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*Original Article*

# Biofilm-forming and antimicrobial resistance traits of staphylococci isolated from goat dairy plants

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## Abstract

Introduction: Biofilm-associated antimicrobial resistance is of increasing importance to the maintenance and spread of foodborne pathogens in the food industry. This study aimed to investigate the ability to form biofilm and the antimicrobial resistance of staphylococci contaminating small-scale goat milk dairy plants.

Methodology: Sixty isolates were tested for antimicrobial resistance against 20 drugs by the microdilution method. Biofilm-forming traits were assessed by the microtiter plate method (MtP), Congo red agar method (CRA), and *icaD* gene detection by polymerase chain reaction (PCR).

Results: High antimicrobial resistance to ampicillin (60/60; 100%), penicillin G (21/60; 35%), and erythromycin (15/60; 25%) was observed, but all isolates were susceptible to amoxicillin/K-clavulanate, ceftriaxone, ciprofloxacin, gentamicin, levofloxacin, linezolid, and moxifloxacin. No resistance to oxacillin or vancomycin was seen among *Staphylococcus aureus*. Twenty-seven isolates (27/60; 45%) were considered to form biofilm according to MtP, and similar biofilm-producing frequencies were observed in coagulase-negative staphylococci (CoNS) (20/44; 45.4%) and *S. aureus* (7/16; 43.7%). The *icaD* gene was observed only in *S. aureus* isolates. There was no association between biofilm production and antimicrobial resistance. A higher frequency of biofilm-producing staphylococci was found in isolates from bulk tank milk and hand swabs. On the other hand, isolates from pasteurized milk showed lower frequency of biofilm formation.

Conclusions: Staphylococci contaminating goat dairy plants are potential biofilm producers. The results suggest no association between the ability to form biofilm and antimicrobial resistance.

**Key words:** antibiotic; biofilm; *ica* gene; slime; *Staphylococcus.*

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## Introduction

Although the global production and commercialization of goat milk and its products are much lower than that of bovine milk, the goat dairy chain plays an important and significant role in the socioeconomic development of certain countries, mainly in poor and semi-arid zones. Northeastern Brazil is a major goat milk-producing region in the country and in Latin America. The goat production chain comprises mostly family producers, and the milk is pasteurized in small-scale dairy plants before being distributed to public schools in the scope of social programs of the federal government [1].

Among other potential foodborne pathogens, a very high staphylococcal contamination was previously reported in bulk goat milk produced in the region [2]. Recently, we also showed that both *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) isolated from different goat milk processing plants harbor classic and novel enterotoxin-encoding genes [3]. *S. aureus* is involved in innumerous cases and outbreaks of food poisoning worldwide, and the consumption of contaminated dairy products has been

reported to be a common source of contamination [4]. According to the Centers for Disease Control and Prevention, more than 240,000 cases of staphylococcal intoxication are reported every year in the United States [5]. In recent years, antimicrobial resistance in bacteria potentially transmitted by foods has been considered a public health concern, and efforts have been made to understand possible factors affecting the emergence and maintenance of antimicrobial-resistant bacteria in the food chain [6-8].

Biofilm production is one of the most important mechanisms that improves the ability of microorganisms to resist adverse factors and colonize the environment. Biofilms are sessile bacterial communities attached to a substrate and embedded in a self-produced extracellular polymeric matrix in which the cells present a different phenotype, metabolism, physiology, and gene expression from planktonic cells [9]. The polysaccharide intercellular adhesin (PIA) is the most studied component that contributes to cell adhesion and slime accumulation in staphylococci [10]. PIA is a N-acetylated β-1,6-glucosaminoglycan, codified by the *ica* operon, which comprises both the *icaADBC* gene cluster and the regulatory *icaR* gene [11]. However, recent studies have highlighted the importance of *ica*-independent mechanisms for biofilm production in staphylococci from milk origin [12].

Biofilm-associated antimicrobial resistance has been reported in *S. aureus* and even CoNS isolated from infected humans and medical utensils in hospitals [13,14]. On the other hand, there is a lack of information about the putative association between antimicrobial resistance and biofilm production in *Staphylococcus* spp. contaminating foods and food processing environments.

Therefore, this study aimed to investigate the ability to form biofilm and antimicrobial resistance of staphylococci contaminating small-scale goat milk dairy plants. To the best of our knowledge, there is no information about the characterization of antimicrobial-resistant staphylococci specifically regarding their potential to produce biofilm in goat milk processing plants.

## Methodology

*Isolates*

*Staphylococcus* spp. samples (n 60) were previously isolated from three small-scale goat dairy plants located in the state of Paraiba from February 2009 to May 2011. Isolates originated from raw (n = 20) and pasteurized milk (n = 13), bulk tank milk (n = 9), equipment (n = 9), and hand swabs from workers (n = 9). The biofilm-producing *S. aureus* ATCC 25923 and the non-biofilm-producing *S. epidermidis* ATTCC 12228 were used as positive and negative controls, respectively [15].

The isolates were cultured in yeast extract trypticase soy agar (0.6 g/100 mL; TSAYE; Himedia Labs., Mumbai, India) at 37°C for 18 hours. Gram staining, catalase, oxidase and coagulase tests were performed to confirm staphylococcal isolates. Species identification was performed in a semi-automated system (Autoscan4, Siemens Medical Solutions USA, Inc, Malvern, USA) using a biochemical-based colorimetric panel (Combo PC33; Siemens) and a commercial software (LabPro Connect; Siemens) [16,17].

*Antimicrobial susceptibility test*

The minimum inhibitory concentration (MIC) was determined by the microdilution method using a semi-automated system (Autoscan 4, Siemens Healthcare, Malvern, USA). Bacterial suspensions were prepared from colonies grown onto TSAYE diluted in pluronic water (Prompt Inoculation System-D, Siemens Healthcare, Malvern, USA) and adjusted to 0.5 McFarland turbidity. The suspensions were dispensed in the microtiter plate panel (Combo PC33, Siemens Healthcare, Malvern, USA) containing 20 different drugs. The following breakpoints ($\mu g/mL^{-1}$) were used, based on the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]: ampicillin (≥ 0.5), ampicillin/sulbactam (≥ 32/16), amoxicillin/K-clavulanate (≥ 8/4), trimethoprim/sulfamethoxazole (≥ 4/76), ceftriaxone (≥ 64), clindamycin (≥ 4), ciprofloxacin (≥ 4), daptomycin (≥ 2), erythromycin (≥ 8), gentamicin (≥ 16), nitrofurantoin (≥ 128), levofloxacin (≥ 8), linezolid (≥ 8), moxifloxacin (≥ 8), oxacillin (≥ 0.5 for CoNS and ≥ 4 for *S. aureus/S. lugdunensis*), penicillin G (≥ 0.25), rifampin (≥ 4), quinupristin/dalfopristin (≥ 4), tetracycline (≥ 16), and vancomycin (≥ 32 for CoNS and ≥ 16 for *S. aureus*). The microtiter plates were incubated at 35°C for 24 hours. Readings were performed using a semi-automated system (Autoscan 4), and interpretation was performed using LabPro software (Siemens Healthcare, Malvern, USA). Strains considered resistant to vancomycin based on MIC values were submitted to E-test confirmation.

*Determination of biofilm production*

Biofilm production was assessed quantitatively by the microtiter plate method (MtP), as described by Stepanovic *et al*. [19], and qualitatively by the Congo red agar method (CRA), as described by Arciola *et al*.

[20]. In the MtP test, a 20 µL bacterial suspension (0.5 McFarland standard) was inoculated in six wells of a polystyrene microtiter plate containing 180 µL of trypticase soy broth (TSB) (Himedia Labs., India) supplemented with glucose (1 g/100 mL) (Himedia Labs., India). Microtiter plates were incubated at 35°C for 18 hours and subsequently washed three times with 200 µL sterile saline solution. After fixation with 150 µL methanol for 20 minutes, the plates were dried at room temperature for 30 minutes. Wells were stained with crystal violet (0.5 g/100 mL) (Himedia labs., India) for 15 minutes and then washed with ethanol (30 minutes). *S. aureus* 25923 and TSB supplemented with glucose were used as positive and negative controls, respectively. The mean optical density (OD) was determined for each isolate by reading the absorbance at 450 nm in a microplate spectrophotometer, XMark (BioRad, Hercules, USA). Biofilm production was assessed as follows: no biofilm production (OD ≤ ODc), weak (ODc ≤ OD ≤ 2.ODc), moderate (2.ODc < OD ≤ 4.ODc), and strong biofilm production (4.ODc < OD), with ODc being the optical density of the negative control plus three times its standard deviation. Three repetitions were performed for each isolate under the same conditions in different moments.

In the CRA method, isolates were streaked onto CRA, incubated at 35°C for 24 hours, and then kept at room temperature for 48 hours. Colony color was determined using a four-color reference scale varying from red to black. Black colonies were considered to be biofilm-producing isolates, while almost-black colonies were considered weak biofilm producers. Red and purple colonies were considered non-biofilm producers.

*Detection of the icaD gene by polymerase chain reaction (PCR)*

The potential of isolates to produce biofilm by means of the *ica*-operon mechanism was tested by PCR targeting the *icaD* gene. *Staphylococcus* spp. DNA was extracted using the phenol/chloroform/isoamyl alcohol protocol as described by Fritsch *et al*. [21]. DNA template (100 ng) was added to the PCR master mix containing 200 µM of each dNTP, 4 mM $MgCl_2$, 0.4 µM oligonucleotide primers, and 1U Taq polymerase (Invitrogen, Carlsbald, USA) in a 25 µL final volume. Forward (5'-ATGGTCAAGCCCAGACAGAG-3') and reverse primers (5'-CGTGTTTTCAACATTTAATGCAA-3') described by Arciola *et al*. [22] were used to amplify a 198 bp DNA fragment corresponding to the *icaD* gene. Amplification was performed in a thermal cycler (T personal, Biometra, Gottingen, Germany) using an initial denaturation step (94ºC for 4 minutes) followed by 30 cycles (94ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 1 minute) and a final extension step (72ºC for 10 minutes). Bands were visualized in agarose gel (2 g/100 mL) stained with GelRed (Biotium, Hayward, USA) under UV light.

Detailed results of the tests for biofilm production and PCR targeting the *icaD* gene for each isolate is available in Table 1S.

*Data analyses*

Descriptive statistics was used to report the frequency and extent of biofilm production and phenotypic antimicrobial resistance in isolates. The kappa agreement test and kappa values (K) were used

**Table 1.** Distribution of isolates based on *Staphylococcus* species and isolation source.

| Isolates | Number of isolates (%) | Isolation sources | | | | |
|---|---|---|---|---|---|---|
| | | Raw milk | Pasteurized milk | Bulk milk tank | Equipment surface | Swabs from handler's hand |
| *S. aureus* | 16 (27%) | 8 | 0 | 5 | 1 | 2 |
| *S. lugdunensis* | 11(18%) | 4 | 1 | 2 | 3 | 1 |
| *S. hyicus* | 9 (15%) | 3 | 6 | 0 | 0 | 0 |
| *S. hominis subsp. hominis* | 8 (13%) | 3 | 2 | 1 | 2 | 0 |
| *S. haemolyticus* | 4 (7%) | 0 | 1 | 1 | 1 | 1 |
| *S. warneri* | 3 (5%) | 0 | 0 | 0 | 0 | 3 |
| *S. saprophyticus* | 2 (3%) | 0 | 1 | 0 | 0 | 1 |
| *S. epidermidis* | 2 (3%) | 1 | 0 | 0 | 0 | 1 |
| *S. auricularis* | 2 (3%) | 1 | 1 | 0 | 0 | 0 |
| *S. cohnii subsp. cohnii* | 2 (3%) | 0 | 1 | 0 | 1 | 0 |
| *S. sciuri* | 1 (2%) | 0 | 0 | 0 | 1 | 0 |
| **Total** | **60 (100%)** | **20** | **13** | **9** | **9** | **9** |

to compare the methods as predictors of the capacity of biofilm production by the isolates, which was interpreted as poor (K < 0.2), fair (K = 0.21–0.40), moderate (K = 0.41–0.60), good (K = 0.61–0.80), and very good (K = 0.81–1) [23]. Fisher's exact test was used to identify possible associations between the predictive traits for biofilm production (CRA method, MtP test, and presence of *icaD* gene) and resistance to the tested antimicrobials (individual or multiresistance [resistance to antimicrobials from three different classes]). Statistical analyses were performed using the computational software SPSS version 20 (SPSS, Chicago, USA).

## Results

*Staphylococcus* spp. isolates used in the present study are shown in Table 1. Except for *S. aureus* (16/60; 26.7%), all other isolates (44/60; 73.3%) were CoNS. Table 2 shows the antimicrobial resistance pattern of *S. aureus* and CoNS isolates to the tested antimicrobials. High antimicrobial resistance was observed for ampicillin (60/60 isolates; 100%), penicillin G (21/60 isolates; 35%) and erythromycin (15/60 isolates; 20%). All isolates were susceptible to amoxicillin/K-clavulanate, ceftriaxone, ciprofloxacin, gentamicin, levofloxacin, linezolid, moxifloxacin, and vancomycin. No resistance to oxacillin was observed in *S. aureus*. Resistance to at least three antimicrobial classes was observed in 11 (18%) isolates. Multiresistance was found in 6 (37%) and 5 (11%) isolates of *S. aureus* and CoNS, respectively.

Biofilm production was shown in 27 of 60 isolates (45%) by the MtP test and in 17 (28%) by the CRA test (Table 3). These two methods showed poor agreement (K = 0.036) in detecting biofilm-producing isolates. The *icaD* gene was not detected in 20 (74%) isolates that showed biofilm formation in the MtP method. On the other hand, *icaD* was detected in 14 (82%) isolates considered biofilm producers by CRA, resulting in a good agreement (K = 0.79) between CRA and *icaD*

**Table 2.** Antimicrobial resistance patterns of *S. aureus* and coagulase-negative staphylococci (CoNS) isolated from different goat milk processing points at small-scale dairy plants.

| Antibiotics | *S. aureus* (n = 16) | | CoNS (n = 44) | |
|---|---|---|---|---|
| | Resistant (%) | Susceptible (%) | Resistant (%) | Susceptible (%) |
| Ampicillin/sulbactam | 4 (25%) | 12 (75%) | 5 (11%) | 39 (89%) |
| Ampicillin | 16 (100%) | 0 (0%) | 44 (100%) | 0 (0%) |
| Clindamycin | 3 (19%) | 13 (81%) | 12 (27%) | 32 (73%) |
| Daptomycin | 2 (12%) | 14 (88%) | 2 (5%) | 42 (95%) |
| Erythromycin | 5 (31%) | 11 (69%) | 10 (23%) | 34 (77%) |
| Nitrofurantoin | 0 (0%) | 16 (100%) | 3 (7%) | 41 (93%) |
| Oxacillin | 0 (0%) | 16 (100%) | 2 (5%) | 42 (95%) |
| Penicillin G | 10 (62%) | 6 (38%) | 11 (25%) | 33 (75%) |
| Rifampin | 2 (12%) | 14 (88%) | 0 (0%) | 44 (100%) |
| Synercid | 0 (0%) | 16 (100%) | 2 (5%) | 42 (95%) |
| Trimethopim/sulfamethoxazole | 0 (0%) | 16 (100%) | 1 (2%) | 43 (98%) |
| Tetracycline | 1 (6%) | 15 (94%) | 1 (2%) | 43 (98%) |
| Vancomycin | 0 (0%) | 16 (69%) | 0 (0%) | 44 (95%) |

**Table 3.** Distribution of *Staphylococcus* isolated from different milk processing points at small-scale goat dairy plants according to their potential to produce biofilm by the microtiter plate method (MtP), Congo red agar (CRA) method, and *icaD* detection by PCR (*icaD*).

| Method of biofilm forming detection | | Raw milk n = 20 | % | Pasteurized milk n = 13 | % | Bulk tank milk n = 9 | % | Surface of equipment n = 9 | % | Handler's hand swabs n = 9 | % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MtP[1] | Negative | 11 | 55 | 10 | 77 | 4 | 44 | 5 | 56 | 3 | 33 |
| | Weak | 5 | 25 | 1 | 8 | 3 | 33 | 2 | 22 | 0 | 0 |
| | Moderate | 1 | 5 | 0 | 0 | 1 | 11 | 2 | 22 | 1 | 11 |
| | Strong | 3 | 15 | 2 | 15 | 1 | 11 | 0 | 0 | 5 | 56 |
| CRA[2] | Negative | 12 | 60 | 13 | 100 | 4 | 44 | 8 | 89 | 6 | 67 |
| | Weak | 3 | 15 | 0 | 0 | 2 | 22 | 1 | 11 | 2 | 22 |
| | Strong | 5 | 25 | 0 | 0 | 3 | 33 | 0 | 0 | 1 | 11 |
| *icaD* | Negative | 12 | 60 | 13 | 100 | 4 | 44 | 9 | 100 | 7 | 78 |
| | Positive | 8 | 40 | 0 | 0 | 5 | 56 | 0 | 0 | 2 | 22 |

[1] Negative (OD ≤ ODc), weak (ODc ≤ OD ≤ 2.ODc), moderate (2.ODc < OD ≤ 4.ODc), and strong biofilm production (4.ODc < OD); [2] Negative (red or purple colonies), weak (almost black colonies), strong (black colonies).

detection. The *icaD* gene was present only in *S. aureus* isolates. However, the frequency of biofilm-producing isolates by MtP test was similar among CoNS (20/44; 45.4%) and *S. aureus* (7/16; 43.7%) isolates.

The frequency of staphylococcal isolates producing biofilm by both MtP and CRA, including *S. aureus* and CoNS, was higher in isolates from bulk tank milk and hand swabs and lower ($p < 0.05$) in isolates from pasteurized milk. No significant association was observed between resistance or multiresistance and biofilm production either by MtP or CRA or *icaD* gene harboring.

## Discussion

The higher frequency of resistance to beta-lactams, mainly ampicillin and penicillin G, in the tested *S. aureus* and CoNS isolates is consistent with previous studies on antimicrobial resistance patterns of staphylococci from the dairy industry [24]. This resistance profile has been associated with the common use of these drugs to prevent and treat infections in farm cattle [25]. The absence of vancomycin or oxacillin resistance in *S. aureus* isolates is interesting, since resistance to these antibiotics has been increasingly detected in isolates from farms and foods [24,26,27].

The high frequency of *S. aureus* or CoNS isolates positive for biofilm formation by MtP might be explained by the high capacity of those organisms to produce biofilm in natural ecosystems, such as those found in milk-processing plants. The biofilm formation by staphylococci on milk contact surfaces and environment of dairy plants is a potential chronic contaminating source to milk and dairy products, posing a risk to consumers [28].

Although increased antimicrobial resistance in biofilm-forming bacteria has been reported as an important factor for the maintenance of pathogenic microorganisms in food processing plants [6,29], no significant association was observed in the present study between antimicrobial resistance or multiresistance and the ability of staphylococci to form biofilms. Indeed, antimicrobial resistance in biofilms formed in dairy plants has been proposed to be more related to the complex extracellular slime avoiding antimicrobial penetration instead of an increased antimicrobial resistance by the biofilm-embedded cells [30,31].

Considering the absence of *icaD* gene detection, it could be proposed that slime production via PIA is not determinant of biofilm production in the tested CoNS isolates; moreover, this could be reinforced because 45% of CoNS isolates were positive for biofilm production by the MtP test. Therefore, the results strongly indicate that *ica*-independent slime mechanisms can be of major importance for biofilm production in the tested staphylococcal isolates. Studies carried out in different ecosystems reported not only the presence of *ica* genes in different *Staphylococcus* species (including CoNS) but also their ability to produce biofilm through PIA synthesis [32,33,34]. Furthermore, other studies indicate that CoNS are capable of producing biofilm by other mechanisms, such as the production of a protein-based matrix [35].

Interestingly, all *S. aureus* isolates were positive for *icaD*, and only two isolates did not show the phenotypical indication of PIA production via the CRA test. Previous studies suggested that some *S. aureus* isolates are not capable of forming biofilm *in vitro* due to point mutations in the *ica* loci or environmental factors [33]. The presence of the *icaD* gene in all *S. aureus* isolates, together with the good agreement ($K = 0.79$) between the presence of *icaD* and the production of biofilm (as detected by the CRA test) also suggests that PIA is a major biofilm-producing mechanism for *S. aureus*. Nevertheless, the detection of biofilm production by MtP in this study was similar ($p > 0.05$) among *S. aureus* and CoNS.

The presence of CoNS has not been as widely reported as the presence of *S. aureus* in goat milk and goat dairy products, but the importance of the former for the goat milk industry is also noteworthy. CoNS are the main mastitis-causing agents in dairy goats, and the presence of enterotoxin-encoding genes in those species has been reported in the Brazilian Northeastern region [3].

A higher potential to produce biofilm was observed in isolates from bulk tank milk and hand swabs from handlers. On the other hand, a lower potential to produce biofilm was seen in staphylococci from pasteurized milk, which was expected because of the lower total bacteria counts in pasteurized milk. Interesting, however, is the fact that the frequency of bacteria harboring biofilm-producing genes showed similar distribution among the source of contamination when analyzed by MtP, CRA, and *icaD* PCR individually, although MtP and CRA showed poor correlation with *icaD* PCR. Curiously, the same percentage of positive isolates (44%) was observed in isolates from bulk tank milk when analyzed by MtP, CRA, and *icaD* PCR. In addition, isolates from pasteurized milk showed a lower frequency of harboring biofilm-producing traits by the three methods. The only exception was related to isolates from hand swabs, in which MtP identified more

positive isolates compared with CRA and *icaD* PCR. These findings suggest that environmental factors might play a major role in triggering biofilm production. The expression of the *ica* operon and the production of biofilm is highly variable among staphylococci. High osmolarity and temperature, glucose concentration, anaerobic conditions, and certain antimicrobials in sub-inhibitory concentrations might increase the expression of the *icaA* gene and biofilm forming in staphylococci [36]. Because the final synthesis of the exopolysaccharide is affected by various mechanisms, the molecular detection of *ica* genes does not warrant phenotypic expression [13].

## Conclusions

Staphylococci contaminating small-scale goat dairy plants are potential biofilm producers, and *ica*-independent mechanisms seem to be involved in the process of slime formation. There is no indication of association between biofilm-producing ability and antimicrobial resistance. No resistance to oxacillin or vancomycin has been detected in *S. aureus*. These findings warrant further investigations to evaluate the impact of biofilms and mechanisms of slime production in the dairy industry.

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### Authors' contributions

Myrella Cariry Lira participated in elaboration, execution and writing of the manuscript. Patrícia Emília Naves Givisiez, Francisca Geovânia Canafistula, and Denis Augusto Spricigo de Sousa fulfilled the analyses of microtiter plate method, Congo red agar method and *icaD* detection by PCR. Marciane Magnani, Evandro Leite de Souza, Wondwossen Abebe Gebreyes, and Celso José Bruno de Oliveira participated in the elaboration, coordination, and writing of the manuscript.

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