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BIOFILM PRODUCTION BY *Staphylococcus* sp ON STAINLESS STEEL CHIPS IN CONTACT WITH BRAZILIAN MINAS CHEESE HOMOGENATES AND BHI BROTH UNDER DIFFERENT EXTRINSIC FACTORS

Nathália C.C. Silva[1], Érika C.R. Bonsaglia[2], Ary Fernandes Júnior[2], Rodrigo T. Hernandes[2]; José C.F. Pantoja[3], Vera L.M. Rall[2]

Address(es): Nathália Silva,
[1]Department of Food Science, Faculty of Food Engineering (FEA), University of Campinas (UNICAMP), 13083-862, Campinas, Sao Paulo, Brazil. Tel. (+55 19) 3251 4012, Fax (+55 19) 3251 0064.
[2]Department of Microbiology and Immunology, Bioscience Institute, Distrito de Rubião Junior S/N, CEP: 18618-970, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil
[3]Department of Hygiene Veterinary and Public Health, Distrito de Rubião Junior S/N, CEP: 18618-970, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil.

*Corresponding author: ncirone@unicamp.br

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ABSTRACT

The aim of this study was to evaluate biofilm production by *Staphylococcus* sp on stainless steel using brain heart infusion (BHI) broth and Minas Frescal cheese broth, at different levels of contamination and under different temperatures, atmospheres. The cheeses were classified according to their microbiota. It was evaluated biofilm formation on stainless steel chips at 8, 15 and 35°C after 48, 72 and 96 h under different conditions. Both *Staphylococcus* isolates formed biofilm on stainless steel chip in BHI broth and cheese broth, under all temperatures and atmospheres studied. In conclusion, BHI broth and Brazilian Minas cheese homogenate performed equally well for biofilm formation tests using *Staphylococcus* strains, and *S. aureus* can produce biofilm on stainless steel at 8ºC, which may be a concern for the dairy industry. Finally, it is important to consider that *S. aureus* can be a good competitor, depending on the nature of the microbiota.

Keywords: *Staphylococcus aureus*; atmosphere; biofilm; temperature; microbiota

INTRODUCTION

Dairy products have been frequently associated with foodborne illness (**Veras *et al.*, 2008; Schmid *et al.*, 2009**). In Brazil, Minas cheese is the most popular cheese, produced by the direct acidification of milk or by adding lactic cultures (**Carvalho *et al*,. 2007**). This cheese can also be handmade on small farms with raw milk, without proper hygiene control, resulting in a product with high levels of bacterial contamination, including *Staphylococcus* sp and coliforms (**Carvalho *et al.*, 2007; Rall *et al.*, 2010**).
Staphylococcus species are commonly found in cheese, with *S. aureus* being the major pathogenic species. Some strains of this genus are able to produce enterotoxin (**Rall *et al.*, 2010**) and form biofilm on abiotic surfaces (**Schlegelová *et al.*, 2008; Gutiérrez *et al.*, 2012**). Flaws in cleaning processes allow the contaminating bacteria to attach to equipment surfaces and become a potential source of contamination in the food industry (**Schlegelová *et al.*, 2008, Gutiérrez *et al.*, 2012**).
The production of polysaccharide intercellular adhesin (PIA) is mediated by the ica (intercellular adhesion) operon (**Cramton *et al.*, 1999**), which consists of the *ica*A, *ica*B, *ica*C and *ica*D genes. *ica*A and *ica*D are of great importance in biofilm production (**Vasudevan *et al.*, 2003**). Other important genes that also produce proteins that regulate biofilm formation are bap (biofilm-associated protein) and agr (accessory gene regulator) (**Melchior *et al.*, 2009**).
The aim of this study was to evaluate biofilm production on stainless steel by *Staphylococcus* sp, using Minas Frescal cheese broth and brain heart infusion (BHI) broth as nutrient media, at different levels of contamination and under different temperatures and atmospheres.

MATERIAL AND METHODS

Microbial quality of cheese samples

We acquired different brands of cheese at retail in the city of Botucatu, SP, Brazil and the temperatures of all purchases (12 – 2 samples per purchase) were measured with a digital infrared thermometer with laser sight (Incoterm, Porto Alegre, Brazil). A total of 24 samples were analyzed, and the samples were classified as having low-level contamination when they had up to 10^3 colony forming units (CFU)/g of mesophilic and psychrotrophic bacteria (PCA medium), in the absence of thermotolerant coliforms (EC broth) and *Staphylococcus* sp. (Baird Parker agar). They were classified as having a high-level of microbiota when we detected more than 10^3 CFU/g of mesophilic and psychrotrophic bacteria and more than 10^3 most probable number (MPN) of thermotolerant coliforms/g, in the absence of *Staphylococcus* sp. All tests were performed with Oxoid culture media (Oxoid, Basingstoke, UK).
Determination of the MPN of thermotolerant coliforms was carried out according to the American Public Health Association (**Kornacki and Johnson, 2001**) in a three-tube series of each dilution. The MPN of thermotolerant coliforms was calculated based on gas production in tubes of *E. coli* broth at 45ºC after 24 h.
Staphylococcus sp counts were determined according to **Lancette and Bennett (2001)**, where serial dilutions of cheese homogenate were plated on Baird Parker agar with 5% egg yolk tellurite emulsion and incubated at 35°C for 48 h. Characteristic colonies were tested for catalase, thermonuclease, and coagulase, and with the Staphytect Plus Dry Spot Kit (OXOID).
The pour plate method was used for mesophilic bacterial enumeration. CFUs were counted after incubation at 35ºC/24 h. Psychrotrophic bacteria were determinated using the spread method, 0.1 mL of each serial dilution was plated on the surface of the plate count agar. The plates were incubated at 4°C for 7 days **(Morton 2001)**.

Production and quantification of biofilm (Stepanovic et al. 2000).

We used stainless steel (AISI 304) chips to detect biofilm production and the temperatures used were 8 (refrigeration temperature by law), 15 (average temperature found in retail), and 35°C. Sterile stainless chips (1 cm diameter) were placed in 24-well plates. The experiment was performed in triplicate.
The positive and negative controls were *Staphylococcus epidermidis* (ATCC 35984 and ATCC 12.228, respectively), and a *S. aureus* strain, isolated from milk and positive for *ica*A, *ica*D, and *bap* genes (data not shown) were used for the tests (**Vasudevan *et al.*, 2003, Cucarella *et al.*, 2001**).
The strains were incubated in BHI broth at 35°C/24 h and the culture was diluted to 10^8 CFU/mL. The Densichek (Biomerieux, l'Etoile, France) was used to measure turbidity. This dilution was employed to inoculate a cheese homogenate, which was produced by homogenization of 5 g of cheese in 45 mL of saline, with

high and low contamination. Aliquots of 300 µL were distributed in triplicate into the wells of the plate and incubated at 8, 15, and 35°C for 96 h. The same was done using bacterial cultures grown in BHI broth. The plates were incubated in aerobiosis and anaerobiosis, using Anaerogen (Oxoid).

We transferred the chips to a new plate to prevent the quantification of biofilm that might have been produced on the plastic surface around the chips, that were washed three times with PBS (pH 7.4) to remove non-adherent bacterial cells, stained with 1% crystal violet for 15 min, and then washed three times. The biofilm was resuspended in 300 µL of glacial acetic acid for 15 min to ensure the homogeneity of the stained material. Next, 200 µL were transferred to a 96-well microplate, which was read in an ELISA reader (Babsystems, EX multiskan) at 570 nm. Non-inoculated BHI and cheese broth were used as blanks to correct the absorbance value. The strains were classified on the basis of biofilm production as non-producers, or weak, moderate, or strong producers, according to Stepanovic et al. (2000).

Statistical Analysis

Initially, the distribution of OD was examined using histograms and normal probability plots. No departures from a normal distribution were found. Thus, a repeated measures model (PROC MIXED, SAS Institute, 2011) was used to compare the mean OD (response variable) between time points (48, 72 or 96 h), sample types (BHI and low contaminant or high contaminant cheese broth), temperatures (8, 15 or 35°C), environment (aerobic or anaerobic) and strains (wild-type or ATCC). Interaction terms between time point and each explanatory variable were included in the model to test the hypothesis that the difference between sample types, temperatures, environments, and strains depended on the time point analyzed. An autoregressive covariance structure was used to model the correlation between the repeated measurements within the same sample. Tukey´s test was used to adjust the P-values resulting from multiple comparisons. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Most commercial establishments evaluated in the current study did not have adequate refrigeration, with storage temperatures ranging from 8.4 to 18.6°C, with a median of 14.6°C, which approximated temperature (15°C) was one of the temperatures used in this study. In Brazil, other authors also found the use of temperatures in supermarkets above that recommended for cold storage, with inadequate temperatures in 70% (**Chesca *et al.*, 2001**) and 66% (**Lima and Fernandes, 2011**) of the establishments studied. Different results were obtained in London, England by **Hobbs and Roberts (1998)**, where only 19.7% of 559 refrigerators did not use a proper temperature. Such observation is probably due to better awareness and greater vigilance on the part of the responsible authorities.

At 96 h, both strains (*S. epidermidis* and *S. aureus*) showed weak biofilm production, according to the definitions of **Stepanovic et al. (2000)** at all temperatures tested (8, 15 and 35°C) and experimental conditions (cheese homogenate with high or low contamination and BHI broth kept under aerobic and anaerobic atmosphere), with no significant difference between the variables, showing that the BHI broth provided similar conditions as cheese broth for biofilm production, at least for the isolates studied, which were classified as weak producers here. Surprisingly, the cheese's microbiota showed no influence on biofilm production, since *S. aureus* is not considered a good competitor (**Rode *et al.*, 2007**). However, it is important to consider that selective pressure may vary according to the microbiota present in the cheese.

The study showed that biofilm formation was higher at 15 ºC and 35 ºC, in comparison to 8 ºC. When 15 ºC and 35 ºC were compared, biofilm formation was similar ($P>0.05$). These results were expected and confirmed that storage temperature is very important to prevent biofilm formation.

This observation may be a warning, since, biofilm formation occurred at 8°C and higher. **Rode *et al.* (2007)** conducted a study on biofilm formation by *S. aureus* in tryptic soy broth (TSB) at temperatures ranging from 20 to 48°C and observed higher biofilm formation at 25 and 30°C. These temperatures is almost the optimal temperature of 35°C studied in this study and it is common these temperatures, mainly in Brazil where the room temperature could be 30°C.

No significant differences were observed between the temperatures and atmospheres employed in the present study. This suggests that temperature does not influence biofilm production. So far, temperatures that really inhibit the biofilm formation are still unknown, where this question will be important in future studies.

Reports using cheese as the nutrient medium for biofilm production were not found, and few other studies have investigated the influence of temperature and atmosphere on biofilm production (**Jaglic *et al.*, 2011**). The most commonly used temperature for investigating biofilm formation is 37°C, since it is the optimal temperature for growth and biofilm production by these bacteria (**Beenken *et al.*, 2003**). However, it is important to consider that 37°C could not correspond to storage and chilled temperatures and this study showed that biofilm could be formed in low temperature.

Some authors, such as **Michu *et al.* (2011)**, used milk to evaluate biofilm formation by *S. epidermidis* on stainless steel chips at different concentrations of NaCl and glucose by analyzing the expression of the *ica*A gene using qPCR at 30°C at 8 and 20 h. This gene was expressed in all milk samples tested, and expression was substantially higher at 20 h. However, it should be emphasized that the presence of mRNA does not imply production of the corresponding protein, which is dependent on mRNA stability and translation rate (**Lodish *et al.*, 2005**).

Jaglic *et al.*, (2011) studied the adherence of *S. epidermidis* to stainless steel, employing milk as a culture medium at different temperatures: 6, 22, and 28°C. In a 6-h assay, they observed that bacterial adherence to stainless steel, when incubated with milk, was higher than that observed in assays performed in TSB. After 6 h at 6°C, adherence to stainless steel was sufficient for biofilm formation, where the temperature investigated in that assay (6°C) was very close to the temperature employed in this study (8°C).

Biofilm formation in stainless steel is a concern in the milk industry, even at low temperatures, at which products are kept (3 to 6°C). This situation also occurs in other kinds of dairy plants, such as those that produce some cheeses and yogurts, where temperatures can reach 30°C. On the surfaces of equipment, where there is cooling pasteurization, biofilm can grow and contaminate other products (Knight et al. 2004). **Schlegelová *et al.* (2008)** and **Melchior *et al.* (2009)** isolated *Staphylococcus* sp. with genes related to biofilm production, including the *ica* cluster and *bap*, from dairy plants.

CONCLUSION

In conclusion, under the conditions tested here, *Staphylococcus* sp. can form biofilms on stainless steel chips with cheese as a nutrient at 8°C, which represents an important problem for the dairy industry. The use of BHI broth allowed us to obtain similar results as those observed with cheese homogenate, indicating that it could be successfully used in cheese researches. Finally, the conception that *S. aureus* is always a poor competitor against competitive microbiota should be reviewed; maybe it depends on the nature of the microbiota.

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