In vitro biofilm production by *Staphylococcus* spp. strains isolated from finger-foods and snacks

Produção *in vitro* de biofilme por *Staphylococcus* spp. isolados de salgadinhos e lanches

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RESUMO
Staphylococcus aureus é um dos principais causadores de doenças de origem alimentar e, embora a Agência Nacional de Vigilância Sanitária – ANVISA não contemple especificamente a detecção de estafilococos coagulase-negativa (ECN) em alimentos, sabe-se que este grupo de bactérias apresenta genes responsáveis pela formação de biofilme e produção de enterotoxinas. No presente estudo foram identificados os *S. aureus* e ECN em amostras de salgados, e sua capacidade de produzir biofilme *in vitro* foi avaliada por duas metodologias: ágar vermelho Congo e placa de microtitulação de poliestireno. Vinte e dois isolados de *Staphylococcus* pertencentes a oito diferentes espécies foram obtidos de 122 salgados, sanduíches e lanches. *S. aureus*, *S. warneri* e *S. haemolyticus* foram as espécies mais frequentemente isoladas. A produção de biofilme foi verificada em sete (31,8 %) isolados pela técnica de ágar vermelho Congo e em três (13,6 %) em placa de microtitulação. Nenhum isolado foi considerado positivo em ambas as metodologias. Apesar do baixo número de isolados, houve concordância de 59,1 % entre os testes. A capacidade de produzir biofilme é importante fator de virulência para *Staphylococcus* spp. e a detecção destes isolados de amostras de alimentos pode ajudar a definir o papel dos ECN como agentes patogênicos transmitidos por alimentos.
Palavras-chave. segurança alimentar, biofilmes, *Staphylococcus*.

ABSTRACT
Staphylococcus aureus is one of the most important food-borne pathogens, and although Brazilian Sanitation Surveillance Agency (ANVISA) does not specifically regards for coagulase-negative *Staphylococcus* (CNS) isolation from foods, it is known that this group of bacteria possesses genes associated with biofilm formation and enterotoxins production. In this context, the present study aimed at identifying the *S. aureus* and CNS in finger-foods and snacks samples, and to evaluate the ability of these strains to produce biofilm *in vitro* by means of two methodologies: Congo Red agar and polystyrene microplates cultures. Twenty-two staphylococcal isolates belonging to eight species were obtained from 122 finger-foods, sandwiches and ready-to-eat (RTE) food products. *S. aureus*, *S. warneri* and *S. haemolyticus* were the most frequent isolates. Biofilm production by *Staphylococcus* spp. was observed in seven (31.8 %) isolates by Congo Red agar technique and three (13.6 %) by polyestirene microplate methodology. There was no positive isolate biofilm producer by both methodologies. Despite the low number of isolates, a concordance of 59.1 % between the tests was found. The ability to produce biofilm is an important virulence factor in *Staphylococcus* spp., and it can support to define the role of CNS as food-borne pathogen.
Keywords. food safety, biofilms, *Staphylococcus*.

INTRODUCTION

Staphylococcus spp. are widespread microorganisms that may play an important role as agents of food-borne diseases[1-4]. *Staphylococcus aureus* and coagulase-negative *Staphylococcus* (CNS) are frequent bacteria on the human skin and mucous membranes, mainly as commensal flora[5,6]. Usually, the interaction between *S. aureus* and its human host appears to be non-hostile, since the bacterial colonization does not cause any symptoms. However, either by an increase in bacterial virulence or by awakenings of the immune system, being as an immune deficiency or an acquired state of immune suppression, *S. aureus* may become a deadly threat[7]. *Staphylococcus* species are the relevant biofilm-forming organisms. The biofilms are defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and they are adherent to an inert or living surface[8]. Biofilms have been well studied in dairy and meat plants process[9,10], cross-contamination[8] and mastitis[11,12,13,14]. There are few studies regarding the biofilm production ability of *Staphylococcus* spp., which are isolated from ready-to-eat, snacks and sandwiches. Snacks are fast foods usually derived from one or more basic food items and they are often eaten between meals[15]. This kind of food is highly consumed as it is often hot food prepared and served quickly, at low cost, and in places usually close to the workplace[16]; and it is consumed by people in both developed and developing countries, and by across-all-age groups[16]. This study aimed at identifying *S. aureus* and coagulase-negative *Staphylococcus* (CNS) in finger-foods and snacks sold in Botucatu, SP, and to evaluate the ability of these isolates in producing biofilm.

MATERIAL AND METHODS

Samples collection

Food samples from 61 groceries, cafes, food trailers, snack bars, bakeries and street vendors, located in important areas in Botucatu, SP, were collected and analyzed twice (from August 2008 to March 2009). Meat, chicken, shrimp, ham-and-cheese, cheese, vegetable finger-food and sandwich samples were collected. These samples were transported in sterile 114 x 229 mm plastic bags (Inlab) under refrigeration, and they were kept in a cooler box until being processed in the same day in the lab.

Microbiological Analysis

Following the methodology recommended by Lancette and Bennett[17], 25 g of food were homogenized in 225 mL of peptone water in plastic bags by using Stomacher Lab Blender 400 for 30 seconds. From this 10^{-1} dilution, further dilutions were made. All of the culture media were purchased from Difco, Becton Dickinson (Sparks, MD).

Staphylococcus spp. were detected and identified according to the methodology described by Murray et al[18]. The phenotypic characteristics of biofilm production by *Staphylococcus* spp. were evaluated by two methodologies: cultivation on BHI agar supplemented with 0.8 % Congo Red[19] and biofilm assay using sterile 96-well bottom polystyrene tissue culture microplates with BHI broth medium[20].

RESULTS AND DISCUSSION

Of 122 meat, chicken, shrimp, cheese, vegetable finger-food and sandwich samples analyzed in this study, *S. aureus* was the most isolated microorganism, which occurred in eight (6.6 %) samples (five chickens, two ham-and-cheese snacks and one meat finger-food). Different species of CNS were detected in 14 (11.4 %) samples. The most frequently isolated CNS were *S. warneri* (from one ham-and-cheese snack, two meat finger-food and one of chicken) and *S. haemolyticus* (from two cheese, two chicken finger-foods), which were found in four samples (3.3 %) each. *S. saprophyticus* was detected in two (1.6 %) samples, being one ham-and-cheese snack and one meat finger-food. The varied CNS species were detected, being each microorganism in each one (0.8 %) sample, being *S. simulans* in ham-and-cheese snack, *S. xylosus* in sandwich, *S. caprae* in cheese finger-food, and *S. schleiferi* subsp. *schleiferi* in chicken finger-food. Co-presence with two different *Staphylococcus* species were observed in two samples: *S. aureus* and *S. warneri* in a meat finger-food, and *S. haemolyticus* and *S. caprae* in a cheese finger-food.

S. aureus might be used as an indicator for assessing the hygiene and sanitary conditions. The isolation of this bacterium from foods evidences the unhygienic conditions during their processing, storage, and other pertinent procedures[21]. This microorganism is a poor competitor but exert a high risk in foods if the normal microbiota has been destroyed or inhibited, e.g. as the cooked and salted meats[22]; also, toxin that causes illness might be produced by foods contaminated with *S. aureus*[23].

Finger-foods and sandwiches pose as potential health risks associated with contamination of raw food

with pathogenic bacteria, contamination by handler during the food preparation, and also through post-cooking handling and cross-contamination[24]. A high tendency in rising the microbial load in snacks depends on the raw materials, the conventional methodologies of food processing and packaging, and the maintenance temperature, which might be a source for causing varied rates of deterioration of the read-to-eat (RTE) food products and snacks in developing countries[15].

Biofilm production was assessed in eight *S. aureus* strains and in 14 CNS isolates using Congo Red agar and polystyrene microplate adherence techniques. Red-Congo was used as pH indicator, and the black coloration indicated that the pH range was from 3.0 to 5.2. The cultures were considered positive, when the black rough colonies were grown[19]. By mean of Congo Red agar technique, the biofilm production was found in seven (31.8 %) strains. On the other hand, by microplate assay, three (13.6 %) strains only were biofilm producer. The results were shown in the Table.

Table 1. Results of biofilm production by *Staphylococcus* spp., using Congo Red agar and polyestirenemicroplate assay

***Staphylococcus* spp.**	**N**	**Biofilm production**	
		Congo Red agar	**Microplate**
S. aureus	8	2	2
S. warneri	4	0	0
S. haemolyticus	4	1	0
S. saprophyticus	2	2	0
S. simulans	1	1	0
S. xylosus	1	1	0
S. caprae	1	0	0
S. schleiferi subs. *schleiferi.*	1	0	1
Total	**22**	**7 (31.8 %)**	**3 (13.6 %)**

None of the strains was positive in both methodologies, which indicated the occurrence of noncompliant results. Of 22 isolated strains, 13 were negative in both techniques; therefore, the overall concordance was of 59.1 % between the two tests (Kappa index = -0.1647), which indicated a good specificity. Due to the low number of isolates, it was not determined which biofilm assay was the most efficient.

S. aureus was the foremost biofilm producer, as 4/8 (50 %) isolates formed biofilm, independently of the employed methodology. Among CNS, 6/14 (42.9 %) were biofilm-forming bacteria. The highest biofilm production by *S. aureus* was not statistically significant, when the CNS rates were considered (p = 0,546).

Very few data on the biofilm production by *Staphylococcus* spp. isolated from finger-foods were found in the specific literature, but biofilms formation in other kind of food were usually analyzed in RTE food samples. Podkowik et al[25] found 67 staphylococcal isolates belonging to 12 species, isolated from 70 RTE food products; *S. aureus* (35.7 %) and *S. epidermidis* (18.5 %) were predominant bacteria. Interestingly, in the present study, no *S. epidermidis* was isolated, even it is one of the most frequent species in the skin microbiota. *S. aureus* was isolated from eight (6.5 %) food samples, being at lower rate than those 35.7 % reported by the above referred authors[25]. These divergent findings could be occurred because different types of food were analyzed in every investigation. Podkowik et al[25] analyzed foods prepared with porcine, bovine, and chicken meat, and the fried or roasted finger-food were the investigated samples in the present study. On that account, the probability of recovering *Staphylococcus* isolates from meat-RTE food is higher, because of the use of different type of preparation, cooking and handling procedures. Podkowik et al[25] performed the detection of virulence-associated genes and the biofilm formation of *S. epidermidis* only; from 13 isolates, five (38.5 %) harbored the *ica* operon, and they formed biofilm. Those values were slightly lower than the findings detected in the present study for *S. aureus* and CNS by means of Congo Red agar assay (31.8 %). Møretrø et al[26] also found lower percentage, using 144 *S. aureus* isolated from three separate Norwegian meat- and poultry-processing plants by swabbing the food cuttings and other contact surfaces and equipment; and 28 (19.5 %) were considered biofilm producers. By means of microplate assay, the findings observed in the present investigation were similar in three samples (13.6 %) only. The finger-foods consumed in Brazil are usually fried food. Thus, considering that the employed temperatures for food frying do not reach the degrees needed to eliminate the microorganisms, it enhances the demands concerning to the precise care for the foods handling during their preparation. These procedures will decrease the risk of contaminating food and avoiding compromising the consumers' health[27].

CONCLUSION

This study verified a considerable quantity of biofilm-producer strains, although the number of isolates was small. These results show the ability of *Staphylococcus* spp. isolated from food in forming

biofilm *in vitro*, and it may represent an advantage in bacterial permanence in the environment, utensils and equipments enhancing the importance of hygienic measures to avoid food contamination and cross contamination. Also, owing to the difficulty in eradicating biofilms, it is crucial to carry on the microbiological control before biofilms are formed, and to make use of hygienic principles for cleaning and sanitizing the utensils and equipments to prevent potential risk of food-borne diseases.

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