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# *In vitro* biofilm formation ability of staphylococci under different growth conditions

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**ABSTRACT**

**The understandings of the various elements that influence the dynamics of biofilms formation are important to contribute to the knowledge of *in vitro* biofilm formation by staphylococci. The aim of this study was to analyze the effect of different growth conditions on biofilm formation by staphylococci. A total of 102 *Staphylococcus* strains, including 60 isolates of coagulase-positives (CoPS) obtained from food and clinical samples and 42 isolates of coagulase-negatives (CoNS) isolated from food, were evaluated. The effects of medium composition on biofilm formation were tested in tryptic soy broth (TSB) with varying concentrations of: Glucose [1% (TSB-1G); 5% (TSB-5G); 10% (TSB-10G)], 0.9% sodium chloride (TSB-NaCl), combination of 5% glucose and 0.9% sodium chloride (TSB-5G/NaCl), or 12.5% of rabbit plasma (TSB-RP). The effects of incubation temperatures (25, 35 and 40°C) was also investigated. The addition of glucose (from 1.0 to 10.0%) did not significantly affect the ability of the food-related CoNS and CoPS strains to adhere on the microplates. Interestingly, clinically-related CoPS strains had a higher rate of strong biofilm formed in TSB-RP (52.94%) (p<0.001). The TSB/NaCl and TSB-RP showed negative and positive effect on the biofilm formation in food-related CoNS strains, respectively. Temperatures of 25 and 40°C had positive effects on the biofilm formation of food-related CoPS strains. The study describes models that could provide relevant insights in staphylococcal biofilm formation for food and clinical research. The present findings also highlighted the need for a careful selection of the assay conditions.**



## INTRODUCTION

*Staphylococcus* species are ubiquitously distributed; they have been isolated from healthy and hospitalized individuals, foods and animals (Sorum and L'Abee-Lund, 2002; Wisplinghoff et al., 2004; Malik et al., 2005). *Staphylococci* can cause food poisoning by releasing enterotoxins into food. They also can cause many forms of infection in humans and other animals, such as: superficial skin lesions and localized abscesses in other sites, deep-seated infections, toxic shock syndrome by release of super-antigens into the blood stream, urinary tract infections and biofilm-associated infection (Cramton et al., 1999; Balaban and Rasooly, 2000; Götz, 2002; Pinchuk et al., 2010).

Members of the *Staphylococcus* genus are divided into two groups: the coagulase-positive staphylococci (CoPS) and the coagulase-negative staphylococci (CoNS) (Lowy, 1998). The CoPS comprises seven species: *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus delphini*, *Staphylococcus intermedius*, *Staphylococcus schleiferi, Staphylococcus coagulans*, *Staphylococcus pseudointermedius* and *Staphylococcus*

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*lutrae* (Euzéby, 2015). Among the CoPS species, *S. aureus* is recognized as one of the major causes of hospital-acquired infection of surgical wounds and causes food poisoning (Moura et al., 2012; Osman et al., 2015). Over more than 50 species of staphylococci belong to CoNS, which includes: *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus warneri*, *Staphylococcus hominis*, *Staphylococcus capitis*, *Staphylococcus chromogenes*, *Staphylococcus schleiferi* and *Staphylococcus simulans* (Euzéby, 2015). *S. epidermidis* is the most important CoNS species and is the major cause of infections associated with prosthetic devices and catheters. Still, some CoNS species are a component of the natural microbiota of food, acting as starters involved in the development and stability of the red color and flavor in fermented meat products (Landeta et al., 2013). Although enterotoxins are related mainly by CoPS species, some studies have demonstrated that CoNS strains can also carry the enterotoxins genes (Moura et al., 2012).

Biofilm formation on biotic or abiotic surfaces of medical devices and food processing constitutes a serious problem for public health's concern. Biofilms constitute potential reservoirs for pathogens, which serve as a continuous source of infections and cross-contaminations (Costerton et al., 1999; Abdallah et al., 2014). Many hospital-acquired infections are associated with biofilm on either native tissues (for example, cartilage, bone) or implanted biomaterials (for example, catheters, orthopedic devices, needles, endoscopes) (Cassat et al., 2007; Weber et al., 2013). *S. epidermidis* and *S. aureus* are the most frequent causes of biofilm-associated infections on indwelling medical devices (Cramton et al., 1999; Götz, 2002; Abdallah et al., 2014). In food processing, staphylococcal biofilms are a potential source of product contamination and may lead to food spoilage and serious fouling problems in equipment (Pinto et al., 2015).

Some environmental parameters have been showed to affect biofilm formation. Nevertheless, there is no consensus and some studies reported that bacterial adhesion and biofilm formation depended upon the bacterial species, the nature of the surface, the growth medium and the association of several environmental conditions (Pompermayer and Gaylarde, 2000; Jerônimo et al., 2012). It also has been reported that nutrient-rich growth media and high temperatures may enhance biofilm formation, due to increasing the expression level of polysaccharide intercellular adhesin (PIA), as well as it is demonstrated that the gene *rbf* regulates biofilm formation in response to glucose and salt (Lim et al., 2004). Moreover, molecules of the human matrix are required for multicellular aggregation in clinical isolates (Chen et al., 2012; Cardile et al., 2014). Many studies on biofilm formation have been performed using clinically relevant bacteria, such as *S. aureus* strains (Lim et al., 2004; Stanley and Lazazzera, 2004; Herrera et al., 2007; Rode et al., 2007; Di Ciccio et al., 2015); however, little is known about the biofilm-forming abilities of other staphylococci, for example, CoNS is isolated from food.

Therefore, it is important to identify the conditions under which microorganisms can survive and produce biofilm in food processing and health care sectors (Lim et al., 2004; Rode et al., 2007; Agarwal and Jain, 2013). The aim of this study was to analyze the effect of different growth conditions that contribute to the current knowledge of *in vitro* biofilm formation by staphylococci.

## MATERIALS AND METHODS

### Bacterial strains

A total of 102 staphylococci (60 isolated CoPS collected from food and clinical samples, and 42 isolates CoNS isolated from food samples) were obtained from the culture collection of the Department of Microbiology, Universidade Federal do Rio Grande do Sul, from March to November 2014 (Table 1). The isolates were identified as genus, species and antimicrobial profiles in previous studies (Antunes et al., 2011; Moura et al., 2012; Martins et al., 2013).

Prior to each experiment, an aliquot of frozen bacterial cells was recovered onto tryptic soy agar (TSA, Oxoid, Thermo Fisher Scientific, Basingstoke, UK), and incubated at 35°C for 24 h. For the experimental procedures, a loopful of the TSA was dispersed in 0.9% saline solution (w/v) sterile until it matched to 0.5 McFarland turbidity standards (approximately $1\times10^8$ CFU/mL).

### Effect of medium composition on biofilm formation

Bacterial strains were propagated in tryptic soy broth (TSB, Oxoid, Thermo Fisher Scientific, Basingstoke, UK). Biofilm formation was analyzed individually for each strain by using the microplate assay with various components in TSB, such as glucose, chloride or plasm, which may be present in foods, beverages, food-processing facilities and humans. For tests in the static biofilm model, six different supplementations of TSB were tested: TSB-1G (TSB was supplemented with 1% glucose, Synth, São Paulo, SP, Brazil); TSB-5G (TSB was supplemented with 5% glucose); TSB-10G (TSB was supplemented with 10% glucose); TSB-5G/NaCl (TSB was supplemented with a combination of 5% glucose and 0.9% sodium chloride, Cromato, Diadema, SP, Brazil); TSB-NaCl (TSB was supplemented with 0.9% sodium chloride); TSB-RP (TSB was supplemented with 12.5% of rabbit plasma, Laborclin, Pinhais, PR, Brazil).

Biofilm formation was evaluated on polystyrene

**Table 1.** *Staphylococcus* spp. used in the study.

| Coagulase phenotype | Strains | Number of strains | Origin | Reference |
|---|---|---|---|---|
| CoNS | *S. saprophyticus* | 23 | Food[1] | Moura et al. (2012) |
| | *S. carnosus* | 9 | Food[1] | Martins et al. (2013) |
| | *S. vitulinus* | 4 | Food[1] | Martins et al. (2013) |
| | *S. cohnii* | 4 | Food[1] | Martins et al. (2013) |
| | *S. equorum* | 1 | Food[1] | Martins et al. (2013) |
| | *Staphylococcus* spp. | 1 | Food[1] | Martins et al. (2013) |
| | Total | **42** | | |
| CoPS | *S. aureus* | 34 | Clinical[2] | Antunes et al. (2011) |
| | *S. aureus* | 23 | Food[3] | Martins et al. (2013) |
| | *S. delphini* | 1 | Food[3] | Martins et al. (2013) |
| | *S. schleiferi* | 1 | Food[3] | Martins et al. (2013) |
| | *S. hyicus* | 1 | Food[3] | Martins et al. (2013) |
| | Total | **60** | | |

[1]Black pudding chilled; [2]Central Venous Catheter; [3]Frozen raw poultry meat.

microplates following the method described by Christensen et al. (1985), with some improvements. Each well of the sterile 96-well flat-bottomed polystyrene microplates was filled with 180 µl of the appropriate culture medium and 20 µl of bacterial inoculum (containing approximately $10^8$ CFU/mL). The microplates were incubated at 35°C for 18 h. The experiments were performed at least eight times for each strain. *S. epidermidis* American Type Culture Collection 35984 was used as the positive control. Wells that contained culture medium without bacteria served as the negative control.

### Effect of temperature on *in vitro* biofilm formation

In order to determine how biofilm density changed over different temperatures, three different temperatures on biofilm formation ability of CoNS and CoPS strains on the microplates was used: 25°C (temperature used in Brazilian Food and Nutrition Services), 35°C (optimal temperature for staphylococci growth) and 40°C (optimum temperature for enterotoxin production) (Vandenbosch et al., 1973). Biofilm formation was determined following the method described by Christensen et al. (1985), with some improvements. Each well of the sterile 96-well flat-bottomed polystyrene microplates were filled with 180 µl of TSB-5G/NaCl and 20 µl of bacterial inoculum (approximately $10^8$ CFU/mL). We chose to use TSB-5G/NaCl medium, since the strains were found to form a similar profile of biofilm production. The microplates were incubated at different temperatures for 18 h. The experiments were performed at least eight times for each temperature evaluated. *S. epidermidis* ATCC 35984 was used as the positive control. Wells that contained only TSB-5G/NaCl served as the negative control.

### Quantification of biofilm formation

Biofilm was quantified by the crystal violet staining method, with some improvements (Stepanovic et al., 2000). The optical density (OD) was measured at 450 nm ($OD_{450}$) in a spectrophotometer (Anthos 2010 Microplates Reader, Austria). Wells containing only the medium were used as background controls. The OD of each strain was determined by comparing the arithmetic mean of the absorbance of the wells with the mean absorbance of the negative controls ($OD_{nc}$). The strains were categorized based on their OD: non-biofilm producers ($OD_s \leq OD_{nc}$), weak biofilm producers ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm producers ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$) and strong biofilm producers ($4.OD_{nc} < OD_s$).

### Statistical analyses

Significance of the association between biofilm formation, effects of culture medium and incubation temperature were assessed by ANOVA followed by the Tukey *post hoc* test. All the variables tested were normally distributed. The results were processed using the Portable Statistic 12.0. Results were considered statistically significant when $p<0.05$.

## RESULTS

### Influence of culture's conditions on *in vitro* biofilm formation

Table 2 shows the biofilm formation ability of CoNS and

**Table 2.** Evaluation of biofilm formation capacity of coagulase-negative (CoNS) and coagulase-positive (CoPS) staphylococci isolated from clinical and food samples growing in TSB medium under different compounds.

| Medium | Biofilm status[1] | Number (%) of biofilm former | | | Total (%) |
|---|---|---|---|---|---|
| | | CoNS | CoPS | | |
| | | Food (*n* =42) | Food (*n* = 26) | Clinical (*n* =34) | |
| TSB-1G | N | 7 (16.67)$^{a,b}$ | 0$^{a}$ | 0 $^{d,e,f}$ | 7 (6.86) $^{b}$ |
| | W | 21 (50) $^{a,b}$ | 2 (7.69) $^{a}$ | 26 (76.47) $^{d,e,f}$ | 49 (48.04) $^{b}$ |
| | M | 13 (30.95) $^{a,b}$ | 1 (3.85) $^{a}$ | 8 (23.53) $^{d,e,f}$ | 22 (21.57) $^{b}$ |
| | S | 1 (2.38) $^{a,b}$ | 23 (88.46) $^{a}$ | 0 $^{d,e,f}$ | 24 (23.53) $^{b}$ |
| TSB-5G | N | 8 (19.05)$^{a,b}$ | 0$^{a}$ | 0 $^{b,c}$ | 8 (7.84) $^{a,b}$ |
| | W | 20 (47.62) $^{a,b}$ | 4 (7.69) $^{a}$ | 13 (38.24) $^{b,c}$ | 37 (36.27) $^{a,b}$ |
| | M | 13 (30.95) $^{a,b}$ | 0 $^{a}$ | 16 (47.06) $^{b,c}$ | 29 (28.43) $^{a,b}$ |
| | S | 1 (2.38) $^{a,b}$ | 22 (84.62) $^{a}$ | 5 (14.71) $^{b,c}$ | 28 (27.45) $^{a,b}$ |
| TSB-10G | N | 8 (19.05)$^{a,b}$ | 0 $^{a}$ | 0 $^{c,e}$ | 8 (7.84) $^{a,b}$ |
| | W | 14 (33.33) $^{a,b}$ | 1 (3.85) $^{a}$ | 16 (47.06) $^{c,e}$ | 31 (30.39) $^{a,b}$ |
| | M | 18 (42.86) $^{a,b}$ | 1 (3.85) $^{a}$ | 18 (52.94) $^{c,e}$ | 37 (36.27) $^{a,b}$ |
| | S | 2 (4.76) $^{a,b}$ | 24 (92.31) $^{a}$ | 0 $^{c,e}$ | 26 (25.49) $^{a,b}$ |
| TSB-NaCl | N | 10 (23.81)$^{a,b}$ | 0$^{a}$ | 3 (8.82)$^{c,f}$ | 13 (12.75) $^{b}$ |
| | W | 23 (54.76) $^{a,b}$ | 3 (11.54) $^{a}$ | 21 (61.76) $^{c,f}$ | 47 (46.08) $^{b}$ |
| | M | 7 (16.67) $^{a,b}$ | 3 (11.54) $^{a}$ | 6 (17.65) $^{c,f}$ | 16 (15.69) $^{b}$ |
| | S | 2 (4.76) $^{a,b}$ | 20 (76.92) $^{a}$ | 4 (11.76) $^{c,f}$ | 26 (25.49) $^{b}$ |
| TSB-5G/NaCl | N | 4 (9.52)$^{a,b}$ | 0 $^{a}$ | 0 $^{a,b}$ | 4 (3.92) $^{a,b}$ |
| | W | 21 (50) $^{a,b}$ | 2 (7.69) $^{a}$ | 9 (26.47) $^{a,b}$ | 32 (31.37) $^{a,b}$ |
| | M | 15 (35.71) $^{a,b}$ | 3 (11.54) $^{a}$ | 14 (41.18) $^{a,b}$ | 32 (31.37) $^{a,b}$ |
| | S | 2 (4.76) $^{a,b}$ | 21 (80.77) $^{a}$ | 11 (32.35) $^{a,b}$ | 34 (33.33) $^{a,b}$ |
| TSB-RP | N | 3 (7.14)$^{a}$ | 0 $^{a}$ | 0 $^{a}$ | 3 (2.94) $^{a}$ |
| | W | 17 (40.48) $^{a}$ | 1 (3.85) $^{a}$ | 1 (2.94) $^{a}$ | 19 (18.63) $^{a}$ |
| | M | 18 (42.86) $^{a}$ | 7 (26.92) $^{a}$ | 15 (44.12) $^{a}$ | 40 (39.22) $^{a}$ |
| | S | 4 (9.52) $^{a}$ | 18 (69.23) $^{a}$ | 18 (52.94) $^{a}$ | 40 (39.22) $^{a}$ |

[1]N, Non-biofilm producers; W, weak biofilm producers; M, moderate biofilm producers; S, strong biofilm producers.
For the same test and bacteria, the same letters do not differ statistically ($p>0.05$).

CoPS strains in different media. In general, food-related CoPS strains showed much higher rates of strong biofilm producer (69.23 to 92.31%) under all conditions tested, than clinical-related CoPS strains (52.94 to 11.76%), and food-related CoNS strains (9.52 to 2.52%). Clearly, all the different media evaluated did not significantly affect the biofilm formation in the food-related CoNS strains ($p>0.05$). In addition, the supplementation of 12.5% rabbit plasma in TSB significantly increases the strong biofilm producer profile in the clinical strains ($p<0.001$).

The addition of glucose in a range from 1.0 to 10.0% in TSB did not significantly affect the ability of the food-related CoNS and CoPS strains to adhere on the microplates (Table 2). A slight increase in the percentage of strains moderate biofilm producer (42.86 %) was detected in TSB-10G, in contrast to TSB-1G (30.95%) and TSB-5G (30.95%); however, the results were not statistically significant ($p>0.05$). The optimal glucose concentration for biofilm formation for food-related CoPS was 10%, where the highest percentage of strong biofilm formed (92.31%) was observed. On the other hand, in clinically-related CoPS strains, the addition of 5% glucose, exhibited significant differences on the biofilm formation ($p<0.05$). An increase in the percentage of strong biofilm producers was detected in TSB-5G (14.71%).

The influence of salt concentration on biofilm production was observed for CoNS and CoPS strains. The presence of 0.9% of sodium chloride in TSB displayed an inhibitory effect on the biofilm production to food-related CoNS and clinically-related CoPS strains, since an increase in the percentage of non-biofilm producers was observed. The addition of sodium chloride also has a negative effect on the biofilm formation of food-related CoPS strains, when a decrease in the frequency of strong producers was observed ($p>0.05$).

The combination of 5% glucose and 0.9% sodium chloride, did not affect the ability of the food-related

**Table 3.** Evaluation of biofilm formation capacity in clinical and food-related staphylococci grown in TSB-5GNaCl medium under different temperatures.

| Temperature (°C) | Biofilm formation[1] | Number (%) of biofilm former | | | Total (%) |
|---|---|---|---|---|---|
| | | CNS | CPS | | |
| | | Food (*n* =42) | Food (*n* = 26) | Clinical (*n* =34) | |
| 25 | N | 15 (35.71)$^{b}$ | 1 (3.85)$^{a}$ | 1 (2.94)$^{c,d}$ | 17 (16.67)$^{b}$ |
| | W | 17 (40.48)$^{b}$ | 1 (3.85)$^{a}$ | 17 (50)$^{c,d}$ | 35 (34.31)$^{b}$ |
| | M | 8 (19.05)$^{b}$ | 0$^{a}$ | 12 (35.29)$^{c,d}$ | 20 (19.61)$^{b}$ |
| | S | 2 (4.76)$^{b}$ | 24 (92.31)$^{a}$ | 4 (11.76)$^{c,d}$ | 30 (29.41)$^{b}$ |
| 35 | N | 4 (9.52)$^{a,b}$ | 0$^{a}$ | 0$^{a,b}$ | 4 (3.92)$^{a,b}$ |
| | W | 21 (50)$^{a,b}$ | 2 (7.69)$^{a}$ | 9 (26.47)$^{a,b}$ | 32 (31.37)$^{a,b}$ |
| | M | 15 (35.71)$^{a,b}$ | 3 (11.54)$^{a}$ | 14 (41.18)$^{a,b}$ | 32 (31.37)$^{a,b}$ |
| | S | 2 (4.76)$^{a,b}$ | 21 (80.77)$^{a}$ | 11 (32.35)$^{a,b}$ | 34 (33.33)$^{a,b}$ |
| 40 | N | 9 (21.43)$^{a,b}$ | 0$^{a}$ | 0$^{c,d}$ | 9 (8.82)$^{b}$ |
| | W | 16 (38.1)$^{a,b}$ | 1 (3.85)$^{a}$ | 21 (61.76)$^{c,d}$ | 38 (37.25)$^{b}$ |
| | M | 13 (30.95)$^{a,b}$ | 1 (3.85)$^{a}$ | 12 (35.29)$^{c,d}$ | 26 (25.49)$^{b}$ |
| | S | 4 (9.52)$^{a,b}$ | 24 (92.31)$^{a}$ | 1 (2.94)$^{c,d}$ | 29 (28.43)$^{b}$ |

[1]N, Non-biofilm producers; W, weak biofilm producers; M, moderate biofilm producers; S, strong biofilm producers.
For the same test and bacteria, the same letters do not differ statistically (p>0.05).

CoNS strains to adhere on the microplates, albeit a reduced percentage of non-biofilm producers (9.52%) was observed (Table 2). For the clinically-related CoPS strains, the combination of 5% glucose and 0.9% sodium chloride exhibited significant differences on the biofilm formation ($p<0.05$). An increase in the percentage of strong biofilm producers was detected in TSB-5G/NaCl (32.35 %), more than that in TSB supplemented with sodium chloride and glucose alone.

The presence of 12.5% of rabbit plasma in the TSB medium, exhibited significant differences on biofilm formation to that of the clinically-related CoPS strains ($p<0.05$). The higher rate of strong biofilm formed was detected in TSB-RP (52.94%) ($p<0.001$). Rabbit plasma showed a slight increase in the biofilm ability of food-related CoNS strains, especially for six non-biofilm producers' strains that changed their phenotype to moderate or weaken the biofilm producers. In contrast, the addition of rabbit plasma showed a negative effect on the biofilm formation by food-related CoPS strains, when a decrease in the frequency of strong producers was observed ($p>0.05$).

### Effect of different temperatures on *in vitro* biofilm formation ability of CoNS and CoPS strains

The temperature also exerted an influence on the biofilm formation as a function of the medium (Table 3). Overall, the percentage of CoNS strains considered as non-biofilm formation was higher at 25°C (35.71%) and 40°C (21.43%), when compared to 35°C (9.52%). Though, at optimum temperature for enterotoxin production (40°C), a slight increase in the frequency of strong biofilm formed in the CoNS strains (9.52%) was observed, however, it was not statistically significant ($p>0.05$).

Otherwise, the temperatures of 25°C and 40°C had positive effects on biofilm formation ability of CoPS isolated from food, where high frequencies of strong biofilm producer (92.31%) were observed for both temperatures. In contrast, a low frequency of strong biofilm producer was observed for CoPS strains isolated from clinical samples at 25°C (11.76%) and 40°C (2.94%). For the clinically-related CoPS strains, the temperature of 35°C was considered optimal for biofilm formation ($p<0.05$). The *S. epidermidis* ATCC 35984 used as the positive control was a strong biofilm producer for all temperatures tested.

## DISCUSSION

The objectives of this research were to determine if there is a difference in biofilm formation between CoPS and CoNS strains under a variety of conditions, and to examine the effects of environmental factors on biofilm formation. It was interesting to observe that food-related CoPS strains were markedly more often classified as strong biofilm formers, than clinically-related CoPS and food-related CoNS strains under all conditions evaluated. In the food industry, it is important to know the conditions under which CoPS strains are able to adhere, since biofilms are a persistent source of microbial contamination that can lead to product spoilage, food

safety problems, and loss of production efficiency. *Staphylococcus saprophyticus* is the predominant species concerning food-related CoNS and it is found to be a common contaminant of food (Jessen and Lammert, 2003; Pinto et al., 2015).

All clinically-related CoPS strains and most of the food-related CoPS strains evaluated belonged to the species *S. aureus* and they displayed different behavior under different supplementation and temperatures. In the present study, the addition of glucose (5%), sodium chloride (0.9%) and the combination of glucose (5%) and sodium chloride (0.9%) in TSB increased the power of clinically-related CoPS strains to adhere to the microplates. Glucose and sodium chloride have been identified to influence the *ica*ADBC expression and PIA production *in vitro* biofilm formation in *S. aureus*. Though, the mechanism has not yet been thoroughly characterized in CoNS, and different mechanisms of biofilm development, irrespective of *ica* operon carriage, has been suggested for *S. epidermidis* (Rachid et al., 2000; Götz, 2002; Knobloch et al., 2002; Agarwal and Jain, 2013; You et al., 2014; Barbieri et al., 2015).

Biofilm formations facilities the spread of horizontal spread of antibiotic resistance determinants nosocomial pathogens by allow the exchange of antibiotic resistance genes. Chronic biofilm infections are recalcitrant to conventional antibiotic therapy. *S. aureus* is currently recognized as a major problem in hospitals throughout the world (Hassanzadeh et al., 2015; Poorabbas et al., 2015). The presence of 12.5% rabbit plasma in the TSB was considered the optimal *in vitro* model for the biofilm formation of clinically-related CoPS strains. In addition, the presence of 12.5% rabbit plasma also showed a slight increase in the biofilm ability of food-related CoNS strains. Few studies have demonstrated that the presence of plasma in the medium enhance the capacity for biofilm formation to *Streptococcus mutans* and *S. aureus* (Chen et al., 2012; Bedran et al., 2013; Cardile et al., 2014). For the clinical strains, some specific modifications, such as, pre-coating of the microtiter plate wells with molecules of human matrix or addition of human serum and the supplementation of the medium with salt and glucose are necessary to stimulate, as much as possible, the *in vivo* situation during infection. The absence of these components, might underestimate the *in vitro* biofilm production, due to a lack of conditioning film, which normally occurs *in vivo*. Several authors showed that bacteria also adhered *in vitro* more extensively to materials that had been conditioned with freshly drawn human blood (Donlan and Costerton, 2002; Götz, 2002; Cassat et al., 2007; Archer et al., 2011; Lebeaux et al., 2013; Otto, 2013).

Even though staphylococci growth in the presence of sodium chloride, non-biofilm former (12.75%) strains were observed in presence of 0.9% sodium chloride. One explanation for this result should be the low concentration of sodium chloride used in the present study. Recently, Lee et al. (2014) demonstrated that *S. aureus* ATCC13565 strain isolated from an outbreak linked to ham consumption showed a significant increase on biofilm formation at higher sodium chloride concentrations and also the expression of the *icaA* genes was also higher at concentrations of 4% and 6% of sodium chloride.

The effect of different incubation temperatures on *in vitro* biofilm formation of *Staphylococcus* sp. remains unclear, as well as the mechanisms which affect biofilm production (Abdallah et al., 2014). The temperatures of 25°C and 40°C negatively influenced the biofilm formation of CoNS strains isolated from food, wherever a high number of non-biofilm producers was observed (35.71% and 21.43%, respectively). It is believed that cell surface hydrophobicity level increased with temperature (Fitzpatrick et al., 2005). Abdallah et al. (2014) showed that higher temperatures did not affect the major content of the biofilm matrix, but it decreased the membrane fluidity of sessile cells. Rode et al. (2007) found that the effect of temperature on biofilm formation was also dependent on the presence of glucose and sodium chloride.

In the optimal growth temperature of staphylococci (35°C), it was observed that 100% of CoPS and 90.48% CoNS strains were biofilm producers. It is not surprising that bacteria form more biofilm at this temperature, since this temperature reproduced the physiological conditions required for growth. Fitzpatrick et al. (2005) found that lower temperatures (30°C compared to 37°C and 42°C) were responsible for increasing the biofilm production in *S. epidermidis*, speculating that this is due to its closeness to the temperature of human skin (33°C).

## Conclusion

The food-related CoPS strains could form biofilm under all conditions tested. The addition of rabbit plasma and a temperature of 35°C were the optimal conditions for CoPS isolated from clinical samples. The food-related CoNS strains showed no difference in the biofilm formation ability under all conditions tested, however the presence of 12.5% rabbit plasma showed a slight increase in the biofilm ability. In addition, CoNS strains displayed distinct biofilm patterns under the tested conditions, but with the temperature of 40°C and the combination of glucose with sodium chloride they showed a synergistic effect. The study indicates the suitability of TSB supplemented with diverse compounds and different temperatures on the biofilm formation of CoPS and CoNS strains, and describes models that could provide relevant insights for biofilm formation in food and clinical research. The present findings also highlight the need for a careful selection of the assay conditions. Further research may

be needed to investigate how biofilm formation genes are expressed in response to different environmental stimuli.

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