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Detection of virulence factors in coagulase-negative *Staphylococcus* spp. strains isolated from Emmental cheese

Detecção de fatores de virulência em cepas de **Staphylococcus** *spp. coagulase negativos isoladas de queijo Emmental*

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ABSTRACT: Food prepared with products derived from animals are involved in most cases of staphylococcal poisoning; therefore, the research of *Staphylococcus* spp. in Emmental cheese is more applicable. The objective of this study was to identify coagulase-negative *Staphylococcus* spp. (CNS) in cheese using biochemical and molecular techniques to detect the presence of nine genes responsible for the production of enterotoxins. From 180 samples analyzed, 204 CNS strains were obtained and identified as being 46 (22.6%) *S. saprophyticus* strains, 27 (13.2%) *S. hominis* spp. *hominis* strains, 22 (10.8%) *S. sciuri* strains, 21 (10.3%) *S. xylosus* strains, 19 (9.3%) *S. epidermidis* strains, 19 (9.3%) *S. haemolyticus* strains, 17 (8.3%) *S. lentus* strains, 17 (8.3%) *S. warneri* strains, 11 (5.4%) *S. equorum* strains and 5 (2.5%) *S. cohnni*. Using the PCRm protocol, 14 (6.9%) strains with the presence of the genes on the enterotoxin E (SEE)11 (78.6%), J (SEJ) 1 (7%), C (SEC) 1 (7%) and I (SEI) 1 (7%) were detected. Based on the results, the type of package is not interfered of growth and isolated that *Staphylococcus* spp. in cheese. It was observed that bacteria capacity to produce coagulase cannot be understood as an indicative of enterotoxigenicity; therefore, the CNS should be considered as a target of importance in the epidemiology of staphylococcal intoxications. It can be concluded that CNS need to be included in bacterial foodborne disease research, since the genes responsible for the production of toxins were detected and none of the studied samples presented *Staphylococcus* spp. counting above the limits allowed by legislation.

KEYWORDS: *Staphylococcus*; enterotoxin; food safety.

RESUMO: Os alimentos preparados com produtos de origem animal são os mais envolvidos em casos de intoxicação alimentar estafilocócica; portanto a pesquisa do *Staphylococcus* spp. em queijos tipo Emmental é relevante. O objetivo foi isolar e identificar espécies de *Staphylococcus* coagulase negativas (CNS)de queijo Emmental acondicionado em vários tipos de embalagem, por meio de técnicas bacteriológicas e bioquímicas e detectar, por PCR, a presença de nove genes responsáveis pela produção de enterotoxinas. Das 180 amostras, foram isoladas 204 cepas de CNS, que foram identificadas por provas bioquímicas como: 46 (22,6%) *S. saprophyticus,* 27 (13,2%) *S. hominis* spp. *hominis,* 22 (10,8%) *S. sciuri,* 21 (10,3%) *S. xylosus,* 19 (9,3%) *S. epidermidis*, 19 (9,3%) *S. haemolyticus*, 17 (8,3%) *S. lentus*, 17 (8,3%) *S. warneri*, 11(5,4%) *S. equorum* e 5 (2,5%) *S. cohnii*. Na PCR multiplex, em 14 (6,9%) isolados foi detectada a presença dos genes para enterotoxina E (SEE), em 11 (78,6%) J (SEJ), em 1 (7%) C (SEC) e em 1 (7%) I (SEI). Com base nos resultados, o tipo de embalagem não interferiu na multiplicação dos *Staphylococcus* spp. isolados dos queijos. Neste estudo, verificou-se que a capacidade para a produção de coagulase pela bactéria não pode ser concebida como indicativa de enterotoxigenicidade, portanto devem-se considerar os CNS como objeto de importância na epidemiologia das intoxicações estafilocócicas, fazendo-se necessária a atenção com relação à pesquisa dos CNS nos alimentos, uma vez que foram detectados genes responsáveis pela produção de toxinas, e nenhuma das amostras apresentou contagem para *Staphylococcus* spp. acima do limite permitido pela legislação.

PALAVRAS-CHAVE: *Staphylococcus*, enterotoxina; segurança alimentar.

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INTRODUCTION

Consumption of cheese in Brazil in 2019 it was 1.12 million tons, considering that Brazil was classified as the fourth largest consumer of cheese, second only to the European Union, United States and Russia (SOUZA, 2020). According to a Brazilian cheese association (Associação Brasileira da Indústria de Queijos, ABIQ), in 2017, the production of cheese exceeded one million tons, an increase of 2% over 2016, and the rate annual consumption is 5.5 kg per capita (kg/population/year), with 2000 dairy industry that producing cheese in Brazil (REDAÇÃO DCI DIGITAL, 2018). Despite the use of different techniques to certify the quality and harmlessness of food, foodborne diseases (FBD) remain a health public problem (GERMANO et al., 1993). The *Staphylococcus* spp. are main agents of food intoxication outbreak, for being usually introduced in food through manipulators and asymptomatic carriers, since they are part of the transient microbiota of humans and animals (STAMFORD et al., 2006). Resolution RDC No. 12/2001 from the Brazilian National Agency of Health Surveillance established the limit of 10^3 colony-forming unit per gram (CFU/g) for coagulase production by staphylococcal strains in fresh or matured cheeses collected from markets (BRAZIL, 2001). However, this resolution did not mention the coagulase-negative *Staphylococcus* spp. (CNS). The coagulase-positive species *S. aureus* is more related to outbreaks of food intoxication due to the characteristic of many strains to produce various types of staphylococcal enterotoxins (SE) (OMOE et al., 2005), but there are many previous studies in the literature (CARDOSO, 1999; SENA, 2000; CARMO et al., 2002; PIMENTEL et al., 2002) proving the toxicity of negative-coagulase species. Therefore, the presence of negative-coagulase species cannot be ignored, especially if they are going to be largely found in food, which indicates mainly poor hygiene practices of manipulators (JAY, 2000). The CNS were considered nonpathogenic bacteria until the discovery of responsible agents for nosocomial infections, although some species can cause infection in humans (KLOOS; BANNERMAN, 1994; CUNHA et al., 2002; CHANG et al., 2003).

The present work aims to identify species of CNS in Emmental cheese using biochemical and molecular techniques to identify the genes association with the production of enterotoxins, and thus evaluate the quality of the final product to costumers.

MATERIAL AND METHODS

Cheese samples

The cheese samples were produced in a dairy industry located in the city of Buritis (MG) according to Normative Instruction No. 62/2003 (BRAZIL, 2003a). The dairy industry follows sanitary norms in relation to the quality of milk used for the production of cheese and meets effective good manufacturing practice (GMP) and sanitation standard operation procedures (SSOP) (BRAZIL, 1996).

Ten batches of Emmental cheese were produced, parted in three portions and packed with three types of package (resin, vacuum and modified atmosphere) each, within six different postproduction periods, at the dairy farm, totaling 180 samples. They were collected from day 0 of production until day 150. The composition of the cheese packing was: the first one had a protection resin (n = 60); the second was vacuum-packed in plastic film (n = 60); and the third part of the cheese was packed only with plastic film and submitted to a modified atmosphere machine (n = 60) that injected a gas mixture (CO_2, O_2 and N_2) into the package. The samples were refrigerated between 4 to 8 °C and transported to the General Bacteriology Laboratory at the Biological Institute, São Paulo, Brazil, for microbiological analyses.

Microbiological analyses

An aliquot of 25 ± 2 g from each cheese sample was prepared and 225 ml of 0.1% peptone water were added in each different package. After that, the samples were vortexed for 60 s in the Stomacher 4000 Circulator (Seward) (10^{-1} dilution). Serial dilutions were performed, from the first dilution (10^{-1}) until 10^{-4}. Selective plating was done using 0.1 ml of each dilution in Baird Parker Agar Difco, using Drigalski handle. Plates were incubated for 36 ± 1 °C for 30-48 h. The suggested colonies were identified *Staphylococcus* spp. using biochemic tests to confirm the genus (catalase and Gram-stained). Coagulase tube method, DNAse test, thermonuclease (TNAse) (BRAZIL, 2003b) and kits of identification API STAPH or VITEK 2 Compact system were used to determine the species according to the availability of the kit in the moment of the analyses.

Statistical analysis

The association of occurrence of *Staphylococcus* spp. and the package type (resin, vacuum or modified atmosphere) was calculated using the nonparametric chi-square test or the G test (SIEGEL; CASTELLAN JUNIOR, 2006) with significant level of 5% ($p < 0.05$), with software BioEstat version 5.03.

PCR for detection of genes (SE)

The methods described by LØVSETH et al. (2004) were used to detect staphylococcal enterotoxin (SE) genes. Amplification of bacterial colonies for staphylococcal enterotoxin genes was performed adding 10 µL of colony suspension to 40 µL of mix of PCR reagents, [1.25 U Taq DNA polymerase, 200 µmol L^{-1} of each dNTP, buffer (10 mmol L^{-1} Tris-HCl,

pH 8.0, 50 mmol L^{-1} KCl), 2 mmol L^{-1} $MgCl_2$]. Two types of mix were prepared because of the large number of primers in the reaction. In the first step, named mix 1, the following primers were used in the concentration of 5 pmol μL^{-1} for each primer targeting the genes: SED, SEE, SEG, and SEI; and, in the second step, named mix 2, with the following primers in the same concentration: SEA, SEB-SEC, SEC, SEH and SEJ. The two types of mix and the following conditions: initial denaturation of 95 °C for 10 min followed by 30 cycles of denaturation at 95 °C for 1 min, hybridization at 68 °C for 1 min, and extension at 72 °C for 1 min, with a final extension of 72 °C for 7 min. A derivative strain of *S. aureus* (ATCC 13565) SEA gene producer was used as positive control, and sterile deionized water as a negative control.

The 10 µL amplified products were homogenized with Blue Juice (Invitrogen) and then submitted to 1.5% agarose gel added TrisBorato EDTA (TBE) 0.5X (0.0045 mol L^{-1} TRIS-Borato e 1 mmol L^{-1} de EDTA pH 8.0) buffer, with added 10000X red gel (Uniscience) at 1:125. Visualization of the bands was performed with an ultraviolet light transducer.

RESULTS

Biochemical identification of Staphylococcus spp.

From the 180 samples of Emmental cheese, none presented colony population above the allowed (< 103 CFU/mL) for *Staphylococcus* spp., but at least one isolate was obtained from each sample. The growth of 208 presumptive isolates of *Staphylococcus* genus was observed. The 208 isolated obtained were biochemically identified (Table 1) by VITECK 2 Compact and/or API STAPH. Four of the 208 isolates were confirmed as *Aerococcus viridans*, which do not belong to genus *Staphylococcus*, so these samples were removed from this study, totaling 204 isolates.

A total of 74 (35.6%) strains of *Staphylococcus* spp. were isolated in the vacuum packages, 67 (32.2%) in the resin packages and 67 (32.2%) in the modified atmosphere package. After statistical analysis, no significant differences ($p = 0.635$) of growth between the types of packages were detected. Table 2 describes the genus and species identified in this study.

Table 1. Regular biochemical tests using API STAPH and/or VITECK 2 Compact for phenotypic identification of isolates.

SPECIES	GLU	FRU	MNE	MAL	LAC	TER	MAN	XLT	MEL	NIT
S. saprophyticus	+	+	-	+	+	+	+	-	-	+/-
S. hominis subsp. *hominis*	+	+	+/-	+	+/-	+	+/-	-	-	+
S. scuiri	+	+	+	+	+/-	+	+	-	-	+
S. xylosus	+	+	+	+	+	+	+	+/-	-	+
S. epidermidis	+	+	+/-	+	+	-	-	-	-	+
S. haemolyticus	+	+	-	+	+	+	+/-	-	-	+
S. lentus	+	+	+	+	+	+	+	-	+	+
S. warneri	+	+	+/-	+	-	+	+/-	-	-	-
S. equorum	+	+	+	+	+	-	+	+/-	-	+
S. cohnii	+	+	+/-	+	-	+	+	+/-	-	-
SPECIES	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE	
S. saprophyticus	-	+	-	-	+	-	+/-	+/-	+/-	
S. hominis subsp. *hominis*	+/-	+/-	-	-	+	-	+/-	+/-	+	
S. scuiri	+/-	+/-	-	-	+	-	+/-	-	-	
S. xylosus	+	+/-	-	+	+	-	+	-	+	
S. epidermidis	+	+/-	-	-	+	-	-	+/-	+	
S. haemolyticus	-	+/-	-	-	+	-	+	+	-	
S. lentus	-	+/-	+	+	+	+/-	+	-	-	
S. warneri	-	+	-	-	+	-	-	+	+	
S. equorum	+/-	+/-	-	-	+	-	-	-	+	
S. cohnii	+/-	+	-	-	-	-	-	-	-	

GLU (D-glucose); FRU (D-fructose); MNE (D-mannose); MAL (D-maltose); LAC (D-lactose); TRE (D-trehalose); MAN (D-mannitol); XLT (xylitol), MEL (D-melibiose); NIT (nitratereduction); PAL (β-naphthylphosphate); VP (Voges-Proskauer); RAF (D-raffinose); XYL (D-xylose); SAC (D-sucrose); MDG (methylα-D-glucopyranoside); NAG (N-acetylglucosamine); ADH (L-arginine) and URE (urea).

Table 2. Number of isolates identified by regular biochemical tests to API STAPH and/or VITECK Compact. Total isolates = 208.

ISOLATES BY PACKAGES TYPES	Nº OF BATCHES	ISOLATES	TYPICAL COLONIES: IDENTIFIED ISOLATES	ATYPICAL COLONIES: IDENTIFIED ISOLATES
Vacuum = 74 (35.6%)	01	08	*06:* S. lentus	*02:* S. sciuri
	02	07	*05:* S. epidermidis	*02:* Aerococcus viridans
	03	09	*07:* S. saprophyticus	*02:* S. equorum
	04	08	*05:* S. saprophyticus	*03:* S. hominis subsp. hominis
	05	09	*06:* S. warneri	*03:* S. xylosus
	06	06	*05:* S. xylosus	*01:* S. epidermidis
	07	05	*05:* S. sciuri	---
	08	08	*06:* S. haemolyticus	*02:* S. equorum
	09	08	*06:* S. hominis subsp. hominis	*02:* S. cohnii
	10	06	*05:* S. saprophyticus	*01:* S. xylosus
Resin = 67 (32.2%)	01	06	*05:* S. lentus	*01:* S. sciuri
	02	06	*05:* S. epidermidis	*01:* Aerococcus viridans
	03	07	*05:* S. saprophyticus	*02:* S. equorum
	04	07	*06:* S. saprophyticus	*01:* S. hominis subsp. hominis
	05	06	*06:* S. warneri	---
	06	07	*05:* S. xylosus	*02:* S. epidermidis
	07	05	*05:* S. sciuri	*01:* S. hominis subsp. hominis
	08	09	*07:* S. haemolyticus	*02:* S. equorum
	09	07	*05:* S. hominis subsp. hominis	*02:* S. cohnii
	10	06	*05:* S. saprophyticus	*01:* S. xylosus
Modified atmosphere = 67 (32.2%)	01	09	*06:* S. lentus	*03:* S. sciuri
	02	06	*05:* S. epidermidis	*01:* Aerococcus viridans
	03	07	*04:* S. saprophyticus	*03:* S. equorum
	04	08	*05:* S. saprophyticus	*03:* S. hominis subsp. hominis
	05	05	*05:* S. warneri	---
	06	06	*05:* S. xylosus	*01:* S. epidermidis
	07	08	06: S. sciuri	*02:* S. hominis subsp. hominis
	08	06	06: S. haemolyticus	---
	09	07	06: S. hominis subsp. hominis	*02:* S. cohnii
	10	05	04: S. saprophyticus	*01:* S. xylosus

PCR for detection of genes related to enterotoxins production

A total of 14 (6.9%) positive strains was observed in the 204 isolates coagulase-negative *Staphylococcus* after molecular detection, and 190 (93.1%) were negative for enterotoxin genes (Table 3).

DISCUSSION

The pattern of bacterial species established in cheeses has been reported in several studies as very diverse, but it is correlated with the results of this study, in which similarities between isolation rates and species were presented; however, this study describes for the first time the occurrence in a different type of cheese, the Emmental cheese.

BORGES et al. (2008), studying the coalho cheese, identified three species of CNS (*Staphylococcus haemolyticus*, *S. hyicus* and *S. xylosus*) in the raw milk as feedstock in the production process; *S. cohnii, S. lentus, S. capitis* and *S. saprophyticus* in pasteurized milk during the process; and *S. epidermidis* (37.5%), *S. xylosus* (25.0%), *S. cohnii* (6.2%), *S. haemolyticus* (6.2%) and *S. lentus* (6.2%) in coalho cheese.

LAMAITA et al. (2005) isolated 31% of CNS in cooled raw milk, where they found three species, *S. epidermidis, S. sciuri,* and *S. cohnii.* In another study, CUNHA, A.;

Table 3. Detection of enterotoxin genes from CNS isolates.

PACKAGE	BATCHES	STRAIN	GENE	TOXIN
ATM	04	*Staphylococcus saprophyticus*	SEE	E
	07	*Staphylococcus hominis* subsp. *hominis*	SEJ	J
	08	*Staphylococcus haemolyticus*	SEE	E
	10	*Staphylococcus xylosus*	SEC	C
	10	*Staphylococcus saprophyticus*	SEE	E
RESIN	04	*Staphylococcus saprophyticus*	SEE	E
	08	*Staphylococcus haemolyticus*	SEE	E
	10	*Staphylococcus saprophyticus*	SEE	E
VACUUM	03	*Staphylococcus equorum*	SEI	I
	04	*Staphylococcus saprophyticus*	SEE	E
	05	*Staphylococcus xylosus*	SEE	E
	06	*Staphylococcus xylosus*	SEE	E
	08	*Staphylococcus haemolyticus*	SEE	E
	10	*Staphylococcus saprophyticus*	SEE	E

CUNHA, M. (2007) evaluated isolates of *Staphylococcus* spp. from food, including milk and dairy products, and reported that the species of major occurrence were *S. epidermidis* (40%), *S. xylosus* (20%), *S. warneri* (20%), *S. saccharolyticus* (15%) and *S. hominis* (5%).

DE LUCA et al. (1997) isolated 313 strains of *Staphylococcus* spp. from 135 sampled cheese of many types, and the predominant species were *S. hominis* (19.5%), *S. xylosus* (19.2%), *S. epidermidis* (14.8%) and *S. cohnii* (16.3%).

FLEMING et al.(2010) found 45 samples of cheese (2 of "*bola*" type, 1 of cheddar type, 1 of *estepe* type, 10 of "*minas*" type, 14 of mozzarella type, 5 of parmesan type, 11 of "*prato*" type and 1 of provolone type) and isolated 37 strains of the following CNS: *S. lentus, S. capitis, S. caprae, S. kloosii, S. gallinarum, S. simulans* and *S. epidermidis.*

SENA (2000) analyzed 90 samples of coalho cheese and isolated 377 strains of *Staphylococcus* spp., 137 strains of CNS, distributed as: 96 (25.5%) of *S. epidermidis*, 41 (10.9%) and *S. hyicus*. VALLE et al. (1990) isolated 342 *Staphylococcus* spp. of goat milk and identified different coagulase-negative species: *S. chromogenes*, *S. warneri*, *S. sciuri*, *S. saprophyticus* and *S. lentus.*

In the present study, a total of 204 isolates were obtained of many types of packages, distributed in 10 species of CNS, and characterized as: 46 (22.6%) *S. saprophyticus*; 27 (13.2%) *S. hominis* subsp. *hominis;* 22(10.8%) *S. sciuri*; 21 (10.3%) *S. xylosus*; 19 (9.3%) *S. epidermidis*; 19 (9.3%) *S. haemolyticus;* 17 (8.3%) *S. lentus;* 17 (8.3%) *S. warneri;* 11 (5.4%) *S. equorum* and 5 (2.5%) *S. cohnii*. It is noteworthy that the *S. equorum* species was isolated in this study, which is not related to any of the other studies described above.

In the present study, 14 (6.9%) staphylococcal enterotoxin (SE) were detected for CNS and 190 (93.1%) were negative for toxins genes. LAMAITA et al. (2005) showed the presence of staphylococcal toxin in cooled raw milk samples using the optimum sensitivity plate (OSP) method, and verified that the pools of 91 (41.3%) isolates from CNS produced a few kinds of toxins as: SEA, SEB, SEC, SED and the *Tsst*-1 toxin. In this study, the major incidence for SE was E type (SEE) in 11 (78.6%) of 14 strains, which highlights the novel results, because the toxin E was detected in samples of Emmental cheese for the first time.

STAMFORD et al. (2006) studied samples of raw milk from Pernambuco public market, of which 109 strains of *Staphylococcus* spp. were isolated, and 32 of them were characterized as CNS using biochemical tests, but their results cannot be entirely compared with the results presented in this study because the 5 strains that tested positive for toxin production did not have the confirmation of toxin type. Although CNS species are not usually considered an object of epidemiological importance for *Staphylococcus* intoxication, it was previously stated that coagulase-negative species may be involved in outbreaks (PEREIRA et al., 2001). Likewise, OLIVEIRA (1999) evaluated samples in powdered milk and cooked ham, obtaining 10 strains of CNS with enterotoxigenic potential belonging to the species: *S. epidermitis*, *S. chromogenes*, *S. hominis, S. hyicus, S. warneri* and *S. xylosus*, from which *S. chromogenes* and *S. Warneri* produced enterotoxin in food.

This study describes that all the *Staphylococcus* spp. were characterized as CNS with the potential of enterotoxin production, mainly for the E, I and J types. The type of package did not inhibit bacterial growth, because in all the packages tested (resin, vacuum and modified atmosphere) there was growth of *Staphylococcus* spp.

Even though the counting for *Staphylococcus* spp. were within the permitted limits for cheese in this study, further

investigation is needed to relate the cases of microorganisms involved as the enterotoxigenic *Staphylococcus* group in food, and correlate the production of coagulase enzymes with capacity of enterotoxigenic production. This statement is corroborated by SU; WONG (1997), who observed that the capacity of coagulase production cannot be designed as a unique indication of enterotoxicity. Therefore, CNS must be considered as an object of importance in the epidemiology of *Staphylococcus* food intoxication and an alert to health authorities on the risk of the presence of microorganisms.

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