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Biofilm formation by *Staphylococcus aureus* on food contact surfaces: Relationship with temperature and cell surface hydrophobicity

P. Di Ciccio, A. Vergara, A.R. Festino, D. Paludi, E. Zanardi, S. Ghidini, A. Ianieri

A b s t r a c t

Staphylococcus aureus (*S. aureus*) is a pathogenic bacterium capable of developing biofilms on food processing surfaces, a pathway leading to cross contamination of foods. The purpose of this study was to evaluate the ability of *S. aureus* to form biofilm on food processing surfaces (polystyrene and stainless steel) with regard to different temperatures (12 and 37 °C) and cellular hydrophobicity. Biofilm assays were performed on n. 67 *S. aureus* isolates from food, food processing environments and food handlers and n. 3 reference strains (*S. aureus* ATCC 35556, *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 12228). A strain-specific variation in biofilm formation within *S. aureus* strains tested was observed. At 37 °C, n. 38/ 67 (56.7%) of strains were biofilm producer in at least one tested surface. A total of n. 25/38 (65.7%) of strains were biofilm producer on polystyrene whereas n. 24/38 (63.1%) were biofilm producer on stainless steel. Moreover, n. 11/38 (28.9%) of strains were biofilm producers on both selected surfaces. The majority of *S. aureus* strains which produced biofilms (n. 17/38=44.7%), were isolated from food environments. At 12 °C, only one *S. aureus* strain from food handler (*S. aureus* 374) was biofilm producer. Cell surface hydrophobicity level increased with temperature. Additionally, a statistically significant difference ($P < 0.001$) was found between hydrophobicity at 37 °C and 12 °C. Finally, the architecture of biofilm formed by *S. aureus* strains on polystyrene and stainless steel surfaces at selected temperatures was observed by scanning electron microscopy. The appearance of thick extracellular products in strongly (*S. aureus* ATCC 35556 e positive control) and the absence of those products in the non-biofilm producer (*S. epidermidis* ATCC 12228 e negative control) is presented.

Introduction

Bacteria most commonly live by adhering to surfaces and forming organized communities called biofilms (Malheiros, Passos, Casarin, Serraglio, & Tondo, 2010). Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adhered to inert or living surfaces (Costerton et al., 1999). The presence of biofilm on food contact surfaces is considered as a health hazard. Microbial biofilms, in fact, may contain a considerable number of both spoilage and pathogenic microorganisms (Giaouris, Chorianopoulos, & Nychas, 2005). Exposure of pathogens to surfaces may take place either by direct contact with contaminated materials or indirectly through airborne microflora (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). The direct contact with raw materials or food products may cause secondary contamination by which the product may become unsafe (Vikova, Babak, Seydlova, Pavlik, & Schlegelova, 2008). Several bacteria are known to form biofilms on different materials (Costerton, Geesey, & Cheng, 1978; Di Ciccio et al., 2012). However, biofilm formation is influenced by the nature of substrate, cell surface charge, presence of flagella and microbial growth phase (Pagedar & Singh, 2012). The majority of surfaces in food processing plants are made of stainless steel that can be easily cleaned and is resistant against chemical agents (Mattila Sandholm & Wirtanen, 1992). However, it was detected by microscopy that even smooth surfaces made from stainless steel can be damaged by mechanical cleaning. Small cracks and scratches are formed on their surfaces and bacteria and organic residues can stick to them (Wirtanen, Husmark, & Mattila-Sandholm, 1996). *S. aureus* is a very adaptable organism and can live in a wide variety of environments as biofilm (Almeida & Oliver, 2001; Rode, Langsrud, Holck, & Moretto, 2007). Additionally, biofilm production is recognized as an important virulence factor for bacteria of the genus *Staphylococcus* (Cucarella et al., 2002; Fox, Zadoks, &

Gaskins, 2005; Vasudevan, Nair, Annamalai, & Venkitanarayanan, 2003). *S. aureus* biofilm on food contact surfaces, in fact, poses a serious risk of food contamination (Gibson, Taylor, Hall, & Holah, 1999). It has been frequently found in surfaces of food processing plants being responsible for outbreaks related to the consumption of fresh and processed foods worldwide (Balaban & Rasooly, 2000; Braga et al., 2005; Hamadi et al., 2005; Marques et al., 2007; Nostro et al., 2004; Oulahal, Brice, Martial, & Degraeve, 2008; Rode et al., 2007). Humans are common asymptomatic carriers of enterotoxigenic *S. aureus* in nose, throat, and skin. Thus, food handlers may contaminate food (Gutierrez et al., 2012). *S. aureus* can produce a multilayered biofilm embedded within a glycocalyx or slime layer with heterogeneous protein expression throughout (Archer et al., 2011). For the food industry it is important to identify the conditions, under which *S. aureus* is able to survive and multiply with regard to food processing. The majority of studies, in fact, have been addressed to clinical aspects related to the biofilm formation by *Staphylococcus* genera such as *Streptococcus intermedius* on catheters and/or medical devices (Silva-Meira, Medeiros-Barbosa, Athayde, Siqueira-Júnior, & Souza, 2012). To date, the literature about the biofilm formation by food-related *S. aureus* strains is still scarce and there is a lack of information about the capacity of *S. aureus* isolated from food, food environments and/or food handlers of forming biofilm when exposed to different environmental conditions simulating those in food processing plants. In order to control the *S. aureus* biofilm in the food industry, the greater understanding of the interactions between microorganisms and food processing equipment is required. Regarding these aspects, this study was carried out with the aim of evaluating the ability of *S. aureus* strains isolated from food, food environments and food handlers, to form biofilms on polystyrene and stainless steel surfaces under different temperatures: 12 and 37 °C. Still, the possible correlation between biofilm formation ability and cell hydrophobicity was examined. Finally, the architecture of biofilm formed by *S. aureus* strains on polystyrene and stainless steel surfaces at selected temperatures was observed by scanning electron microscopy (SEM).

Materials and methods

Bacterial strains

The experiment was conducted on n.67 *S. aureus* strains: n. 19, n. 26 and n. 22 strains isolated from food, food environments and food handlers, respectively. The biofilm production was also examined using three *S. aureus* reference strains. In particular, *S. aureus* ATCC 35556 is a reported biofilm producer that has been shown to form a strong biofilm (Cramton, Gerke, Schnell, Nichols, & Goetz, 1999; Seidl et al., 2008), whereas *S. epidermidis* ATCC 12228 was used as negative biofilm producer (Atshan et al., 2012; Lee et al., 2013). Finally, a known additional *S. aureus* reference strain (ATCC 12600) was used to define into different categories the *S. aureus* isolates (n.67). Prior to the conduction of experiments, all strains were activated by culturing twice in 10 mL tryptic soy broth (TSB e Oxoid S.p.A., Milan, Italy) at 37 °C for 24 h. Biofilm production assay A previously described method was used (Di Bonaventura et al., 2008). Polystyrene tissue culture plates (961 mm²) and AISI 304 stainless steel chips (530 mm²) were used for biofilm formation assays at 12 and 37 °C. These two temperatures were selected by their relevance to the food industry (12 °C) and in infectious disease (37 °C). Moreover, polystyrene and stainless steel were selected because they are the most widely used material in the construction of food processing equipment and they have different physico-chemical characteristics: hydrophilic for stainless steel and hydrophobic for polystyrene. Briefly, Stainless steel chips were degreased before use by overnight immersion in ethanol, then rinsed thoroughly in distilled water and autoclaved for 15 min at 121 °C. Cultures of *S. aureus* were prepared, from overnight TSA-growth, in TSB by incubating at selected

temperatures: 12 and 37 °C. Cultures were then washed three times with phosphate-buffered saline (PBS; pH 7.3) (SigmaAldrich S.r.l., Milan, Italy) and diluted with fresh TSB to reach a concentration of about 108 CFU mL⁻¹ by reading the optical density (OD) level at 550 nm (UV Mini-1240 e Shimadzu). Three milliliters (mL) of the standardized inocula were then added to polystyrene tissue culture plates (35 mm diameter) and stainless steel chips. Samples were then incubated at 12 °C and at 37 °C. After 24 h incubation, non-adherent cells were removed by dipping each sample three times in sterile PBS. Samples were fixed at 60 °C for 1 h and stained with 3 mL of 2% crystal violet solution in 95% ethanol for 15 min. After staining, samples were washed thrice with distilled water. Negative controls underwent the same treatment but without inoculation. The quantitative analysis of biofilm production was performed by adding 3 mL of 33% acetic acid to destain the samples. From each sample 200 µl were transferred to a microtiter plate and the OD level of the crystal violet present in destaining solution was measured at 492 nm (Varian SII Scan Cary 100). Considering different growth area of tested surfaces (polystyrene: 961 mm² and stainless steel: 530 mm²), results were normalized by calculating the biofilm production indices (BPIs) as follows: $BPI [OD \text{ mean biofilm surface (mm}^2\text{)}^{-1}] \times 1000$. Two independent sets of all experiments were performed in triplicate.

Cell surface hydrophobicity assay

S. aureus hydrophobicity was evaluated, at selected temperatures: 12 and 37 °C, by microbial adherence to n-hexadecane (MATH) test according to Mattos-Guaraldi, Formiga, and Andrade (1999), with slight modification. Briefly, 4 mL of standardized inocula in PBS (OD₅₅₀ 0.8) were overlayed with 0.4 mL of n-hexadecane (SigmaAldrich). After 1-min agitation by vortexing, the phases were allowed to separate for 15 min at room temperature. The results were expressed as the proportion of the cells which were excluded from the aqueous phase, determined by the equation as follows: $[(A_0 - A)/A_0] \times 100$, where A_0 and A are the initial and final optical densities of the aqueous phase, respectively. *S. aureus* strains were classified as: highly hydrophobic, for values >50%; moderately hydrophobic, for values ranging from 20 to 50% and hydrophilic, for values <20%. All experiments were carried out in triplicate and repeated in two independent sets of experiments. The data were analyzed by using one way ANOVA followed by Newman-Keuls multiple comparison test (set at 5%).

SEM of *S. aureus* biofilms

For visualization of *S. aureus* biofilm architecture, SEM images were taken. For SEM analysis, the reference strains (*S. aureus* ATCC 35556 and *S. epidermidis* ATCC 12228) were selected. Biofilms were prepared, as described above, at selected temperatures (12 °C and 37 °C), for 24 h on polystyrene tissue plates and stainless steel chips, then washed by dipping three times in sterile PBS to remove non-adherent cells. Samples were dehydrated in ethanol-water mixtures, with increasing ethanol concentrations (65%, 75%, 85%, 95% and 100%) and finally overnight air-dried. Dehydrated specimens were coated with gold-palladium by Polaron E5100 II (Polaron Instruments Inc., Hatfield, CA). After processing, samples were observed with a Philips XL30CP scanning electron microscope in the high-vacuum mode at 15 kV (Philips, Eindhoven, Netherlands). The images were processed for display using Photoshop (Adobe Systems Inc., San Jose, CA, USA) software.

Results

Biofilm-forming ability of *S. aureus* strains

Biofilm formation, expressed as BPI, was compared with reference strains: *S. aureus* ATCC 35556 (positive control - BPIPC), *S. aureus* ATCC 12600 (reference strain e BPI12600) and *S. epidermidis* ATCC 12228 (negative control - BPINC) for each isolate. In particular, the BPI value of *S. aureus* ATCC 12600 (reference strain e BPI12600) was half the BPI value of positive control (ATCC 35556) on both surfaces tested at 37 °C (Table 1). All isolates (n.67) were defined into different categories on the basis of their BPIs values. The cutoff point for the biofilm production was the BPI value obtained by negative control on polystyrene (BPINC $\frac{1}{4}$ 0.294) and stainless steel (BPINC $\frac{1}{4}$ 0.149). *S. aureus* strains showing ability to produce biofilms were classified as weak (BPINC < *S. aureus* BPIs < BPI12600), moderate (BPI12600 < *S. aureus* BPIs < BPIPC) or strong (*S. aureus* BPIs > BPIPC). Considerable variations in biofilm-forming ability were observed between the n.67 *S. aureus* strains tested under different temperatures (12 and 37 °C) and surfaces (polystyrene and stainless steel). At 37 °C, n. 38/67 (56.7%) of *S. aureus* strains were biofilm producer in at least one tested surface. A total of n. 25/38 (65.7%) of *S. aureus* strains were biofilm producer on polystyrene whereas n. 24/38 (63.1%) were biofilm producer on stainless steel. Moreover, n. 11/38 (28.9%) of *S. aureus* strains were biofilm producers on both selected surfaces. Fig. 1 shows the ability of the *S. aureus* strains to produce biofilm on polystyrene and stainless steel at 37 °C whereas summarized results of *S. aureus* isolates from different sources are shown in Table 2 and Table 3. The majority of *S. aureus* strains which produced biofilms (n. 17/38=44.7%), were isolated from food environments. Among the *S. aureus* strains from food environments, n. 2/17 (11.7%) were classified as moderate biofilm producer (*S. aureus* 193 and *S. aureus* 194) on polystyrene whereas they were no biofilm producer on stainless steel. Anyway, at 37 °C the highest BPI value (BPI 1.019), which was greater than BPIPC (BPI 0.758), was showed on polystyrene by food isolated strain (*S. aureus* 281), although this one was a weak biofilm producer on stainless steel. At 12 °C, only one *S. aureus* strain isolated from food-handler (*S. aureus* 374) was biofilm producer and it was classified as weak biofilm producer on both selected surfaces. This strain at 37 °C was classified as moderate biofilm producer on polystyrene and weak bio-film producer on stainless steel.

Cell surface hydrophobicity: effect of temperature and association with biofilm formation

Cell surface hydrophobicity level increased with temperature. As a matter of fact, a statistically significant ($P < 0.001$) difference was found between hydrophobicity at 37 °C and those produced at 12 °C. In particular, among the 67 strains tested at 37 °C, n. 43 (64.1%) strains resulted highly hydrophobic, n. 21 (31.3%) strains moderately hydrophobic and n. 3 (4.4%) strains hydrophilic. At 12 °C, n. 21 (31.3%) strains resulted highly hydrophobic, n. 25 (37.3%) strains moderately hydrophobic and n.21 (31.3%) strains hydrophilic.

SEM analysis of *S. aureus* biofilm

Representative micrographs of biofilms produced by two reference strains (*S. aureus* ATCC 35556 and *S. epidermidis* ATCC 12228) are shown in Fig. 2. In particular, a scanning electron micrograph illustrating the appearance of thick extracellular products in strongly biofilm producer strain (*S. aureus* ATCC 35556) and the absence of those products in the non-biofilm producer strain (*Staphylococcus epidermidis* ATCC 12228) is presented. Biofilm formation by *S. aureus* ATCC 35556 was clearly observed on both polystyrene and stainless steel after 24 h incubation period at 37 °C, as shown in Fig. 2(aeb). On the contrary, at 37 °C the negative biofilm producer (*S. epidermidis* ATCC 12228) showed an absence of extracellular products on selected surfaces as shown in Fig. 2(ced).

Discussion

Microbial adhesion and biofilms are of great importance for the food industry and occur on a high variety of food contact surfaces (Di Ciccio et al., 2012; Donlan & Costerton, 2002; Flemming, Meier, & Schild, 2013; Simões, Simões, & Vieira, 2010). The biofilms enhance the ability of bacteria to survive stresses and constitute potential reservoirs for pathogens such as *S. aureus* (Abdallah, Benoliel, Drider, Dhulster, & Chihib, 2014). Several authors have reported the ability of bacteria to form biofilms on materials commonly used in the food sector, such as the stainless steel, glass, rubber, polycarbonate, polyurethane, polystyrene, polypropylene, titanium, aluminum, and ceramic (Donlan, 2001; Hamadi et al., 2014; Simões et al., 2010; Va'zquez-S'anchez, Habimana, & Holck, 2013). The contaminated surfaces, in fact, in spreading pathogens to foods is already well established in food processing, catering and domestic environment (Giaouris et al., 2014; Silva-Meira et al., 2012). The environmental conditions encountered in this sector, such as temperature, nutrient availability, surface type, pH and humidity, provide for the bacterial growth and their biofilm formation. Moreover, some authors underlined the presence of biofilms on the food contact surfaces despite the use of disinfection procedures (Gounadaki, Skandamis, Drosinos, & Nychas, 2008; Gutierrez et al., 2012; de Jesus Pimentel-Filho, Martins, Bicalho Nogueira, Cuquetto Mantovani, & Dantas Vanetti, 2014). *S. aureus* and its problem and little is known about the ability of wild *S. aureus* strains isolated from food, food-handlers and food environments to form biofilms when they are exposed to conditions simulating those in food processing plants (Leite de Souza et al., 2014). In addition, several studies have tested a limited number of food-related strains. In the present study, 3 standard type strains and 67 food-related strains were employed and the biofilm formation was compared at 12 °C and 37 °C. These two temperatures were selected by their relevance to the food industry (12 °C), as stated by Regulation (EC) n.853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on biofilm formation are recognized as a serious clinical the hygiene of foodstuffs, and in infectious disease (optimum growth temperature: 37 °C). Polystyrene and stainless steel were selected because they are the most widely used materials in the food sector. In particular, stainless steel is the most frequently used material in the construction of food processing equipments due to its mechanical strength, corrosion resistance and resistance to damage caused by the cleaning process, whereas polystyrene is one of the most commonly plastic used in the food industry, mainly for packaging (de Jesus Pimentel-Filho et al., 2014). Based on our results, considerable variations in the ability to form biofilms on polystyrene and stainless steel were shown among *S. aureus* isolates. At 37 °C the formation of biofilms by *S. aureus* occurred preferentially on polystyrene (65.7%) compared to stainless steel (63.1%). These findings are in accordance with the results of Pagedar, Singh, and Batish (2010) who suggested that hydrophobicity is also a relevant factor in the formation of biofilms by *S. aureus* strains. Moreover, in the present study only one *S. aureus* strains isolated from food-handler showed the ability to form biofilm on polystyrene and stainless steel at 12 °C. The ability of *S. aureus* to colonize surfaces at low temperatures used in the food industry may contribute to the persistence of the bacterium in food processing environments, consequently increasing cross-contamination risks. Based on our results, at 37 °C biofilm was produced at higher levels than at 12 °C. In particular, at 37 °C, the highest amount (BPI 1.019) of biofilm was formed on polystyrene by a food isolated strain (*S. aureus* 281). However, that *S. aureus* strain showed a BPI greater than BPIPC (BPI 0.758) on polystyrene, although it was a weak biofilm producer (BPI 0.198) on stainless steel. Several studies have shown that the temperature changes, which take place in both food and medical environments, affect biofilm formation (Cerca & Jefferson, 2008; Nilsson, Ross, & Bowman, 2011). Anyway, the effect of temperature changes remains unclear on the biofilm formation of *S. aureus*. Results obtained by

Va'zquez-Sa'nchez et al. (2013) on n.26 *S. aureus* isolated from seafood and n.2 *S. aureus* reference strains showed that most of the strains had a higher biofilm production at 37 °C than at 25 °C on polystyrene. Similar results were obtained by Choi, Kim, Bae, and Lee (2013). However, Pagedar et al. (2010) reported a higher cell count of the *S. aureus* biofilm at 25 °C in contrast to that obtained at 37 °C on stainless steel. Otherwise, Silva-Meira et al. (2012) assessed the biofilm formation by n.3 *S. aureus* from food services on stainless steel and polypropylene surfaces at 7 and 28 °C. The isolates of *S. aureus* revealed high capability to adhere and form biofilm on the tested surfaces in both assayed incubation temperature after three days of cultivation. These authors showed that there is no clear effect of the incubation temperature on the biofilm formation of *S. aureus*. This discrepancy may reflect the difference in experimental conditions. In fact, Oulahal et al. (2008) found that the effect of the growth temperature on the formation of *S. aureus* biofilm is affected by several environmental factors such as nutrient availability and surface type. Biofilm formation depends on the characteristics of surface, the bacterial cell, the growth medium and other environmental factor (de Jesus Pimentel-Filho et al., 2014). On the other hand, in a study carried out by Rode et al. (2007), the biofilm formation on polystyrene was estimated for n.10 *S. aureus* strains incubated at various temperatures and the results indicated that biofilm production is higher at sub-optimal temperatures. In addition, the authors also found that the effect of temperature on biofilm formation was dependent on the presence of glucose and NaCl (Rode et al. 2007). Finally, in another investigation performed in 2014 by Va'zquez-Sa'nchez, Cabo, Ibusquiza, and Rodríguez-Herrera (2014), the biofilm-forming ability of *S. aureus* strains (n.26) isolated from fish products was assessed on stainless steel at 25 °C. Most strains showed a biofilm-forming ability higher than the reference strain. Some studies have found that the biofilm formation on hydrophobic substrata occurred to a greater extent than that on hydrophilic ones (Cerca, Pier, Vilanova, Oliveira, & Azeredo, 2005; Pagedar et al., 2010). Anyway, Da Silva-Meira et al. (2012) have stated that stainless steel (hydrophilic) and polystyrene (hydrophobic) have no significant effect on the biofilm formation of *S. aureus*. Besides hydrophobicity and surface tension parameters, the surface roughness has been found as an essential factor affecting biofilm formation including those of *P. aeruginosa* and *S. aureus* (Arnold & Bailey, 2000; Giaouris et al., 2014; de Jesus Pimentel-Filho et al., 2014; Katsikogianni, Spiliopoulou, Dowling, & Missirlis, 2006; Lee et al., 2013; Tang et al., 2011). As far as the bacterial cell hydrophobicity is concerned, our results suggest that growth temperature may influence the hydrophobicity in *S. aureus*. In particular, the cell surface hydrophobicity level increases with temperature. A statistically significant difference, in fact, was found between hydrophobicity at 37 °C and those showed at 12 °C. In order to evaluate the architecture of the biofilms, the SEM analysis on *S. aureus* strains was carried out. The scanning electronic microscopy allows the observation of bacteria/ surface interaction and may be used as a semi-quantitative technique. Thus, in this study to confirm the presence of an extracellular polysaccharide and glycoprotein network layer, the SEM analysis was used. Biofilm formation by *S. aureus* ATCC 35556 was clearly observed on both surfaces after 24 h incubation period at 37 °C as shown in Fig. 2aeb. An important factor observed was the production of a considerable amount of exopolysaccharide matrix by positive reference strain (*S. aureus* ATCC 35556) on both polystyrene and stainless steel while the negative reference strain (*S. epidermidis* ATCC 12228) did not produce extracellular polysaccharides. Moreover, at 37 °C, biofilm exhibited a complex organization, in term of cell number and extracellular polysaccharides produced. In particular, the polystyrene surface was totally colonized by the positive reference strain (ATCC 35556) and cells were embedded in a large thick layer while the stainless steel was partly colonized and cells were aggregated in clusters (Fig. 2aeb). Finally, at 37 °C negative biofilm producer (ATCC 12228) showed an absence of network layers on both surfaces such as polystyrene and

stainless steel (Fig. 2ced). Our study attempted to investigate the biofilm formation, expressed as BPI, by wild isolates of *S. aureus* and to correlate the BPI values with the SEM images. It can be concluded that some assayed isolates of *S. aureus* presented highlighted capacity to form biofilm on polystyrene and/ or stainless steel surfaces. Further studies focusing on the capability of *S. aureus* isolates from food services to form biofilm when they are exposed to conditions simulating those in food processing plants are needed to confirm these initial findings.

Conclusions

Microbial biofilms enhance the ability of bacteria to survive stress and can cause problems in the food industry. In fact, the persistence of biofilm on food contact surfaces, and equipment, may constitute a continuous source of contamination. Our results suggest that the biofilm formation of *S. aureus* is influenced by environmental conditions relevant for the food industry such as temperature and cell surface properties. In the present study, several *S. aureus* strains from food, food environments and food-handlers were biofilm producers in at least one assay. This fact is of public health concern because it indicates a potential source for persistence of *S. aureus* contamination in the food industry. Based on our results, in fact, the majority of

S. aureus strains that showed the ability to form biofilm on the tested surfaces were isolated from food environments. The prevention and control of *S. aureus* biofilms in food processing environment should be based on an integrated efforts. A regular cleaning and disinfection of all equipment and food contact surfaces, also during processing, and an ambient temperature of not more than 12 °C in the food processing plants are essential to avoid or reduce the risk of the

S. aureus biofilm formation in the food industry. In addition, the processing equipments should be designed with high standards of hygiene in mind. In conclusion, in order to reduce the microbiological risk related to the biofilm formation, a better understanding of how *S. aureus* attaches and form biofilm when it is exposed to conditions simulating those in food processing plants is needed. Moreover, it is of importance to improve hygienic conditions to control the emergence of biofilms in the food sector.

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References

- Abdallah, M., Benoliel, C., Drider, D., Dhulster, P., & Chihib, N. E. (2014). Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. *Archives of Microbiology*, 196, 453e472.
- Almeida, R. R., & Oliver, S. P. (2001). Interaction of coagulase-negative *Staphylococcus* species with bovine mammary epithelial cells. *Microbial Pathogenesis*, 31, 205e212.
- Archer, N. K., Mazaitis, M. J., Costerton, J. W., Leid, J. G., Powers, M. E., & Shirtliff, M. E. (2011). *Staphylococcus aureus* biofilms Properties, regulation and roles in human disease. *Virulence*, 2(5), 445e459.
- Arnold, J. W., & Bailey, G. W. (2000). Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. *Poultry Science*, 79, 1839e1845.

Atshan, S. S., Shamsudin, M. N., Lung, L. T., Sekawi, Z., Ghaznavi-Rad, E., & Pei, C. P. (2012). Comparative characterisation of genotypically different clones of MRSA in the production of biofilms. *Journal of Biomedicine and Biotechnology*, 7. Article ID 417247.

Balaban, M. S., & Rasooly, A. (2000). Staphylococcal enterotoxins. *International Journal of Food Microbiology*, 61, 1e10.

Braga, L. C., Shupp, J. W., Cummings, C., Jett, M., Takahashi, J. A., Carmo, L. S., et al. (2005). Pomegranate extract inhibits *Staphylococcus aureus* growth and subsequent enterotoxin production. *Journal of Ethnopharmacology*, 96, 335e339.

Cerca, N., & Jefferson, K. K. (2008). Effect of growth conditions on poly-N-acetylglucosamine expression and biofilm formation in *Escherichia coli*. *FEMS Microbiology Letters*, 283, 36e41.

Cerca, N., Pier, G. B., Vilanova, M., Oliveira, R., & Azeredo, J. (2005). Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. *Research in Microbiology*, 156, 506e514.

Choi, N. Y., Kim, B. R., Bae, Y. M., & Lee, S. Y. (2013). Biofilm formation, attachment, and cell hydrophobicity of foodborne pathogens under varied environmental conditions. *Journal of the Korean Society for Applied Biological Chemistry*, 56, 207e220.

Costerton, J. W. (1999). Introduction to biofilm. *International Journal of Antimicrobial Agents*, 11, 217e221.

Costerton, J. W., Geesey, G. G., & Cheng, K. J. (1978). How bacteria stick. *Scientific American*, 238, 86e95.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W., & Götz, F. (1999). The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infection and Immunity*, 67, 5427e5433.

Cucarella, C., Tormo, M. A., Knecht, E., Amorena, B., Lasa, I., Foster, T. J., et al. (2002). Expression of the biofilm-associated protein interferes with host protein receptors of *Staphylococcus aureus* and alters the infective process. *Infection and Immunity*, 70, 3180e3186.

De Jesus Pimentel-Filho, N. de F., Martins, M. C., Bicalho Nogueira, G., Cuquetto Mantovani, H., & Dantas Vanetti, M. C. (2014). Bovicin HC5 and nisin reduce *Staphylococcus aureus* adhesion to polyethylene and change the hydrophobicity profile and Gibbs free Energy of adhesion. *International Journal of Food Microbiology*, 190, 1e8.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., et al. (2008). Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. *Journal of Applied Microbiology*, 104, 1552e1561.

Di Ciccio, P., Conter, M., Zanardi, E., Ghidini, S., Vergara, A., Paludi, D., et al. (2012). *Listeria monocytogenes*: biofilm in food processing. *Italian Journal of Food Science*, 24(3), 203e213.

Donlan, R. M. (2001). Biofilms and device-associated infections. *Emerging Infectious Diseases*, 7, 277e281.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15, 167e193.

Flemming, H. C., Meier, M., & Schild, T. (2013). Mini-review: microbial problems in paper production. *Biofouling*, 29, 683e696.

Fox, L. K., Zadoks, R. N., & Gaskins, C. T. (2005). Biofilm production by *Staphylococcus aureus* associated with intramammary infection. *Veterinary Microbiology*, 107, 295e299.

Giaouris, E., Chorianopoulos, N., & Nychas, G. J. (2005). Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica enteritidis* PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection*, 68, 2149e2154.

Giaouris, E., Heir, E., He'braud, M., Chorianopoulos, N., Langsrud, S., Møretrø, T., et al. (2014). Attachment and biofilm formation by foodborne bacteria in meat processing environments: causes, implications, role of bacterial interactions and control by alternative novel methods. *Meat Science*, 97, 298e309.

Gibson, H., Taylor, J. H., Hall, K. E., & Holah, J. T. (1999). Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial bio-films. *Journal of Applied Microbiology*, 87(1), 41e48.

Gounadaki, A. S., Skandamis, P. N., Drosinos, E. H., & Nychas, G. J. (2008). Microbial ecology of food contact surfaces and products of small-scale facilities producing traditional sausages. *Food Microbiology*, 25, 313e323.

Gutierrez, D., Delgado, S., V'azquez-Sa'nchez, D., Martínez, B., Lo'pez Cabo, M., Rodríguez, A., et al. (2012). Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food Industry surfaces. *Applied and Environmental Microbiology*, 78(24), 8547e8554.

Hamadi, F., Asserne, F., Elabed, S., Bensouda, S., Mabrouki, M., & Latrache, H. (2014). Adhesion of *Staphylococcus aureus* on stainless steel treated with three types of milk. *Food Control*, 38, 104e108.

Hamadi, F., Latrache, H., Mabrouki, M., Elchmari, A., Outzourhit, A., Ellouali, M., et al. (2005). Effect of pH on distribution and adhesion of *Staphylococcus aureus* to glass. *Journal of Adhesion and Science of Technology*, 19, 73e85.

Katsikogianni, M., Spiliopoulou, I., Dowling, D. P., & Missirlis, Y. F. (2006). Adhesion of slime producing *Staphylococcus epidermidis* strains to PVC and diamond-like carbon/silver/fluorinated coatings. *Journal of Materials Science: Materials in Medicine*, 17, 679e689.

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology*, 85, 227e236.

Lee, S. H. I., Mangolin, B. L. C., Gonçalves, J., Neeff, D. V., Silva, M. P., Cruz, A. G., et al. (2013). Biofilm-producing ability of *Staphylococcus aureus* isolates from Brazilian dairy farms. *Journal of Dairy Science*, 97, 1812e1816.

Leite de Souza, E., Silva Meira, Q. G., de Medeiros Barbosa, I., Alves Aguiar Athayde, A. J., da Conceição, M. L., & de Siqueira Júnior, J. P. (2014). Biofilm formation by *Staphylococcus aureus* from food contact surfaces in a meat-based broth and sensitivity to sanitizers. *Brazilian Journal of Microbiology*, 45, 67e75.

Malheiros, P. S., Passos, C. T., Casarin, L. S., Serraglio, L., & Tondo, E. C. (2010). Evaluation of growth and transfer of *Staphylococcus aureus* from poultry meat to surfaces of stainless steel and polyethylene and their disinfection. *Food Control*, 21, 298e301.

Marques, S. C., Rezende, J. D. G. O. S., Aparecida de Freitas Alves, L., Silva, B. C., Alves, E., Ronaldo de Abreu, L., et al. (2007). Formation of biofilms by *Staphylococcus aureus* on stainless steel and glass surface and its resistance to some selected chemical sanitizers. *Brazilian Journal of Microbiology*, 38, 538e543.

Mattila Sandholm, T., & Wirtanen, G. (1992). Biofilm formation in the industry: a review. *Food Reviews International*, 8(4), 573e603.

Mattos-Guaraldi, A. L., Formiga, L. C. D., & Andrade, A. F. B. (1999). Cell surface hydrophobicity of sucrose fermenting and nonfermenting *Corynebacterium diphtheriae* strains evaluated by different methods. *Current Microbiology*, 38, 37e42.

Nilsson, R. E., Ross, T., & Bowman, J. P. (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. *International Journal of Food Microbiology*, 150, 14e24.

Nostro, A., Blanco, A. R., Cannatelli, M. A., Enea, V., Flamini, G., Morelli, I., et al. (2004). Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. *FEMS Microbiology Letters*, 230, 191e195.

Oulahal, N., Brice, W., Martial, A., & Degraeve, P. (2008). Quantitative analysis of survival of *Staphylococcus aureus* or *Listeria innocua* on two types of surfaces: polypropylene and stainless steel in contact with three different dairy products. *Food Control*, 19, 178e185.

Pagedar, A., & Singh, J. (2012). Influence of physiological cell stages on biofilm formation by *Bacillus cereus* of dairy origin. *International Dairy Journal*, 23, 30e35.

Pagedar, A., Singh, J., & Batish, V. K. (2010). Surface hydrophobicity, nutritional contents affect *Staphylococcus aureus* biofilms and temperature influences its survival in preformed biofilms. *Journal of Basic Microbiology*, 50, S98eS106.

Regulation (EC) n.853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. G.U.L. n.139 del 30/04/2004.

Rode, T. M., Langsrud, S., Holck, A., & Moretto, T. (2007). Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. *International Journal of Food Microbiology*, 116, 372e383.

Seidl, K., Stucki, M., Ruegg, M., Goerke, C., Wolz, C., Harris, L., et al. (2008). *Staphylococcus aureus* CcpA affects biofilm formation. *Infection and Immunity*, 76, 2044e2050.

Silva-Meira, Q. G., Medeiros-Barbosa, I., Athayde, A. J. A. A., Siqueira-Júnior, J. P., & Souza, E. L. (2012). Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers. *Food Control*, 25, 469e475.

Simoes, M., Simoes, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT-Food Science Technology*, 43, 573e583.

Tang, L., Pillai, S., Revesbech, N. P., Schramm, A., Bischoff, C., & Meyer, R. L. (2011). Biofilm retention on surfaces with variable roughness and hydrophobicity. *Biofouling*, 27, 111e121.

Vasudevan, P., Nair, M. K., Annamalai, T., & Venkitanarayanan, K. S. (2003). Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. *Veterinary Microbiology*, 92, 179e185.

Va'zquez-S'anchez, D., Cabo, M. L., Ibusquiza, P. S., & Rodríguez-Herrera, J. J. (2014). Biofilm-forming ability and resistance to industrial disinfectants of *Staphylococcus aureus* isolated from fishery products. *Food Control*, 39, 8e16.

Va'zquez-S'anchez, D., Habimana, O., & Holck, A. (2013). Impact of food-related environmental factors on the adherence and biofilm formation of natural *Staphylococcus aureus* isolates. *Current Microbiology*, 66, 110e121.

Vlkova, H., Babak, V., Seydlova, R., Pavlik, I., & Schlegelova, J. (2008). Biofilms and hygiene on dairy farms and in the dairy industry: sanitation chemical products and their effectiveness on biofilmsea Review. *Czech Journal of Food Science*, 26, 309e323.

Wirtanen, G., Husmark, U., & Matilla-Sandholm, T. (1996). Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. *Journal of Food Protection*, 59(7), 727e733.