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. Silva¹, Érika C.R. Bonsaglia², Ary Fernandes Junior², Rodrigo T. Hernandez³, José C.F. Pantojã³, Vera L.M. Rall⁴

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

*Corresponding author: ncrone@unicamp.br

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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; Gutiérrrez 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; Gutiérrrez et al., 2012). The production of polysaccharide intercellular adhesion (PIA) is mediated by the ica (intercellular adhesion) operon (Cramton et al., 1999), which consists of the icaA, icaB, icaC and icaD genes. icaA and icaD 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 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 8°C, 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

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 10² colony forming units (CFU)/g of mesophilic and psychrotrophic bacteria (PCA medium), in the absence of thermodurable coliforms (EC broth) and Staphylococcus sp. (Baird Parker agar). They were classified as having a high-level of microbiota when we detected more than 10³ CFU/g of mesophilic and psychrotrophic bacteria and more than 10² most probable number (MPN) of thermodurable coliforms/g, in the absence of Staphylococcus sp. All tests were performed with Oxoid culture media (Oxoid, Basingstoke, UK). Determination of the MPN of thermodurable 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 thermodurable coliforms was calculated based on gas production in tubes of E. coli broth at 45°C 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 Staphytycent Plus Dry Spot Kit (OXOID). The pour plate method was used for mesophilic bacterial enumeration. CFUs were counted after incubation at 35°C/24 h. Psychrotrophic bacteria were determined 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 icaA, icaD, 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 10³ 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

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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 8°C, 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
been 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 new plates 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, 250 μL were transferred to a 96-well microplate, which was read in an ELISA reader (Babysystems, 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. So far, temperatures that really inhibit the biofilm formation are still unknown, where this question will be important in future studies.

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 icaA gene using qPCR at 30°C and 40°C, which genes was expressed in all milk samples tested, and expression was substantially higher at 40°C. 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).

Jagle et al. (2011) studied the adherence of S. epidermidis to stainless steel, employing milk as a culture medium at different temperatures, mainly in Brazil where the room temperature could be 30°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). Schlegeleva et al. (2008) and Melchior et al. (2009) showed that 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 microflora should be reviewed; maybe it depends on the nature of the microbiota.

REFERENCES


