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

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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 o 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 o 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. 17/38e44.7%), were isolated from food envi- ronments. At 12 o 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 differ- ence ($P < 0.001$) was found between hydrophobicity at 37 o C and 12 o 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 com- munities 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 consid- ered as an health hazard. Microbial biofilms, in fact, may contain a considerable number of both spoilage and pathogenic microor- ganisms (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 (Vlkova, 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 subtrata, 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, & Matilla- 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, bio- film 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 sur- faces, in fact, poses a serious risk of food contamination (Gibson, Taylor, Hall, & Holah, 1999). It has been frequently found in sur- faces 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 (Gutierezz et al., 2012). S. aureus can produce a multilayered biofilm embedded within a glycocalyx or slime layer with heterogenous 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 pro- cessing. 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 in- teractions between microorganisms and food processing equip- ment is required. Regarding these aspects, this study was carried out with the aim of evaluating the ability of S. aureus strains iso- lated from food, food environments and food handlers, to form biofilms on polystyrene and stainless steel surfaces under different temperatures: 12 and 37 o 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 examinated 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, & Go€tz, 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 oC 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 o C. These two temperatures were selected by their relevance to the food industry (12 o C) and in infectious disease (37 o 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 hy- drophobic 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 oC. Cultures of S. aureus were prepared, from overnight TSA- growth, in TSB by incubating at selected

temperatures: 12 and 37 oC. Cultures were then washed three times with phosphate-buffered saline (PBS; pH 7.3) (SigmaeAldrich 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 Shimadsu). Three milliliters (mL) of the stan- dardized inocula were then added to polystyrene tissue culture plates (35 mm diameter) and stainless steel chips. Samples were then incubated at 12 o C and at 37 oC. After 24 h incubation, non-adherent cells were removed by dipping each sample three times in sterile PBS. Samples were fixed at 60 o 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 ml 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 mean biofilm surface (mm2)-1] 1000. Two independent sets of all ex- periments were performed in triplicate.

Cell surface hydrophobicity assay

S. aureus hydrophobicity was evaluated, at selected tempera- tures: 12 and 37 o 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 (SigmaeAldrich). After 1-min agitation by vortexing, the phases were allowed to separate for 15 min at room tempera-ture. The results were expressed as the proportion of the cells which were excluded from the aqueous phase, determined by the equation as follows: [(A0 A)A-0 1] 100, where A0 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 NewmaneKeuls 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 o Ce37 oC), 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 etha- nolewater mixtures, with increasing ethanol concentrations (65%, 75%, 85%, 95% and 100%) and finally overnight air-dried. Dehy- drated specimens were coated with goldepalladium 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 photo- shop (Adobe Systems Inc., San Jose, CA, USA) software.

Results

Biofilm-forming ability of S. aureus strains

Biofilm formation, expressed as BPI, was compared with refer- ence 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 o 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 ob- tained by negative control on polystyrene (BPINC ¼ 0.294) and stainless steel (BPINC ¼ 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 oC) and surfaces (polystyrene and stainless steel). At 37 oC, n. 38/67 (56.7%) of S. aureus strains were biofilm producer in at least one tested sur- face. 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 o C whereas summarized re- sults of S. aureus isolates from different sources are shown in Table 2 and Table 3. The majority of S. aureus strains which pro- duced biofilms (n. 17/38e44.7%), were isolated from food envi- ronments. 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 o 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 o 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 o C was clas- sified 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 oC and those produced at 12 o C. In particular, among the 67 strains tested at 37 o 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 oC, 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 refer- ence 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 for- mation by S. aureus ATCC 35556 was clearly observed on both polystyrene and stainless steel after 24 h incubation period at 37 o C, as shown in Fig. 2(aeb). On the contrary, at 37 oC 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; Simo~es, Simo~es, & Vieira, 2010). The biofilms enhance the ability of bacteria to survive stresses and consituite 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; Simoes 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 for- mation. Moreover, some authors underlined the presence of bio- films on the food contact surfaces despite the use of disinfection procedures (Gounadaki, Skandamis, Drosinos, & Nychas, 2008; Gutie'rrez et al., 2012; de Jesus Pimetel-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, n.3 standard type strains and n.67 food-related strains were employed and the biofilm formation was compared at 12 o C and 37 oC. These two temperatures were selected by their relevance to the food industry (12 oC), 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 oC). 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 re- sults, considerable variations in the ability to form biofilms on polystyrene and stainless steel were shown among S. aureus iso- lates. At 37 oC 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 hydropho- bicity 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 o 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 o C biofilm was produced at higher levels than at 12 oC. In particular, at 37 oC, the highest amount (BPI 1.019) of biofilm was formed on polystyrene by a food isolated strain (S. aureus 281). However, that S. aureusstrain 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 re- mains 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 o C than at 25 o 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 oC in contrast to that ob- tained at 37 o 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 oC. 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 pres- ence of glucose and NaCl (Rode et al. 2007). Finally, in another investigation performed in 2014 by Va'zquez-S'anchez, 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 o 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 for as the bacterial cell hydrophobicity is concerned, our results suggest that growth temperature may influence the hy- drophobicity in S. aureus. In particular, the cell surface hydropho- bicity level increases with temperature. A statistically significant difference, in fact, was found between hydrophobicity at 37 o C and those showed at 12 o 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 tech- nique. 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 o C as shown in Fig. 2aeb. An important factor observed was the pro- duction 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 poly- saccharides. Moreover, at 37 oC, biofilm exhibited a complex or- ganization, 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 oC 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 con- ditions 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 oC 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 emer- gence of biofilms in the food sector.

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