plate (RYSER; SCHUMAN, 2015).

PSYCHROTROPIC BACTERIA

From the 10^{-1} dilution of Minas Frescal Cheese, dilutions were made up to 10^{-4}, in tubes containing 9 mL of saline. Using the Spread-Plate method, aliquots of 0.1 mL of each serial dilution were spread onto PCA and incubated at 4 °C/7 days. The final concentration was calculated as described above (VASAVADA; CRITZER, 2015).

DETECTION OF ENTEROTOXIN GENE

S. aureus ATCC 13565 (*sea*), ATCC 14458 *(seb*), ATCC 19095 (*sec*) and FRI 361 (*sed*), were used as positive controls for the enterotoxins production. *S. aureus* strains, previously isolated from cheese were analyzed for the presence of genes responsible for those enterotoxins production. Two isolates encoding each enterotoxin gene were used, besides positive control. DNA was extracted using the MiniSpin Kit (GFX Healthcare) according to the manufacturer's recommendations. PCR protocol and the *primers* were previously described by (CÂNDIDO et al., 2020).

PRODUCTION OF ENTEROTOXIN IN CHEESE AND BHI

All the strains used belong to the collection of the microbiology laboratory of the Paulista State University (UNESP), Botucatu - São Paulo. The study was carried out in triplicate and performed with 3 strains producing enterotoxin, one standard strain ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), FRI 361 (*sed*) and two isolated *S. aureus* strains positive for each gene encoding *sea*, *seb*, *sec,* and *sed*, previously isolated from cheese, from a previous study. The SEE toxin was not researched because the kit TD 0900 lacks it.

The strains that were stored frozen in glycerol, were activated in BHI broth at 36 °C / 24 hours. After growth, they were standardized on the McFarland 0.5 scale (10^8 CFU / mL) and diluted in saline to 10^5 CFU / mL. Individual experiments were carried out inoculating a single strain producing each enterotoxin, for all the analyzed variables.

An inoculum of 10^5 CFU / mL was homogenized in vortex and inoculated in low and high contamination cheese and in BHI. 10g of cheese were packed in sterile bag and the inoculum was added. The cheese and the inoculum were homogenized, and the samples were incubated at 8°C, 15°C (more frequent temperature observed in 10 supermarkets, as shown in table 1), and 35°C for 24, 48 and 72 hours in the aerobic and anaerobic environment, to guarantee anaerobiosis, the anaerobiosis jar was used. The detection and quantification of enterotoxins were tested using reverse passive latex agglutination (TD 0900, Oxoid), according to manufacturer's instructions. The results of enterotoxin production were expressed in dilution.

STATISTICS

The statistical analysis were performed by the SAS System (Copyright (c) 2002-2010 by SAS Institute Inc., Cary, NC, USA). ANOVA test was performed to compare all tested variables. The Tukey´s test was used to adjust the p-values resulting from multiple comparisons. Statistical significance was declared for $p< 0.05$.

RESULTS AND DISCUSSION

In Table 1, it is possible to observe the results of the microbiological analyzes for the 25 samples of Minas Frescal Cheese, and the temperature of the samples when they were collected, only 22 samples were used in the enterotoxin production experiments, as the 3 samples that present *Staphylococcus spp.*count.

Table 1. Averages and their respective standard deviations for the counting of thermotolerant coliforms, *Staphylococcus* spp., mesophilic bacteria, psychrotrophic bacteria, and temperature (°C) in the moment of purchase.

Sample	Coliforms (log MPN/g)	*Staphylococcus* spp. (log CFU/g)	Mesophilic (log CFU/g)	Psychrotrophic (log CFU/g)	Temperature (°C)
1	-	-	-	-	9.0
2	6.04	-	6.58	4.11	9.0
3	-	-	-	-	9.0
4	7.04	-	8.36	5.00	12.4
5	-	-	-	-	12.4
6	7.66	-	7.11	3.60	12.4
7	-	-	-	-	16.9
8	5.04	-	5.18	3.48	16.9
9	6.04	-	6.60	4.78	16.9
10	-	-	-	-	10.4
11	-	-	-	-	10.4
12	5.66	-	5.78	3.60	10.4
13	-	-	-	-	11.6
14	5.04	-	6.30	3.48	11.6
15	-	-	-	-	11.6
16	-	-	-	-	11.6
17	-	-	-	-	15.2
18	5.32	0.60	5.60	3.48	15.2
19	5.38	-	5.85	2.30	15.2
20	-	-	-	-	13.1
21	-	-	-	-	13.1
22	-	-	-	-	13.1
23	4.66	-	5.90	3.60	12.2
24	5.38	1.48	6.85	4.48	12.2
25	6.04	2.30	7.48	5.48	12.2
Average+ SD	6.39±6.76	0.97±1.60	7.07±7.66	4.32±4.80	12.4±2.5

NOTE: MPN: Most Probable Number; CFU: Colony Forming Unit; SD: standard deviations, -: Did not show growth.

There was no enterotoxin production at 8 °C in all treatment, consequently, no statistically significant difference was observed.

Table 2 shows the significant difference among each treatment to enterotoxin SEA, SEB, SEC and SED comparing all parameters.

Table 2. Results of enterotoxin production with a significant difference of 0.05% between high-contamination, low-contamination cheeses and BHI production, under different conditions.

Enterotoxin	Temperature	Aerobic condition			Anaerobic condition		
		24h	48h	72h	24h	48h	72h
A	15 °C	BHI>LC	BHI>LC	BHI>LC	-	-	-
	35 °C	BHI>LC	-	-	-	-	-
B	15 °C	-	-	BHI>LC	-	-	-
	35 °C	-	-	-	-	BHI>LC	BHI>LC and BHI>HC
C	15 °C	-	-	-	-	-	BHI>HC
D	15 °C	-	-	BHI>HC	-	BHI>HC	BHI>HC
	35 °C	BHI>HC	BHI>HC	BHI>HC	BHI>HC	BHI>HC	BHI>HC

NOTE: LC = lightly contaminated cheese; HC = highly contaminated cheese; BHI = Brain Heart Infusion broth; - = There is no significant difference.

Regarding the production of enterotoxin A, at a temperature of 15 °C, the production of enterotoxin A was higher in BHI broth than in low contamination cheeses, for the 3 aerobic incubation times, but for the other treatments there is no statistical difference significant ($p < 0.05$) at 15 °C. Regarding the production at 35 °C only in the 24-hour aerobic time, it is possible to verify a higher production of enterotoxin A in BHI broth than in low contamination cheeses, with no statistical difference for the other treatments at 35 °C.

Regarding SEB, in aerobic condition at 15 °C after 72 hours, the BHI broth showed significantly higher production than the low contaminated cheese. At 35 °C under the anaerobic atmosphere, this difference was observed in 48 and 72 hours. SEC production did not show a significant difference, except anaerobically at 15 °C, and the production was higher in BHI than in cheese with a high contamination. Surprisingly, when is considered the matrix, the enterotoxin D, in both aerobic and anaerobic condition had a significant difference at 15 °C in 72 hours among the BHI broth and contaminated cheese. At 35 °C and in both atmosphere condition, the production of SED in BHI was higher than contaminated cheese.

When is considered the atmosphere condition, no relevant difference was found ($p<0.05$), showing that *S. aureus* is capable to produce enterotoxin in both. This result was expected since *S. aureus* is well-known as a facultative microorganism in terms of O_2 requirement (TYNER; PATEL, 2016; ZEDEN et al., 2018).

Some differences were observed taking into account the medium, temperature and time. In BHI broth, the strains produced more enterotoxin than in both levels of contamination in cheese and the higher production was observed in 35 °C followed by 15°C. Regarding the time, differences among 24 hours and 72 hours for SEA and SED were observed, however for SEB the difference was between 24 hours, 48 and 72 hours. Enterotoxin C production had no difference between BHI medium and cheese presenting a low contamination however, its production in highly contaminated cheese was significantly lower than both BHI medium and cheese with a low contamination.

Staphylococcus aureus is commonly isolated in food products (CRAGO et al., 2012) and outbreak caused by staphylococcal enterotoxin have been observed, including in cheese (JOHLER et al., 2015; SOLANO et al., 2013). This species can

grow between 6 and 48 °C, with an optimum of 35–41 °C, and can produce enterotoxins between 10 and 46 °C, with an optimum of 34–40 °C, with undetectable production of SE at temperatures below 10 °C (SCHELIN et al., 2011).

There are studies that only test the ability of *S. aureus* to produce toxins in BHI broth, and using the optimum growth temperature (35-37 °C), without using a food matrix (ROSENGREN; LINDBLAD; LINDQVIST, 2013). But, in foods, these parameters are different, due to the storage under refrigeration. The present study investigates the toxin production in three temperatures: according to the Brazilian law requirement (8 °C), media of real temperatures observed in supermarkets (15 °C) and the optimal temperature for *S. aureus* growths. These temperatures allowed better evaluation about enterotoxin production in real circumstances than studies that only evaluated in optimal temperature.

For all enterotoxin studied, its production were significantly different among the temperatures, observing just this parameter. When the inoculum was incubated at 35 °C, the enterotoxins quantification was higher than at 15 °C, and at 8 °C this production was not observed, showing the importance of an adequate refrigeration. Necidová et al. (2012) confirmed that *S. aureus* growth was inhibited at 8 °C as well as the enterotoxin production. On the other hand, some researchers demonstrated that the production of enterotoxins started at 10 °C (ABOLGHAIT et al., 2020; ANGELIDIS et al., 2020), corroborating with our research.

Despite the results showing that at 8 °C there was no enterotoxin production, temperature required in Brazilian laws, the production, storage and transport process of cheeses may not respect the law requirement for temperature, as reported for some studies (LIMA, 2011). In the present study, product storage temperatures ranged from 9 to 17 ° C, as shown in table 1.

Another important aspect is the production of enterotoxin in BHI broth to be higher than in cheese. This study shows that the conditions found in food differ from the optimal conditions of a culture medium, suggesting that food matrices show a more realistic view of enterotoxin production than BHI medium. The difference of enterotoxin production between artificial media and cheese could be explained by the salt, pH and nutrient availability (ANGELIDIS et al., 2020), being that in the culture media, there are adequate and sufficient amounts of nutrients and salt for a good growth and development of the microorganisms, and the pH adjusted to its optimum condition, and in the cheeses there may be less nutrients, or quantities of salt that are not compatible with the type of bacteria and the pH of the food may not be ideal for its growth. Other studies have shown that food matrix impacts on enterotoxin production of strains, and that production in lab broths is frequently higher when compared with that of food matrices (PEXARA et al., 2012; SCHUBERT et al., 2016; VIÇOSA et al., 2019).

S. aureus produced more SEC in a cheese with low contamination than in one more contaminated, confirming that *S. aureus* is not a good competitor (ASPERGER; ZANGERL, 2011). The cheese microbiota could affect *S. aureus* metabolisms and stress response once is common to these microorganisms acidify the media, causing proteolysis and decreasing the redox potential of the cheese matrix (ANGELIDIS et al., 2020; CRETENET et al., 2011). For example, in a study in which *Lactococcus lactis* was used in cheese, were verified, the ability to alter the production of enterotoxins, produced by *S. aureus*, highlighting the importance of environmental conditions in the expression of virulence (CRETENET et al., 2011).

Studies observed a significant decrease in the production of SEC and the expression of genes that encode SEC in milk to a laboratory media (VALIHRACH et al., 2014; VALIHRACH; ALIBAYOV; DEMNEROVA, 2013). The present study found a bigger enterotoxin C production at 15 °C/72 hours in anaerobic condition in broth, than in cheese, this result was also observed in other enterotoxin and parameters. This underscores the difference between studies in laboratory media and food matrices.

CONCLUSIONS

We observed that *S. aureus* produced a different range of enterotoxin according to the substrate. In BHI the production was higher than in cheese, suggesting that food substrates are more appropriate to analyze the real capacity of enterotoxin production. Besides, the microbiota presents can interfere in its production. Finally, this study reinforces the importance in using the adequate temperature on production, transportation, and storage of dairy products.

Análise da produção de enterotoxinas por *Staphylococcus aureus* no Queijo Minas Frescal sob diferentes fatores intrínsecos e extrínsecos

RESUMO

Staphylococcus aureus é um patógeno associado de origem alimentar, principalmente produtos lácteos, devido à produção de enterotoxinas. Este estudo analisou a produção de toxinas clássicas em queijo por *S. aureus*, em diferentes temperaturas, ambientes e níveis de contaminação. O Queijo Minas Frescal foi classificado de acordo com sua microbiota. As amostras de queijo foram classificadas em baixo ou alto nível de contaminação de acordo com a concentração daquela bactéria. Uma alíquota de 10^5 UFC de cada cepa (produtora de enterotoxina A, B, C ou D) em BHI foi inoculada em dois tipos de queijos comerciais de baixa e alta contaminação. Portanto, as amostras foram incubadas a 8 °C, 15 °C e 35 °C por 24, 48 e 72 horas em condição aeróbia e anaeróbia. A detecção e quantificação das enterotoxinas foram testadas contra as toxinas A, B, C e D usando aglutinação reversa passiva em látex. Não houve produção de enterotoxina a 8 °C em todos os tratamentos, conseqüentemente, nenhuma diferença estatisticamente significativa foi observada. A 15 °C, a produção de SEA foi significativamente maior no caldo BHI do que no queijo com baixa contaminação nos três tempos avaliados. Em relação ao SEB, em condição aeróbia a 15 °C após 72 horas, o caldo BHI apresentou produção significativamente superior ao queijo pouco contaminado. *S. aureus* produziu uma gama diferente de enterotoxinas de acordo com o substrato. No BHI, a produção foi maior do que no queijo, sugerindo substratos alimentares mais adequados para analisar a real produção de enterotoxinas. Além disso, a microbiota presente pode interferir na sua produção.

PALAVRAS-CHAVE: temperatura; SE; microbiota; atmosfera.

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Correspondência:
Nathália Cristina Cirone Silva
Universidade Estadual de Campinas (UNICAMP), Cidade Universitária Zeferino Vaz, Barão Geraldo, Campinas, CEP 13083-970, São Paulo, Brasil.