

Detection of exopolysaccharide production and biofilm-related genes in *Staphylococcus* spp. isolated from a poultry processing plant

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Abstract

Staphylococcus spp. can survive in biofilms for long periods of time, and they can be transferred from one point to another and cause environmental contamination in food processing. The aim of this study was to detect *Staphylococcus* strains isolated from a poultry processing plant by the presence of adhesion genes and the phenotypic production of exopolysaccharide. In the present study, the production of exopolysaccharide and the presence of adhesion genes in 65 strains of *Staphylococcus* spp. were evaluated. All strains of *Staphylococcus* spp. produced exopolysaccharide, as confirmed by formation of black and opaque colonies in Congo Red Agar. The variation of sucrose content was critical for the production of exopolysaccharide in Congo Red Agar since at low sucrose concentrations all strains presented a characteristic result, i.e., there was no exopolysaccharide production. The *atl* gene was found in all strains, and the *icaA* and *icaD* genes were found in 97% of them. The data obtained suggest that *Staphylococcus* spp. isolated from the poultry processing plant evaluated has a potential for biofilm formation. An efficient control of this microorganism in food processing environment is necessary as they may represent a potential risk to consumers.

Keywords: *Staphylococcus aureus*; biofilm; adhesion genes; *ica* genes; Congo red agar.

1 Introduction

Several types of foods are indicated as the cause of gastrointestinal poisoning by *S. aureus*, often being related to the consumption of meat and its products because of their favorable water activity, pH, and nutrient characteristics and extensive manipulation during processing (Ananou et al., 2005). This microorganism can remain viable in biofilms over long periods of time, and they can be transferred from one point to another and cause environmental contamination in food processing (Shale et al., 2005).

The cells released from cell aggregates or biofilms maintain high resistance to antimicrobial agents, and may contain sufficient numbers of bacteria to represent a potential infectious dose. Thus, it is assumed that cell aggregates or biofilm formation by *S. aureus* can be a potential risk for food cross-contamination (Souza et al., 2014).

The ability of *S. aureus* to produce biofilm represents an important virulence factor and is dependent on the environmental conditions, suggesting that there is a mechanism that detects and responds to environmental signals related to the expression of genes involved in adhesion processes (Toledo-Arana et al., 2005).

Several genes are involved in the production of staphylococcal biofilm formation (Cerca et al., 2008; Valle et al., 2003); however, the regulatory mechanisms are poorly understood (Rode et al., 2007). There is a consensus that the main determinants in the mechanism of biofilm formation by *Staphylococcus* are mediated by the production of capsular polysaccharide adhesin (PS/A)

and intercellular polysaccharide adhesin (PIA) or poly-N-succinyl- β -1,6-glucosamine, whose synthesis is encoded by the gene products of the locus *ica* operon of *ica*ABCD (Yazdani et al., 2006; Stanley & Lazazzera, 2004; Beenken et al., 2003).

The genes and products of the locus *ica* are considered critical for biofilm formation and virulence of the *Staphylococcus* genus, and they are regulated in response to environmental factors such as glucose, anaerobiosis, high osmolarity, high temperature, ethanol, and iron limitation (Yazdani et al., 2006). Several studies have reported higher frequency of distribution of the *ica* genes in *Staphylococcus* isolates from foods (Melo et al., 2013; Rumi et al., 2013; Ciftci et al., 2009; Rode et al., 2007; Møretro et al., 2003).

Understanding the processes of biofilm formation by *S. aureus* can facilitate the study on the ability of wild strains to adhere and to form biofilms when they are exposed to conditions simulating the environments found in food processing plants, and it allows a better definition of control strategies (Souza et al., 2014).

PCR analysis only reveals the genetic predisposition for biofilm formation and expression of *ica* genes; thus, the real biofilm formation must be confirmed by additional phenotypic methods (Melo et al., 2013). The aim of this study was to detect *Staphylococcus* strains isolated from a poultry processing plant by the presence of adhesion genes and the phenotypic production of exopolysaccharide.

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2 Materials and methods

2.1 Bacterial species

Strains of *Staphylococcus* spp. belonging to the culture bank of the Food and Water Source Pathogen Microbiology Laboratory at the Federal University of Viçosa, Brazil (Table 1) were used in the present study.

2.2 Phenotypic analysis of exopolysaccharide production

Exopolysaccharide (EPS) production was assessed by cultivating the strains on Congo Red Agar (CRA) using a method modified by Freeman et al. (1989). The CRA allows the detection of exopolysaccharide production by variation in the color of colonies in the medium. The medium was prepared adding 0.8 g of the Congo red dye (Merck®) in 1L of Brain and Heart Infusion Agar (BHI, Himedia®) supplemented with different amounts of sucrose (50 g, 25 g, 12.5 g, 6.25 g, and 3.13 g) without the addition of sucrose to evaluate the influence of the concentration of sucrose on the phenotypic production of exopolysaccharide. The CRA proposed by Freeman et al. (1989) contains 50 g.L⁻¹ of sucrose. The bacterial isolates were streaked onto Congo Red Agar plates and incubated at 37 °C for 24 hours, followed by incubation at 25 °C for 24 hours to evaluate the influence of temperature. The *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228) strains were also used for control purposes, positive and negative, respectively.

In the present study, a six-color reference scale was used for a fine classification of colonies colors, according to Arciola et al. (2002). The six-color tones of the scale were as follows: very black; black; and almost black, which were considered as positive results, and Bordeaux; red; and very red; considered as negative results.

2.3 Identification of adhesion genes

Genomic DNA extraction

The isolates of *Staphylococcus* spp. were incubated in B.O.D. incubator (Fanem®) at 37 °C for 18 hours in 5 mL of BHI syrup (Himedia®). The DNA of the isolates was extracted from one

milliliter of the bacterial culture, which was centrifuged (Jouan® CR3i) at 12000 x g at 4 °C for 2 minutes. The supernatant was discarded, and 60 µL of lysis solution (20% sucrose, Na₂HPO₄ 0.01 mol/l, pH 7.0) and lysozyme (USB®) (0.1 g/ml added at the moment of the extraction) were added to the sediment with incubation in water bath at 37 °C for 1 hour. After that time, 3 µL of K proteinase (Sigma®) (20 mg/ml), 507 ml of 10:1 TE buffer (Tris-HCl 10 mmol/l, pH 7.5, EDTA 1 mmol/l), 30 ml of SDS (Amresco®) 10% were added, mixed, and incubated at 37 °C for 30 minutes. Later, 5 ml of RNase (Sigma®) were added and the tube containing the DNA extracted by the cells was incubated at 25 °C for 2 minutes; next, 100 ml of NaCl (Vetec®) 5 mol/l, 80 ml of hexadecyltrimethylammonium (CTAB (Sigma®) 2%: 0.7 M NaCl (1:1) were added, and the tube was incubated at 65 °C for 10 minutes. Afterwards, 750 ml of chloroform:isoamyl alcohol (24:1) were added, and the suspension was stirred for 1 minute and centrifuged (Jouan®) at 12000 x g for 15 minutes. The supernatant was transferred to another tube, to which 750 ml of chloroform: isoamyl alcohol (24:1) were added followed by centrifugation for 10 minutes. The supernatant was again transferred to another micro tube, to which approximately 500 ml of 100% isopropanol were added and stored at -20 °C for 18 hours. After that, it was again centrifuged at 12000 x g for 15 minutes, and the supernatant was discarded; 1.5 ml of 75% ethanol was added, mixed gently for 5 minutes, centrifuged again for 2 minutes, and the supernatant discarded. This procedure was repeated twice. The pellet was oven-dried at 37 °C for 5 min (Fanem®), and then added to 100 ml of 10:1 TE buffer (Tris-HCl 10 mmol/L, pH 7.5, EDTA 1mmol/L). The DNA was quantified using a spectrophotometer (Nanodrop®).

PCR reaction for identification of the *icaAD* and *atl* genes

The primers (Sigma®) used for the amplification of the *icaD* (F – GGA TCC TTA GTG TTA CAA TTT T; R – TGA CTT TTT GGT AAT TCA AGG TTG TC), *icaA* (F – TGA ACC GCT TGC CAT GTG; R – CAC GCG TTG CTT CCA AAG A) and *atl* (F – GCC TGT TGC AAA GTC AAC AA; R – CAC CGA CAC CCC AAG ATA AG) genes were reacted separately using the parameters modified for the amplification by Korem et al. (2010), Rode et al. (2007), McLaughlin & Hoogewerf, (2006),

Table 1. List of strains of *Staphylococcus* spp. evaluated.

Strain (s)	Number	Coagulase		Origin
		positive	negative	
<i>Staphylococcus aureus</i>	35	22	13	Manipulator ^a
<i>Staphylococcus aureus</i>	5	3	2	Surface ^b
<i>Staphylococcus aureus</i>	14	12	2	Poultry carcass ^c
<i>Staphylococcus aureus</i>	4	4	-	Utensil ^d
<i>Staphylococcus</i> spp.	3	1	2	Manipulator ^a
<i>Staphylococcus</i> spp.	1	1	-	Surface ^b
<i>Staphylococcus</i> spp.	3	3	-	Utensil ^d

Reference Strains

Staphylococcus aureus (ATCC 6538)

Staphylococcus aureus (ATCC 25923)

Staphylococcus epidermidis (ATCC12228)

Strains isolated from: ^a hands of handlers of a poultry slaughterhouse; ^b AISI 304 stainless steel surfaces; ^c poultry carcass; ^d plates and knives used in the poultry slaughterhouse.

respectively. The reactions consisted of a mixture of 0.4 mmol/l of each primer, 0.3 mmol/l dNTP, 1.5 mM of MgCl₂, 1x of buffer of Taq DNA polymerase (Promega®) enzyme, 1 µl (50 ng) of the genomic DNA, and 1 U of Taq DNA polymerase (Promega®) to a final volume of 25 µl. The reaction was conducted using a thermal cycler (Axigen®) and the amplification conditions consisted of a denaturation step at 94 °C for 5 min, followed by 30 thermal cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds, with a final extension step at 72 °C for 5 min. A *S. aureus* (ATCC 6538) strain was used as positive control. Five microliters of the amplified product were visualized in a transilluminator using a Quantum ST4 – 1000/26mX gel documentation system after conducting electrophoresis with 1% agarose gel. A 100 pb molecular marker (BioLabs®) was used.

3 Results

3.1 Phenotypic assay for detection of the exopolysaccharide production

All strains tested positive for production of EPS because they formed black and opaque colonies on Congo Red Agar (CRA) with 50 g/l of sucrose in the first 24 hours of incubation. After 24 hours, the black colonies became reddish (center). However, they are still considered positive as almost black colonies, according to the colorimetric scale proposed by Arciola et al. (2002) (Figure 1).

The colonies appeared red and smooth at concentrations of or below 3.13 g/l sucrose in the medium, which indicates a negative result for EPS production (Figure 2). However, 48 hours after incubation, at a 6.25 g/l sucrose concentration, a decrease in diffusivity of black pigment of the colonies was observed (Figure 3).

3.2 Presence of the *atl*, *icaA*, and *icaD* genes

All strains showed that *atl* genes are predominant in the *Staphylococcus* genus (Figure 4A) with amplification products of 600 bp. The presence of the *icaA* and *icaD* genes (Figure 4B and 4C) with an amplification product of 300 bp was found in 97% of the strains of *Staphylococcus spp.* However, among the tested strains, 3% did not amplify *icaA* and *icaD* genes, but they showed characteristics of EPS production *in vitro*.

4 Discussion

The color of the colonies ranged from very dark to almost black, according to the classification described by Arciola et al. (2002), and were considered as positive for the production of EPS. However, 48 hours after incubation, the concentration of 6.25 g/l of sucrose in the medium was a limiting factor for EPS production due to decreased diffusivity in the black pigment of the colonies incubated in the culture medium containing low concentration of sucrose for a longer time.

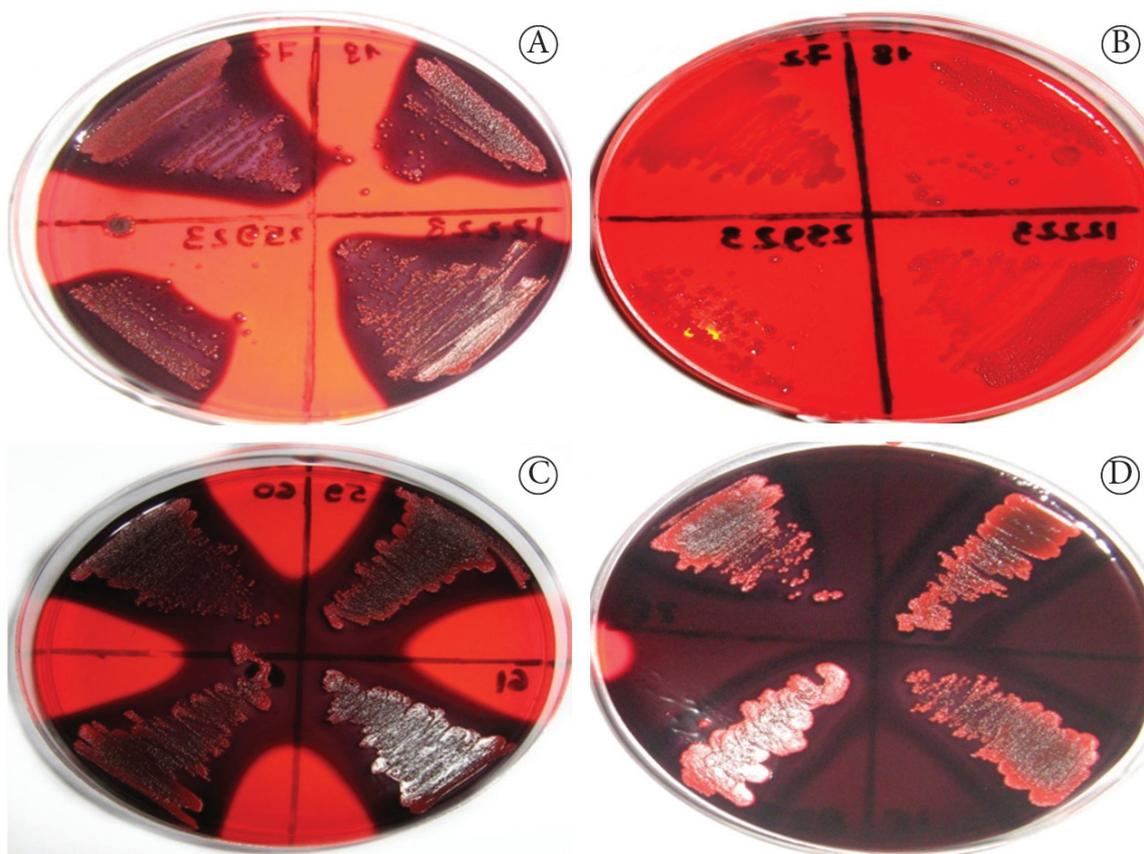


Figure 1. Colonies of *Staphylococcus* strains producing exopolysaccharide on Congo red agar with 50 g/l of sucrose. A - black or almost black colonies (positive), B - red and smooth colonies (negative), C - positive colonies after 24 hours of incubation, D - positive colonies after 48 hours of incubation.

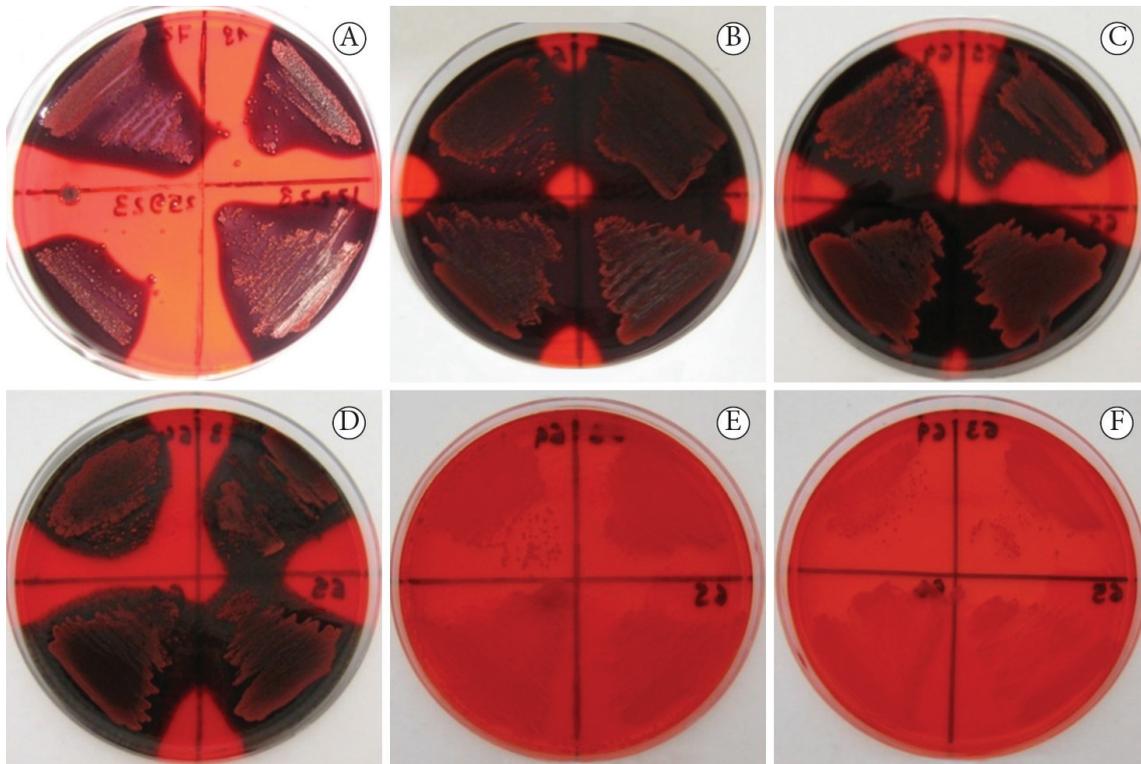


Figure 2. Production of exopolysaccharide according to the sucrose content of the Congo red agar. A - 50 g/l of sucrose, B - 25 g/l of sucrose, C - 12.5 g/l of sucrose, D - 6.25 g/l of sucrose, E - 3.13 g/l of sucrose, and F - without sucrose.

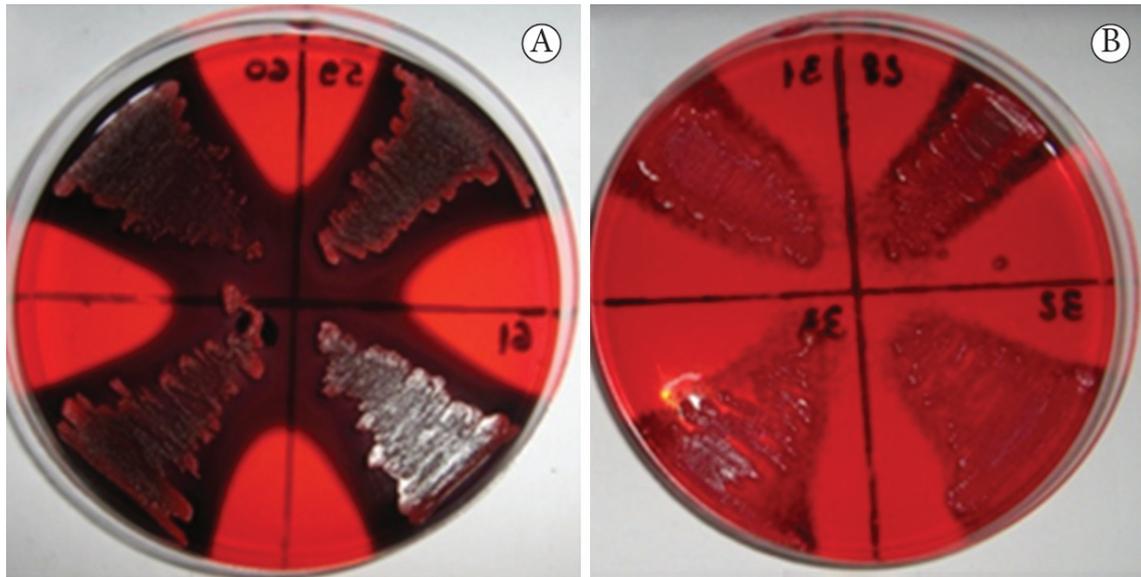


Figure 3. Reduction in the diffusivity of dark pigments at the concentration of 6.25 g/l sucrose. A - 24 hours of incubation, B - 48 hours of incubation.

This result demonstrates that the concentration of a carbon source can limit the gene expression involved in the production of PIA and PS/A. Researchers suggest that the PIA synthesis can be regulated by the presence of a carbohydrate source in the medium that expresses positive phenotypes in biofilm formation in *Staphylococcus* (Dobinsky et al., 2003; Baldassarri et al., 2001; Rachid et al., 2000).

The biofilm formation by strains of *S. aureus* can be affected by environmental conditions relevant for the food industry

due to the constant presence of carbohydrate residues formed during processing.

The phenotypic production of EPS by *icaAD*-negative strains used in the present study suggests that staphylococcal biofilm development may have occurred via an *ica*-independent pathway. According to O’Gara (2007), the *atl* gene, plays an important role in the primary attachment phase of biofilm development. The *atl* gene was present in all strains evaluated. This alternative mechanism of biofilm synthesis relies on the

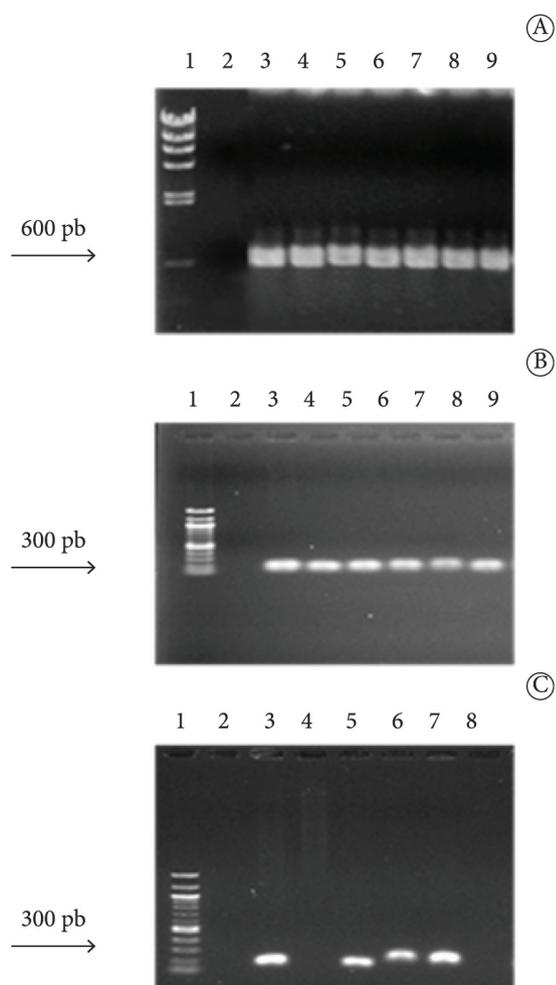


Figure 4. Molecular identification of genes involved in the adhesion of *Staphylococcus* spp. A - Molecular identification of the *atl* gene (Line 1: molecular marker; Line 2: negative control; Line 3 to line 9: positive results), B - Molecular identification of *icaA* gene (Line 1: molecular marker; Line 2: negative control; Line 3 to line 9: positive results), C - Molecular Identification of *icaD* gene (Line 1: molecular marker; Line 2: negative control; Lines 3: positive result; Line 4: negative result; Line 5 to line 7: positive results; Line 8: negative result).

ability of *S. aureus* to express a variety of adhesion proteins that allows cells to attach and to colonize a large number of different surfaces.

Furthermore, it cannot be determined, based on the detection of only one *ica* gene, whether the strain has the potential to produce biofilm since there is a genetic variation among *Staphylococcus* strains.

Møretrø et al. (2003) suggest that it is more appropriate to use the biofilm formation and not the presence or absence of the *locus ica* as one of the criteria for determining potentially virulent strains because biofilm formation on inert surfaces is highly sensitive to environmental and nutritional conditions, such as the presence of ethanol, iron, varying glucose and sodium chloride concentrations, among others. Kaiser et al. (2013) developed a new CRA formula containing glucose,

sodium chloride, and vancomycin, and they confirmed the capacity to detect biofilm in 210 *S. epidermidis* strains isolates, including only 76 (36.2%) *icaAB* gene-positives strains. The use of glucose or sodium chloride or combination of both enhances biofilm producing capacity of staphylococcal isolates irrespective of the presence or absence of *ica* operon (Agarwal & Jain, 2013).

This demonstrates that one should be careful before drawing general conclusions about gene presence in *S. aureus* when it comes to biofilm formation. The results are relevant for food safety as they indicate that food processing conditions could promote biofilm formation by *S. aureus* regardless of the expression of *ica* genes.

The mechanisms involved in the transcriptional regulation of the *ica* locus are only partially understood, and different regulatory mechanisms are known for *S. aureus* and *S. epidermidis* (Arciola et al., 2012).

The high prevalence of EPS production by the tested strains in this study emphasizes the responsibility of professionals involved in the food production chain to adopt hygienic and sanitary measures since the formation of biofilm in the industrial environment by *Staphylococcus* spp. should occur within hours. Accordingly, the increasing automation of the industries and the employment of sophisticated equipment can complicate the cleaning process and create a large number of niches for the adhesion and multiplication of microorganisms, which contribute to biofilms formation with consequent impairment of food safety and equipment efficiency.

Some studies have evaluated the effect of different growth conditions on the adherence and biofilm formation of *S. aureus* isolates in food industries. The assayed strains revealed high capacities to adhere and form biofilms on polypropylene and stainless steel surfaces under different growth conditions, and the cells in biofilm matrixes were resistant to total removal when exposed to the sanitizers such as sodium hypochlorite and peracetic acid (Souza et al., 2014; Jerônimo et al., 2012).

Several studies have reported a higher frequency of distribution of the *ica* genes and slime production in *Staphylococcus* isolates from milk (Melo et al., 2013; Rumi et al., 2013; Ciftci et al., 2009), meat, and contact surfaces in processing plants (Schlegelová et al., 2008), indicating the virulence potential of the strains evaluated.

The combination of phenotypic and genotypic methods employed in the present study allowed the screening of strains potentially capable of expressing biofilm-forming *Staphylococcus* genus genes. Nevertheless, there is still a need for further studies on regulatory mechanisms of biofilm formation by *Staphylococcus* in the processing environment, mainly owing to genetic variations between the strains evaluated. Thus, professionals involved in food production should be aware of the importance of controlling biofilm formation by *Staphylococcus* and that it can occur under conditions commonly used in the food industry representing a potential risk to consumers.

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