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ORIGINAL ARTICLE

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Non-biofilm-forming commensal *Staphylococcus epidermidis* isolates produce biofilm in the presence of trypsin

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Abstract

Epidemiological studies comparing clinical and commensal *Staphylococcus epidermidis* isolates suggest that biofilm formation is a discriminant biomarker. A study showed that four non-biofilm-forming clinical *S. epidermidis* isolates could form an induced biofilm by trypsin treatment, suggesting that *S. epidermidis* can form biofilms in a protease-independent way and in a trypsin-induced way. In this study, the trypsin capacity to induce biofilm formation was evaluated in non-biofilm-forming *S. epidermidis* isolates (n = 133) in order to support this mechanism and to establish the importance of total biofilms (meaning the sum of protease-independent biofilm and trypsin-induced biofilm). *Staphylococcus epidermidis* isolates from ocular infections (OI; n = 24), prosthetic joint infections (PJI; n = 64), and healthy skin (HS-1; n = 100) were screened for protease-independent biofilm formation according to Christensen's method. The result was that there are significant differences ($p < .0001$) between clinical (43.2%) and commensal (17%) protease-independent biofilm producers. Meanwhile, non-biofilm-forming isolates were treated with trypsin, and biofilm formation was evaluated by the same method. The number of commensal trypsin-induced biofilm producers significantly increased from 17% to 79%. In contrast, clinical isolates increased from 43.2% to 72.7%. The comparison between clinical and commensal total biofilm yielded no significant differences (p = .392). A similar result was found when different isolation sources were compared (OI vs. HS-1 and PJI vs. HS-1). The genotype *icaA*$^-$/*aap*$^+$ was associated with the trypsin-induced biofilm phenotype; however, no correlation was observed between *aap* mRNA expression and the level of trypsin-induced biofilm phenotype. Studying another group of commensal *S. epidermidis* non-biofilm-forming isolates (HS-2; n = 139) from different body sites, it was found that 70

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isolates (60.3%) formed trypsin-induced biofilms. In conclusion, trypsin is capable of inducing biofilm production in non-biofilm-forming commensal *S. epidermidis* isolates with the *icaA*$^{-}$/*aap*$^{+}$ genotype, and there is no significant difference in total biofilms when comparing clinical and commensal isolates, suggesting that total biofilms are not a discriminant biomarker.

KEYWORDS
non-biofilm-forming, protease-independent biofilm, *Staphylococcus epidermidis*, total biofilm, trypsin

1 | INTRODUCTION

Staphylococcus epidermidis is responsible for a large number of infections, such as bacteremia, endocarditis, and endophthalmitis, as well as infections associated with medical devices (Otto, 2009; Schoenfelder et al., 2010). Recently, coagulase-negative staphylococci (CoNS) have become important opportunistic infectious agents (Magill et al., 2014; Weiner et al., 2016). Together with *Staphylococcus aureus*, CoNS are the primary agents responsible for nosocomial infections and represent the most common source of infections associated with medical devices (Darouiche, 2004), causing a problem for the public health system. Molecular epidemiological studies reveal a sizeable genetic diversity among staphylococci (Conlan et al., 2012; Schoenfelder et al., 2010); in isolates from ocular infections (OI), prosthetic joint infections (PJI), and healthy skin (HS), there is no genetic association among them (Flores-Páez et al., 2015; Ortega-Peña et al., 2019, in press). For the case of isolates from ocular infections (OI), biofilm formation is a discriminant biomarker compared to commensal isolates (Duggirala et al., 2007; Jain & Agarwal, 2009; Okee et al., 2012; Suzuki, Kawamura, Uno, Ohashi, & Ezaki, 2005); however, there is evidence that the number of isolates from prosthetic joint infections with biofilm formation capacity is similar to commensal isolates (Hellmark, Söderquist, Unemo, & Nilsdotter-Augustinsson, 2013).

Most infections caused by *S. epidermidis* involve biofilm formation, as do infections caused by other staphylococci. Biofilms are bacterial agglomerations adhered to biotic or abiotic surfaces, with bacteria embedded within an extracellular matrix. Biofilms confer resistance to antibiotics and the host immune response (Otto, 2009), and they represent the main mechanism underpinning CoNS infections. The development of a biofilm begins with the adhesion of cells to a surface and their subsequent intercellular aggregation (Otto, 2009). Usually, biofilm-forming strains of *S. epidermidis* produce poly-*N*-acetylglucosamine (PNAG), which is encoded by the *icaADBC* operon (Ziebuhr et al., 1997); PNAG surrounds the bacterium and promotes the formation of a PNAG-dependent biofilm. However, some clinical isolates of *S. epidermidis* lacking *icaADBC* operon show the ability to form a biofilm (Dice et al., 2009; Hellmark et al., 2013; Klug, Wallet, Kacet, & Courcol, 2003; Rohde et al., 2007; Ziebuhr et al., 1997). In these isolates, the accumulation-associated protein (Aap) plays a vital role in protein-dependent biofilm formation (Hennig, Nyunt Wai, & Ziebuhr, 2007; Hussain, Herrmann, Eiff, Perdreau-Remington, & Peters, 1997; Rohde et al., 2004).

Clinical studies report that most CoNS isolates (ca. 50%–60%) are biofilm producers (Hellmark et al., 2013; Juárez-Verdayes et al., 2013). The remaining clinical isolates (ca. 50%–40%) are non-biofilm producers, and these are less studied. Rohde et al. (2004) obtained a revertant mutant of a non-biofilm-forming *S. epidermidis* 5179 strain isolated from a cerebrospinal fluid infection that could form a biofilm. This mutant strain expressed a truncated 140 kDa isoform of the 220 kDa Aap (full-length Aap). In contrast, expression of full-length Aap did not lead to a biofilm-positive phenotype; the full-length Aap must be proteolytically processed through proteases. Furthermore, they found that treatment with trypsin induced the biofilm formation in four non-biofilm-forming *S. epidermidis* isolates from patients with infected joint prostheses, suggesting that protease-mediated induction of the biofilm formation seems to be a general mechanism in CoNS (Rohde et al., 2004). These results set a precedent for *S. epidermidis* biofilms, suggesting that there are two types of biofilms: biofilms where proteases are not required for biofilm formation (protease-independent biofilms) and protease-induced biofilms. However, a large number of clinical and commensal isolates must be analyzed in epidemiological studies in order to establish the importance of this protease-induced biofilms mechanism. In previous studies, we demonstrated that collections of isolates from OI and PJI are genetically different and there are no clonal associations between them; it also occurs with commensal isolates (Flores-Páez et al., 2015; Ortega-Peña et al., 2019). Thus, the objective of this work was to evaluate the trypsin biofilm induction capacity of genetically different *S. epidermidis* isolates from clinical (OI and PJI) and commensal (HS) sources.

2 | MATERIALS AND METHODS

2.1 | CoNS isolates

Commensal and clinical CoNS isolates used in this work were those reported previously by our research group. Twenty-four clinical

isolates of patients with OI from 2000 to 2010 were obtained from "Instituto de Oftalmología Fundación Conde de Valenciana" (Flores-Páez et al., 2015), and 64 clinical isolates of patients with PJI from 2011 to 2015 were from "Instituto Nacional de Rehabilitación Luís Guillermo Ibarra Ibarra" (Ortega-Peña et al., 2019).

On the other hand, commensal CoNS isolates of 100 unrelated individuals were obtained from healthy skin (HS-1; Flores-Páez et al., 2015). Likewise, 5 healthy conjunctiva (HC) isolates were included (Flores-Páez et al., 2015). All CoNS isolates were confirmed by MALDI-TOF analysis Vitek™ MS (bioMérieux).

One hundred eighty-eight isolates of *S. epidermidis* (24 of OI, 64 of PJI, and 100 of HS-1) and a group of 23 non-epidermidis CoNS (1 of OI, 11 of PJI, 6 of HS-1, and 5 of HC) were studied. Non-epidermidis CoNS was composed as follows: 11 *Staphylococcus hominis*; 5 *Staphylococcus warneri*; 2 as *Staphylococcus capitis*; and 1 of each of *Staphylococcus lentus*, *Staphylococcus caprae*, *Staphylococcus sciuri*, *Staphylococcus haemolyticus*, and *Staphylococcus lugdunensis*. Genotypic features of OI, PJI, and HS-1 isolates were previously determined (MLST, *agr* type, SSC*mec* type), showing high diversity among them (Flores-Páez et al., 2015; Ortega-Peña et al., 2019).

Also, a new collection consisting of 139 *S. epidermidis* isolates were collected from healthy skin (HS-2) of different body parts (head, nostril, axillae, and conjunctiva) of five healthy subjects and included in this work. Similarly, isolates were identified by MALDI-TOF Vitek™ MS (bioMérieux), and the clonality was determined by pulse-field gel electrophoresis (PFGE).

2.2 | Pulsed-field gel electrophoresis (PFGE)

To evaluate the clonality, a representative sample of trypsin-inducible and trypsin-uninducible biofilms isolates from each body part was prepared. Genotyping of 50 isolates was made by a PFGE protocol for *S. aureus* described by The Centre for Disease Control and Prevention, Atlanta, USA. Chromosomal DNA of each strain was extracted and digested with *Sma*I restriction endonuclease (New England Biolabs). Restriction fragments were resolved in a CHEF GenePath System (Bio-Rad®). Classification of the clones was based on Tenover criteria (Tenover et al., 1995), and the percentage of relatedness was determined by the Dice coefficient (Dice, 1945). Isolates with cut-off values of 85% were considered as belonging to the same clone.

2.3 | Detection of protease-independent biofilm and protease-induced biofilm formation in isolates

Detection of protease-independent biofilm formation (defined in this paper as biofilm formed without the addition of protease to the culture medium) was performed according to standard Christensen's method (Christensen et al., 1985). All CoNS isolates were inoculated in tryptic soy broth (TSB; Sigma-Aldrich) and incubated for 24 hr at 37°C. They were then inoculated into 96-well tissue culture plates (Nunc, Thermo Fisher Scientific) in TSB medium (1:200 dilution). The plates were incubated for 24 hr at 37°C. Following the incubation, the plates were washed vigorously with 1× phosphate-buffered saline (PBS), dried for 30 min at 55°C, and stained with 0.5% (w/v) crystal violet solution. After staining, the plates were washed with 1× PBS. The absorbance (A_{492}) of adhered, stained cells was measured using a Multiskan GO Microplate spectrophotometer (Thermo Fisher Scientific).

The average A_{492} values were calculated for all tested isolates. *S. epidermidis* RP62A and *S. epidermidis* ATCC 12228 were used as positive and negative controls, respectively, and all tests were performed in triplicate and repeated three times. The cut-off value ($A_{492}c$) was established. It is defined as three standard deviations (*SD*) above the mean A_{492} of the negative control: $A_{492}c$ = average A_{492} of negative control + (3 × *SD* of negative control). Final A_{492} value of a tested strain was expressed as average A_{492} value of the strain reduced by $A_{492}c$ value (A_{492} = average A_{492} of a strain − $A_{492}c$). The $A_{492}c$ value was calculated for each microtiter plate separately.

Detection of trypsin-induced biofilm formation was performed as described above, but supplementing different concentrations of trypsin (0.2, 2, and 20 μg/ml; Gibco, Thermo Fisher Scientific) to the medium. The plates were incubated for 24 hr at 37°C. Following the incubation, biofilm formation and cut-off values were determined as described above.

2.4 | Scanning electron microscopy (SEM)

Isolates were inoculated as previously mentioned except that polystyrene Petri dishes with clean glass coverslips were used. Biofilm formation was induced, adding 20 μg/ml of trypsin and with incubation at 37°C for 24 hr, allowing the biofilm formation on the coverslips. The dishes were then washed three times with 1× PBS and dried at 25°C. For SEM analysis, the samples were fixed in 2.5% glutaraldehyde/formaldehyde for 30 min and then rinsed in 1× PBS before being fixed in osmium tetroxide. The samples were then rinsed three times with 1× PBS and were dehydrated serially in 30%, 50%, 70%, 90%, and 100% ethanol. The material was mounted and processed using the gold-plating method. A Denton Vacuum Desk II sputter coater was used to cover samples which were deposited onto specimen mounts and observed with a JEOL JSM 5800-LV microscope at an accelerating voltage of 15 kV.

2.5 | Bacterial viability testing after ciprofloxacin treatment

The viability of cells in both planktonic and trypsin-induced biofilm forms in the presence of ciprofloxacin was measured by MTT reduction assay reported by Brambilla, Endo, Cortez, and Filho (2017) with some modifications (Brambilla et al., 2017). For the planktonic state, the isolates were first grown in blood agar for 24 hr. Bacterial suspensions were then prepared and adjusted to 0.5 value on the McFarland standard scale. The dilutions (10 μl) were deposited in wells of 96-well flat bottom microplates (Nunc) containing 100 μl of Mueller Hinton Broth (MHB, Sigma-Aldrich) supplemented with

different concentrations of ciprofloxacin (1, 8, 64, and 512 μg/ml). The microplates were statically incubated for 24 hr at 37°C; the cells were recovered and centrifuged, the medium was removed, the cells were washed with 0.85% sodium chloride solution, and 100 μl of 1% (w/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO) was immediately added; the plates were incubated for 2 hr at room temperature. Cells were recovered again and centrifuged, and MTT was then replaced with 100 μl of dimethyl sulfoxide (DMSO), and the plates were incubated at room temperature in the dark for 15 min. Finally, sample color intensity was determined at 600 nm using a Multiskan GO Microplate spectrophotometer (Thermo Fisher Scientific).

For the evaluation of bacterial viability within trypsin-induced biofilms, biofilm formation was first induced on plates with MHB supplemented with 20 μg/ml of trypsin, for 24 hr at 37°C. The culture medium was removed, and the plates were washed gently twice with 0.85% sodium chloride solution to remove planktonic cells. Mueller Hinton Broth (100 μl) was supplemented with ciprofloxacin as specified above. The microplates were incubated for 24 hr at 37°C, and the culture medium was removed and replaced with 1% MTT; the plates were then treated, and the results were read as described above. The experiments were performed in triplicate; biofilm-forming *S. epidermidis* RP62A strain was included as a positive control.

2.6 | Amplification of *ica* and *aap* genes by PCR

Bacterial DNA was isolated, as described by Catalanotti et al. (2005). The *icaA* and *aap* genes were amplified using the primers listed in Table 1. PCR amplifications were performed using 1 μl of DNA template (100 ng), 1× buffer, 1 mM $MgCl_2$, 5 mM of each dNTPs, 1 U of Taq DNA polymerase (Invitrogen), and 0.2 μM of each specific primer. PCR conditions were as follows: 30 cycles of 30 s at 92°C, 40 s at 60°C, and 30 s at 72°C. PCR products were analyzed on agarose gels.

2.7 | RT-qPCR of *aap*

Overnight cultures in TSB of trypsin-inducible and trypsin-uninducible *S. epidermidis* isolates were diluted 1:200 in new TSB with or without trypsin (20 μg/ml) in 24-well tissue culture plates (Nunc) and incubated for 24 hr at 37°C. Cells were collected afterward, and the trypsin-induced biofilms were scraped from the bottom of the wells. RNA purification and RT-qPCR were performed as previously described (Martínez-García et al., 2019). Briefly, cells were washed with 1× PBS, and total RNA was extracted with TRIzol (Invitrogen), treated with DNase I (Invitrogen), and re-extracted. For the reverse transcriptase (RT) reaction, total RNA (3 μg) with 0.5 μg of oligo-hexamers (Invitrogen) was denatured at 70°C for 10 min. Then, 1× single strand buffer, 0.5 mM DTT, 10 mM of each dNTPs, and 200 U of MMLV reverse transcriptase (Invitrogen) were added. Reverse transcriptase reactions were performed at 42°C for 1 hr. The expression of *16SrRNA*, used as a control, and *aap* was determined using the primers of Table 1. Relative expression was determined by the $2^{-\Delta\Delta Ct}$ method. The results shown are expressed as the average of triplicates, and the standard deviation is represented by error bars.

TABLE 1 Primers used in this study

Gene	Sequence (5′→3′)	Reference
icaA	Fw: TCTCTTGCAGGAGCAATCAA Rv: AGGCACTAACATCCAGCA	Catalanotti et al. (2005)
aap	Fw: AGAAACAAGCTGGTCAAG Rv: CTGCGTAGTTAAGAAAATC	Juárez-Verdayes et al. (2013)
16SrRNA	Fw: AGGAGTCTGGACCGTGTCTC Rv: GCGTAGCCGACCTGAGAG	Juárez-Verdayes et al. (2013)

2.8 | Statistical analysis

Differences between controls and trypsin-induced biofilm formation were analyzed using one-way ANOVA with Tukey's test. Analyses of proportions were performed using Fisher's exact test. The odds ratio was used to associate between genotypes and trypsin-induced biofilm producers. The analyses were done employing GraphPad Prism software version 5.0.

3 | RESULTS

3.1 | Detection of protease-independent biofilm and trypsin-induced biofilm formation in *S. epidermidis* isolates

Christensen's method was used to detect protease-independent biofilm formation in all *S. epidermidis* isolates (n = 188) to compare clinical and commensal isolates. Of 88 clinical isolates (OI and PJI), 38 isolates (43.2%) were protease-independent biofilm producers. Of 100 commensal isolates (HS-1), 17 isolates (17%) were protease-independent biofilm producers. This comparison gave a statistically significant difference (p < .0001). The remaining 133 isolates were non-biofilm producers.

Both clinical and commensal non-biofilm-forming isolates (n = 133) were treated with trypsin to determine which of them showed a trypsin-induced biofilm phenotype. A total of 88 isolates (88/133, 66.1%) formed biofilm after treatment with trypsin. 52% of clinical isolates showed a trypsin-induced biofilm formation, while 74.7% of commensal isolates showed it as well (p = .0023). The comparison between protease-independent biofilm (17%) and trypsin-induced biofilm (74.7%) of commensal isolates had a significant difference (p < .0001). In clinical isolates, this comparison was not significantly different (p = .3757; Table 2). These results suggest a significant increase in the number of commensal isolates showing a trypsin-induced biofilm phenotype.

Rohde et al. (2004) concluded that protease-induced biofilm formation by *S. epidermidis* is another biofilm formation mechanism. However, this result was obtained with only four non-biofilm-forming isolates. In this work, a significant number of isolates were included

TABLE 2 Detection of protease-independent biofilm and trypsin-induced biofilm formation in *Staphylococcus epidermidis* isolates

Biofilm *n* = 188	Clinical Protease-independent biofilm (A) *n* = 88	Commensal Protease-independent biofilm (B) *n* = 100	Clinical trypsin-induced biofilm (C) *n* = 50	Commensal trypsin-induced biofilm (D) *n* = 83	Clinical total biofilm (E) *n* = 88	Commensal total[a] biofilm (F) *n* = 100
Positive biofilm (%)	38 (43.2)	17 (17)	26 (52)	62 (74.7)	64 (72.7)	79 (79)
Negative biofilm (%)	50 (56.8)	83 (83)	24 (48)	21 (25.3)	24 (27.3)	21 (21)
Significant difference	A/B $p < .0001$ A/C $p = .3757$		C/D $p = .0087$	D/B $p < .0001$	E/F $p = .392$; A/E $p < .0001$	B/F $p < .0001$

[a]Total biofilm formation value is obtained from the sum of protease-independent biofilm and trypsin-induced biofilm.

to support the suggested mechanism. Thus, we established two categories of biofilm for *S. epidermidis*: a biofilm where proteases are not required (denominated protease-independent biofilm in this paper) and a protease-induced biofilm (Rohde et al., 2004). Likewise, we introduced the term "total biofilm" for data management, meaning the sum of protease-independent biofilm and trypsin-induced biofilm isolates. Comparisons between clinical and commensal isolates showed that the total biofilm for the clinical isolates and commensal isolates was 72.7% and 79%, respectively, without statistically significant difference between these two groups ($p = .1298$). In contrast, the comparison between the protease-independent biofilm phenotype with the total biofilm of the clinical isolates was statistically different ($p < .0001$). Similarly, this occurred between commensal groups ($p < .0001$; Table 2). Overall, these results support those reported by Rohde et al., in the sense that the trypsin-induced biofilm formation is an important mechanism to be considered. Besides, there was a significant increase in the number of commensal isolates with the trypsin-induced biofilm phenotype.

3.2 | Concentration of trypsin and structure-function of trypsin-induced biofilm

According to Rohde et al. (2004), four PJI isolates were treated with five different concentrations of trypsin (0.02, 0.2, 2, 20, and 200 μg/ml), and only concentrations between 0.2 and 20 μg/ml were able to induce biofilms; they did not demonstrate whether these concentrations affected bacterial growth or if the structure and function of the trypsin-induced biofilm is altered.

To confirm that trypsin does not hurt microbial growth at these concentrations, bacterial growth curves were performed. Trypsin concentrations of 0.2, 2, and 20 μg/ml did not affect microbial growth, but growth inhibition was observed at 2000 μg/ml of trypsin (Figure 1). Thus, these results demonstrate that concentrations between 0.2 and 20 μg/ml of trypsin do not affect bacterial growth.

SEM was used to evaluate the structure of trypsin-induced biofilms in comparison with those formed by protease-independent biofilms (Figure 2). The protease-independent biofilm showed a dense, uniform, and thick layer (100× magnification, Figure 2a) compared with trypsin-induced biofilms, which showed a nonuniform structure (100× magnification, Figure 2c). At 10,000× magnification, an extracellular matrix was observed in the protease-independent biofilm (Figure 2b), while in the trypsin-induced biofilm, there is no extracellular matrix observable (Figure 2d). In the absence of trypsin, biofilm formation was not observed, and only a few cells adhered to the glass surface could be observed (Figure 2e,f).

A biofilm confers specific properties to bacteria that they do not have in the planktonic state, including resistance against antibiotics. A cell survival assay was made to demonstrate that trypsin-induced biofilm formation also protects the cells within the biofilm. Survival of protease-independent biofilm-forming *S. epidermidis* RP62A in the presence of different concentrations of ciprofloxacin was higher than the cell survival in the planktonic state. This was also observed in trypsin-induced biofilms of *S. epidermidis* (Figure 3). These results

demonstrate that trypsin-induced biofilms in the tested concentrations do not cause microbial death, and the biofilms formed from this treatment have a structure and function similar to protease-independent biofilms.

We searched for a single trypsin concentration to induce biofilm formation. The optimal trypsin concentration for the induction of biofilm formation in *S. epidermidis* isolates was first determined. It was observed that non-biofilm-forming *S. epidermidis* isolates treated with different concentrations of trypsin (0.2, 2, and 20 μg/ml) formed biofilms. Nevertheless, a single trypsin concentration capable of inducing biofilm formation in all the isolates tested was not found because the isolates from different sources had different proportions of trypsin-induced biofilms at various concentrations of trypsin (Table 3). The results suggested the need to test the three concentrations to get a more significant number of isolates capable of produce trypsin-induced biofilms.

3.3 | Trypsin-induced biofilms in samples of different sources

Examining trypsin-induced biofilm data from different sources showed that the OI isolates have 45.8% of protease-independent biofilms, 92.3% of trypsin-induced biofilms and 95.8% of total biofilms; significant differences were found in protease-independent biofilms and trypsin-induced biofilms (p = .0111) but not in trypsin-induced biofilms or total biofilms (p = 1). Similarly, HS-1 commensal isolates had a significant increase in trypsin-induced biofilms; this result was statistically significant compared with protease-independent biofilms (p < .0001) but not with total biofilms (p = .234). In contrast, PJI isolates showed no increase in trypsin-induced biofilm (37.8%) in comparison with protease-independent biofilms (42.2%; p = .8336), but there was a significant difference when compared to total biofilms (64.1%; p = .0134; Table 4).

Comparing the protease-independent biofilm-forming OI isolates (45.8%) with the protease-independent biofilm-forming HS-1 isolates (17%), a significant difference was found (p = .0052). However, no significant difference was found between the percentage of total biofilm of OI isolates (95.8%) and HS-1 isolates (79%, p = .0722). The same pattern was observed for PJI isolates: The significant difference was found in protease-independent biofilm-forming PJI isolates (42.2%) and protease-independent biofilm-forming HS-1 isolates (17%; p = .0006) but not to the percentage of total biofilm of PJI (64.1%) and HS-1 isolates (79%; p = .0669; Table 4).

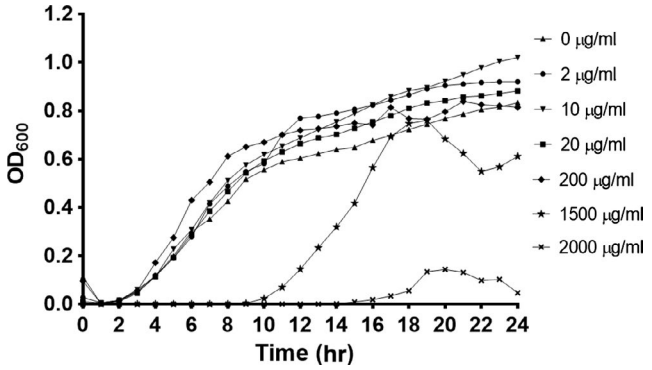

FIGURE 1 Growth of *Staphylococcus epidermidis* in the presence of trypsin. Microbial growth was determined in triplicate and was monitored by measuring changes in optical density at 600 nm at different concentrations of trypsin

All isolates used in this work have been characterized genotypically (PFGE, *agr* type, and SSC*mec*), showing a high diversity among them (Flores-Páez et al., 2015; Ortega-Peña et al., 2019). When including phenotypic data (production of protease-independent biofilm, trypsin-induced biofilm or no production of trypsin-induced biofilm) in the phylogenetic trees reported, we did not find a correlation between the isolates and different phenotypes.

3.4 | Commensal isolates from different body sites

Due to the significant increase of trypsin-induced biofilms in the commensal HS-1 isolates of healthy individuals, we sought to observe the effect on isolates from different body sites of a single individual. *S. epidermidis* was isolated from the head, nose, axillae, and healthy conjunctiva of five individuals (HS-2; n = 139). Twenty-three isolates produced protease-independent biofilm (16.5%), and 116 (83.5%) were non-biofilm-forming isolates. Of these, 70 isolates (70/116; 60.3%) were trypsin-induced biofilm producers.

The trypsin-induced biofilm-forming isolates were higher than the protease-independent biofilm-forming isolates in each individual (p < .05), except for individual 3 (Table 5). By body sites of the same individual, it was found variations in trypsin-induced biofilm-forming isolates (ranging from 10% to 100%). A high percentage of trypsin-induced biofilm-forming isolates was observed in different body sites of the individuals, except for axillae (10%) of individual 2 and the head of individual 5 (20%). This result suggests that *S. epidermidis* isolates from different body sites can produce biofilm by treatment with trypsin, although the pattern was inconsistent in the same individual and among different individuals.

In order to determine clonality of the isolates from a body site of same individual, PFGE was used with a representative sample of trypsin-induced and uninduced biofilm-forming isolates. Pulse-field gel electrophoresis was performed with the isolates 8, 12, 12, 8, and 10 from the individuals 1, 2, 3, 4, and 5, respectively. The results showed the presence of 6 out of 8, 7 out of 12, 8 out of 12, 6 out of 8, and 6 out of 10 different clones in individuals 1, 2, 3, 4, and 5, respectively (Figure 4). Some clones (14, 28, and 24 clones) were identified in different body sites of the same individual. Only a pair of isolates belonging to the clone 8 were identified in two different individuals (2 and 4). Interestingly, there was no association between clonality and the capability of isolates to form biofilm in the presence of trypsin, since isolates belonging to the same clone showed these two phenotypes indistinctly.

3.5 | Association between genotype and trypsin-induced biofilm formation

Association between trypsin-induced biofilm producers and the presence of *icaA* and *aap* genes was studied. As shown in Table 6,

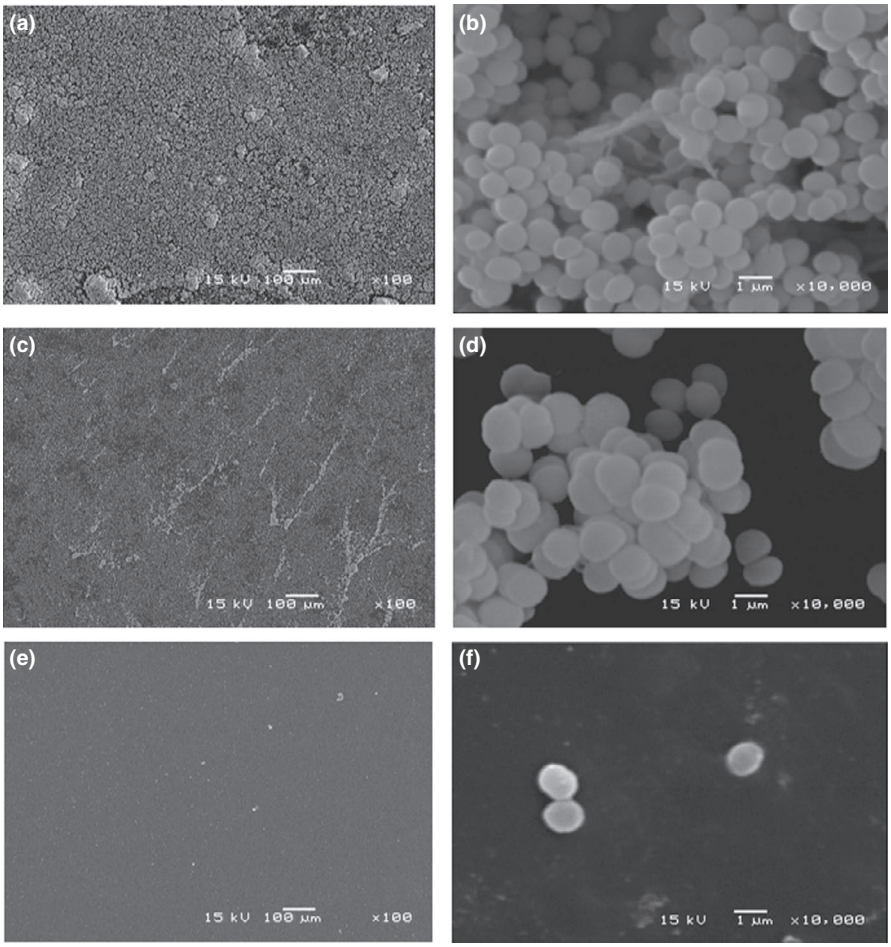

FIGURE 2 Microscopic characteristics of trypsin-induced biofilms. SEM images of *Staphylococcus epidermidis* biofilms. (a) protease-independent biofilm of *S. epidermidis* at 100× and (b) 10,000×. (c) trypsin-inducible biofilm of *S. epidermidis* treated with 20 µg/ml of trypsin at 100× and (d) 10,000×. (e) Trypsin-inducible biofilm of *S. epidermidis* without trypsin treatment at 100×, and (f) 10,000×

trypsin-inducible OI isolates showed a heterogeneous distribution in *icaA*$^+$ (30.7%) and *icaA*$^-$ (61.5%), and there was a low frequency of *icaA*$^+$ in trypsin-uninducible isolates. The opposite was observed for the PJI isolates: 8.1% of the trypsin-inducible isolates were *icaA*$^+$, and 51.3% of the trypsin-uninducible isolates were *icaA*$^-$. The same occurred in HS-1 trypsin-inducible isolates, which were *icaA*$^-$ and in a more significant proportion.

Considering the *aap* gene, OI and HS-1 isolates showed a high frequency of *aap*$^+$ genotype and a low proportion of *aap*$^-$ genotype. In PJI isolates, the proportion of *aap*$^+$ genotype was different from that of trypsin-inducible (37.8%) and trypsin-uninducible (29.7%) isolates ($p < .05$). 32.4% of the isolates were trypsin-uninducible with the *aap*$^-$ genotype.

Trypsin-inducible isolates ($n = 88$) and trypsin-uninducible isolates ($n = 45$) were analyzed by odds ratio test to associate *icaA* and *aap* genes with the biofilm-inducible phenotype. The value of the odds ratio for *icaA*$^+$ and the trypsin-inducible isolates group was 1.285 (95%; 0.4294–3.844), indicating the lack of an association. Nevertheless, for *aap*$^+$ and the trypsin-inducible isolates group, the odds ratio was 6.365 (95%; 2.233–18.14), indicating a strong association between the *aap*$^+$ gene and trypsin-inducible biofilm phenotype.

To determine whether the *aap* expression level was affected by trypsin, mRNA expression levels in a subset of trypsin-inducible and trypsin-uninducible isolates were measured. The trypsin treatment did not increase the *aap* mRNA expression levels in both groups of isolates (trypsin-inducible and trypsin-uninducible isolates), suggesting that trypsin does not affect *aap* expression at the transcriptional level. Besides, no correlation was observed between the *aap* mRNA expression and the level of trypsin-induced biofilm (Figure 5), since the isolates with high trypsin-inducible biofilm level (2HS, 9HS 10HS) had low *aap* mRNA expression, and the isolates with low trypsin-inducible biofilm level (30HC, 14HC) had high *aap* mRNA expression.

3.6 | Trypsin-induced biofilms in non-epidermidis CoNS

Non-epidermidis CoNS isolates ($n = 23$; non-biofilm-forming isolates) were assayed with three concentrations of trypsin. Overall, 21.7% of non-epidermidis CoNS isolates were trypsin-induced biofilm producers (Table 7). Regarding the species, *S. hominis*, *S. warneri* (1.6%), and *S. capitis* (0.8%) were trypsin-induced biofilm producers. Besides, *S. hominis* was found only in HC, while *S. warneri* was found in HS-1 and OI. *S. lentus*, *S. caprae*, *S. sciuri*, *S. haemolyticus*, and *S. lugdunensis* strains isolated from PJI did not produce trypsin-induced biofilms.

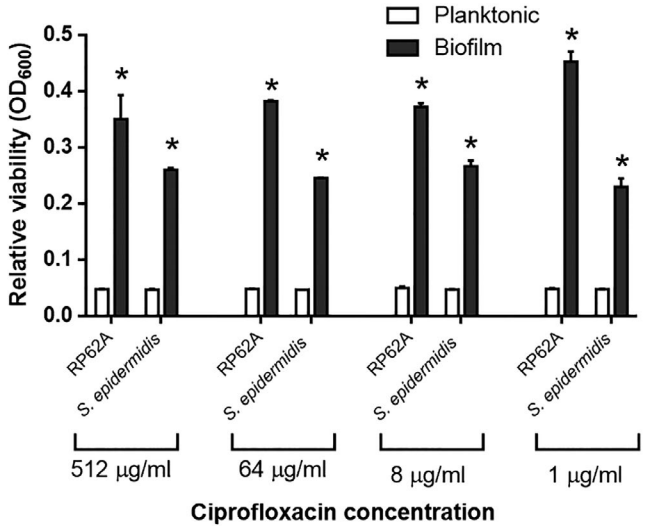

FIGURE 3 Bacterial survival in the presence of ciprofloxacin. Bacteria growing in planktonic and biofilm modes were exposed to different concentrations of ciprofloxacin; bacterial survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The experiments were performed in triplicate. The results were analyzed using a one-way ANOVA with Tukey's test. $p < .0001$ is marked with asterisks

4 | DISCUSSION

Approximately 40%–60% of clinical *S. epidermidis* isolates from peripheral blood infections (Mertens & Ghebremedhin, 2013), prosthesis infections (Hellmark et al., 2013), and ocular infections (Flores-Páez et al., 2015; Juárez-Verdayes et al., 2013) generate in vitro biofilms (defined as protease-independent biofilms in the present work) as determined by Christensen's method. This result is the basis of epidemiological studies demonstrating that clinical isolates of *S. epidermidis* produce more biofilms than commensal isolates, indicating that biofilm formation is a discriminant factor in these two bacterial populations. Rohde et al. demonstrated that four PJI non-biofilm-forming *S. epidermidis* isolates are able to produce biofilm after treatment with trypsin. In this study, more clinical and commensal isolates were included to establish the potential of trypsin-induced biofilm formation. Trypsin-induced biofilm formation was evaluated in 133 non-biofilm-forming *S. epidermidis* isolates from OI, PJI, and HS-1, and 66.1% (88/133) showed the induced phenotype. This result is essential for biofilm formation studies in *S. epidermidis*. The main conclusion of Rohde's report was that protease-mediated induction of biofilm formation can be a general mechanism in *S. epidermidis*, and our results support this idea, indicating the presence of a significant staphylococcal population that requires external proteases for the induction of biofilm formation. Thus, Rohde's work and ours suggest a new approach of biofilm formation in *S. epidermidis*. This paper suggests that protease-induced biofilm formation should be considered in further studies.

It is known that a variety of stimuli influence biofilm expression, including oxygen, salt, glucose, ethanol, nitrites, and many antibiotics

TABLE 3 Proportion of trypsin-induced biofilm formation at different concentrations of trypsin

Trypsin concentration	OI *n* = 12 (%)	PJI *n* = 14 (%)	HS–1 *n* = 62 (%)
0.2 μg/ml	30.7	28.6	6.4
0.2–2 μg/ml	15.4	0	23.4
2 μg/ml	0	0	8.5
2–20 μg/ml	7.7	0	21.3
20 μg/ml	7.7	50.0	6.4
0.2–20 μg/ml	38.5	21.4	34.0

(McCann, Gilmore, & Gorman, 2008). Glucose, ethanol, and salt are mainly known to increase PNAG-dependent biofilm formation (Knobloch et al., 2001). Regarding the protein-dependent biofilms, Hennig et al. reported that ethanol increase biofilm formation in a nonproducing PNAG strain, along with its *aap* transcription, while the addition of NaCl inhibits biofilm formation and *aap* transcription (Hennig et al., 2007). It is important to note that other protein, the extracellular matrix binding protein (Embp), can mediate cell aggregation and biofilm formation in *S. epidermidis* (Christner et al., 2010). Under standard in vitro conditions, Embp is not expressed, but Embp production is induced in the presence of serum (Schommer et al., 2011). Our results demonstrate that trypsin is another stimulus to induce biofilm formation. Also, it has been recently demonstrated that *S. epidermidis* SepA protease can process Aap, leading to biofilm formation (Paharik et al., 2017). Importantly, trypsin might not be the inducing protease of biofilms at *S. epidermidis* infection sites in the human body, but the possibility that proteases acting similarly to trypsin should not be discarded. In this way, Rohde et al. also found that cathepsin G and elastase (neutrophil protease) work similarly to trypsin (Rohde et al., 2004).

The impact of trypsin-induced biofilms on *S. epidermidis* isolates can be seen when comparing clinical and commensal isolates. Epidemiological studies compare protease-independent biofilms in both populations, showing significant differences between them and suggesting that protease-independent biofilms are a discriminant biomarker (Duggirala et al., 2007; Jain & Agarwal, 2009; Okee et al., 2012; Suzuki et al., 2005). The same result was found in our work. However, when comparing total biofilms (the sum of protease-independent biofilms and trypsin-induced biofilms), the significant difference is lost between clinical and commensal isolates, and this also occurs between clinical entities (OI vs. HS-1 and PJI vs. HS-1). This result assumes that if the total biofilms of *S. epidermidis* are compared, biofilms are not a discriminant biomarker between clinical and commensal isolates, suggesting that trypsin-induced biofilm formation must be considered for further epidemiological studies. Harris et al. reported limitations in the use of techniques for the testing of specific pathogenic biomarkers of *S. epidermidis* associated with chronic infections, for example, biofilm formation and expression of RNAIII and PSMγ (Harris et al., 2017). Another important finding was the significant increase in the number of

TABLE 4 Percentages of trypsin-induced biofilm formation in different samples

Biofilm	OI protease-independent biofilm (A) *n* = 24	OI trypsin-induced biofilm (B) *n* = 13	OI total biofilm (C) *n* = 24	PJI protease-independent biofilm (D) *n* = 64	PJI trypsin-induced biofilm (E) *n* = 37	PJI total biofilm (F) *n* = 64	HS–1 protease-independent biofilm (J) *n* = 100	HS–1 trypsin-induced biofilm (K) *n* = 83	HS–1 total biofilm (L) *n* = 100
% Positive biofilm	11 (45.8)	12 (92.3)	23 (95.8)	27 (42.2)	14 (37.8)	41 (64.1)	17 (17)	62 (74.7)	79 (79)
% Negative biofilm	13 (54.2)	1 (7.7)	1 (4.2)	37 (57.8)	23 (62.2)	23 (35.9)	83 (83)	21 (25.3)	21 (21)
Significant difference	A/B* A/J**	B/C, NS	C/L, NS	D/E, NS D/J***	E/F*	F/L NS	J/K***	K/L, NS	

Abbreviation: NS, not significant difference.
*$p = .01$.
**$p = .001$.
***$p = .0001$.

commensal trypsin-inducible *S. epidermidis* isolates. These isolates have been considered refractory for biofilm formation since they do not carry the *ica* operon, which is an important element for biofilm formation. Nevertheless, trypsin-induced biofilms in commensal isolates suggest that *S. epidermidis* dwelling on healthy skin can produce biofilm but require an exogenous protease, suggesting the existence of a control mechanism in the skin that prevents biofilm-mediated *S. epidermidis* infection. Also, HS *S. epidermidis* has been considered as a possible contaminant capable of producing ocular infections and prosthetic joint infections; from the data obtained in this work, it is possible to suggest that HS *S. epidermidis* can produce a protease-induced biofilm to establish an infection. This idea is supported by the finding that *S. epidermidis* isolated from different body parts of the same individual (head, nose, axillae, and conjunctiva) can form biofilm after trypsin treatment. We found that a significant proportion of commensal *S. epidermidis* isolates (60.3%) can produce trypsin-induced biofilms in healthy individuals. We also found that isolates from the same body site and different individuals correspond to different clones, as reported by Conlan et al. (2012). This finding is important because the high distribution of *S. epidermidis* in the human skin may be the leading cause of contamination of medical devices compared with *S. aureus* (Dimick et al., 2001).

Usually, biofilm-forming strains of *S. epidermidis* produce PNAG, with a biosynthesis directed by the *icaADBC* operon (Heilmann et al., 1996). Some clinical *S. epidermidis* isolates lack the *icaADBC* operon and can still form a biofilm (Dice et al., 2009; Hellmark et al., 2013; Klug et al., 2003; Rohde et al., 2007; Ziebuhr et al., 1997). It has been determined that the Aap protein plays a vital role in the formation of protein-type biofilms by these isolates (Hennig et al., 2007; Hussain et al., 1997; Rohde et al., 2004). Isolates capable of producing a trypsin-induced biofilm were associated with *icaA*$^-$ and *aap*$^+$ genotypes, suggesting that Aap could be involved in trypsin-induced biofilm formation since isolates with the *aap*$^-$ genotype did not form trypsin-induced biofilms. However, *aap* expression levels were the same with or without trypsin in inducible and uninducible isolates, suggesting that the participation of Aap in the trypsin-inducible biofilm formation is not at the transcriptional level. Rohde et al. reported that the 220 kDa Aap protein of *S. epidermidis* 5179 can be digested by trypsin, resulting in a 140 kDa truncated isoform (Rohde et al., 2004). We performed the SDS-PAGE of cell wall proteins of trypsin-inducible and trypsin-uninducible isolates trying to find similar results, but our findings were not conclusive (Appendix 1), suggesting that Aap does not seem to be involved at the transcriptional level, but at Aap processing level. Thus, studies focused on Aap processing must be conducted in the future.

We propose a new systematic procedure to detect *S. epidermidis* biofilms. Protease-independent biofilms are initially determined by the commonly used Christensen's method in all isolates. For those that do not produce biofilms, modified Christensen's method is used, consisting of adding three different trypsin concentrations (0.2, 2, and 20 μg/ml) to the culture media and subsequently assessing biofilm formation. To confirm, *icaA/aap* genotyping of the trypsin-induced biofilm isolates should be *aap*$^+$.

TABLE 5 Trypsin-induced biofilm formation in different parts of human body (HS-2 group)

Individuals	Body sites	Protease-independent biofilm *n* (%)	Negative biofilm *n* (%)	Trypsin-induced biofilm *n* (%)	Percentage
1	Head	0	3	2	66.6
	Nostrils	0	4	3	75
	Axillae	0	4	2	50
	Conjunctiva	1	2	2	100
	Total (*n* = 14)	1 (7.1%)	13 (92.8)	9 (69.2)	*p = .0013
2	Head	0	10	6	60
	Nostrils	1	9	4	44.4
	Axillae	0	10	1	10
	Conjunctiva	1	2	2	100
	Total (*n* = 33)	2 (6%)	31 (93.9)	13 (42)	*p = .0009
3	Head	4	6	3	50
	Nostrils	6	4	2	50
	Axillae	0	7	3	42.8
	Conjunctiva	1	2	2	100
	Total (*n* = 30)	11 (36.6)	19 (63.3)	10 (52.6)	p = .3759
4	Head	3	7	5	71.4
	Nostrils	0	10	10	100
	Axillae	0	9	9	100
	Conjunctiva	0	3	3	100
	Total (*n* = 32)	3 (9.3)	29 (90.7)	27 (93.1)	*p < .0001
5	Head	0	10	2	20
	Nostrils	0	10	7	70
	Axillae	6	1	1	100
	Conjunctiva	0	3	3	100
	Total (*n* = 30)	6 (20)	24 (73.4)	11 (54.2)	*p = .0116

*p is between protease-independent biofilm and trypsin-induced biofilm.

The use of three concentrations of trypsin is necessary because it allows us to obtain the highest number of trypsin-inducible isolates. It is necessary to point out that the employed concentrations are safe because they do not affect the cell growth of *S. epidermidis*.

When this procedure was used in non-epidermidis CoNS, the proportions of isolates that could or could not form a trypsin-induced biofilm were not significantly different, suggesting that trypsin is not adequate for biofilm induction in these isolates. Information concerning biofilm formation in non-epidermidis CoNS is limited, and the molecular mechanisms mediating biofilm formation are less studied (Frank & Patel, 2007; Fredheim et al., 2009; Szczuka, Telega, & Kaznowski, 2014; Yokoi et al., 2016). A mechanism concerning a protease that favors biofilm formation has not yet been found. *S. lugdunensis* and *S. haemolyticus* biofilms are composed mainly of proteins and not by polysaccharides, but such proteins and the mechanisms involved have not been identified (Frank & Patel, 2007; Fredheim et al., 2009). Since it is highly probable that *S. epidermidis* Aap is involved in trypsin-induced biofilm formation as reported by Rohde et al. (Rohde et al., 2004), we performed a BLAST search of Aap-like proteins in other CoNS using *S. epidermidis* Aap amino acid sequence as a query. Percentages of identity found for other *Staphylococcus* species were 54% for *S. hominis* (Sequence ID: WP_053084241.1), 38% for *S. warneri* (Sequence ID: WP_058660273), 52% for *S. capitis* (Sequence ID: AKL93117.1), and 61% for *S. simulans* (Sequence ID: WP_001208316.1), indicating that Aap-like proteins produced by other staphylococci species are different from those produced by *S. epidermidis*. Therefore, it might partially explain why trypsin is not adequate for the process.

In conclusion, the current study provides evidence that trypsin has a high biofilm induction capacity in non-biofilm-forming clinical and commensal *S. epidermidis* isolates, suggesting a general mechanism of *S. epidermidis*. Besides, trypsin can significantly increase trypsin-induced biofilm formation in commensal isolates. Our results show that there is no significant difference when comparing total biofilms of clinical and commensal isolates, suggesting that biofilms are not a determinant biomarker. We also propose a modification of Christensen's method and a systematic procedure to determine total biofilms in *S. epidermidis*.

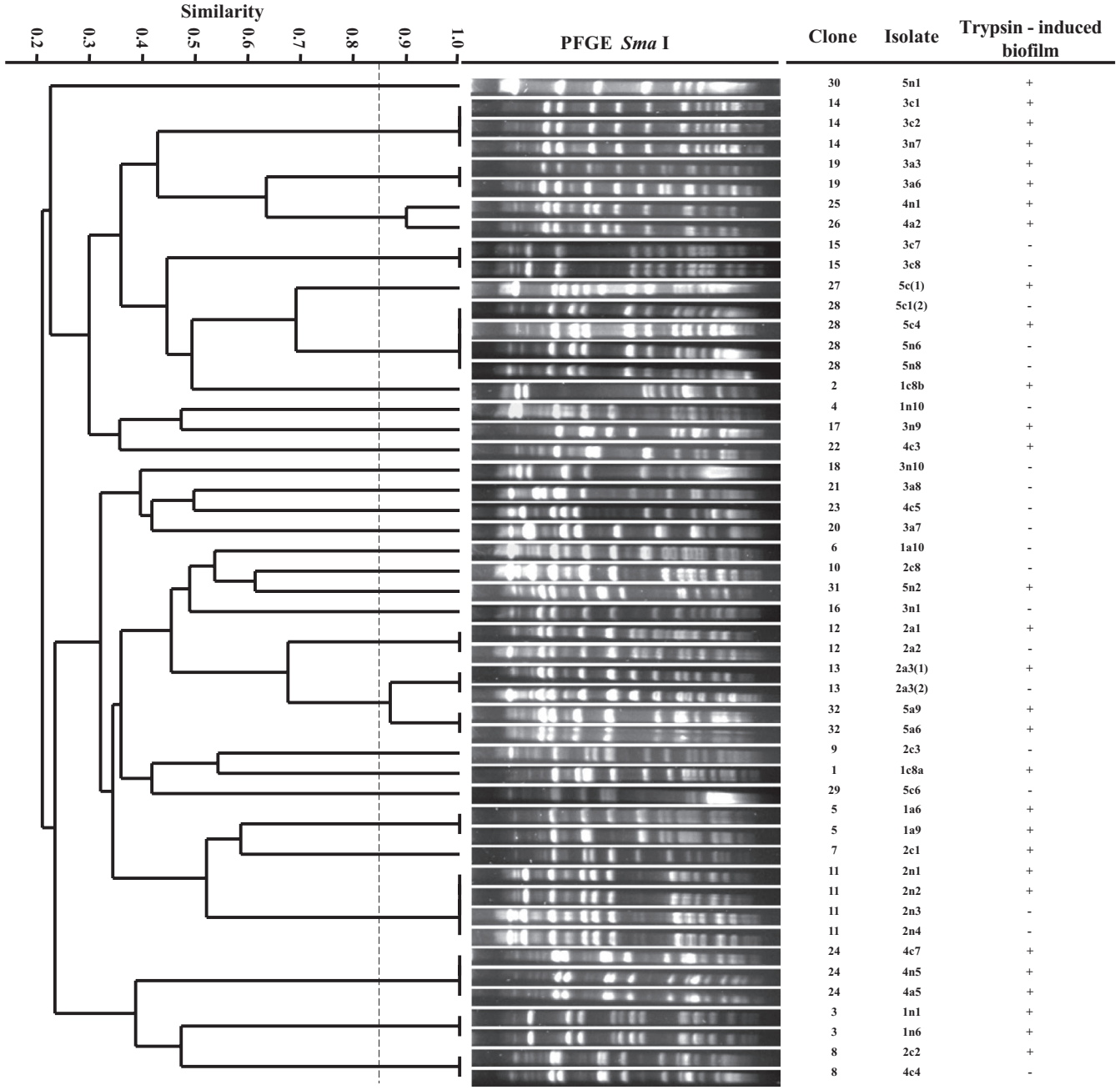

FIGURE 4 Clonality of trypsin-inducible and trypsin-uninducible isolates. Pulsed-field gel electrophoresis (PFGE) dendrogram and genetic relatedness of 50 *Staphylococcus epidermidis* isolated from different body sites of five healthy subjects (HS-2). The letter in the isolate's codes indicates the body part from where they were obtained (n, nostril; a, axillae; c, head)

TABLE 6 *icaA* and *aap* genotypes in trypsin-induced biofilm isolates

Genotype	Inducible OI isolates (*n* = 12)	Uninducible OI isolates (*n* = 1)	Inducible PJI isolates (*n* = 14)	Uninducible PJI isolates (*n* = 23)	Inducible HS−1 isolates (*n* = 62)	Uninducible HS−1 isolates (*n* = 21)
icaA^{+} (%)	4 (33.3)	0 (0)	3 (21.4)	4 (17.4)	8 (12.9)	1 (4.7)
icaA^{-} (%)	8 (66.7)	1 (100)	11 (78.6)	19 (82.6)	54 (87.1)	20 (95.3)
aap^{+} (%)	12 (100)	1 (100)	14 (100)[a]	11 (47.8)	56 (90.3)	20 (95.3)
aap^{-} (%)	0 (0)	0 (0)	0 (0)	12 (52.2)	6 (9.7)	1 (4.7)

[a]Statistically significant difference by Fisher's exact test between induced biofilm and uninducible biofilm groups.

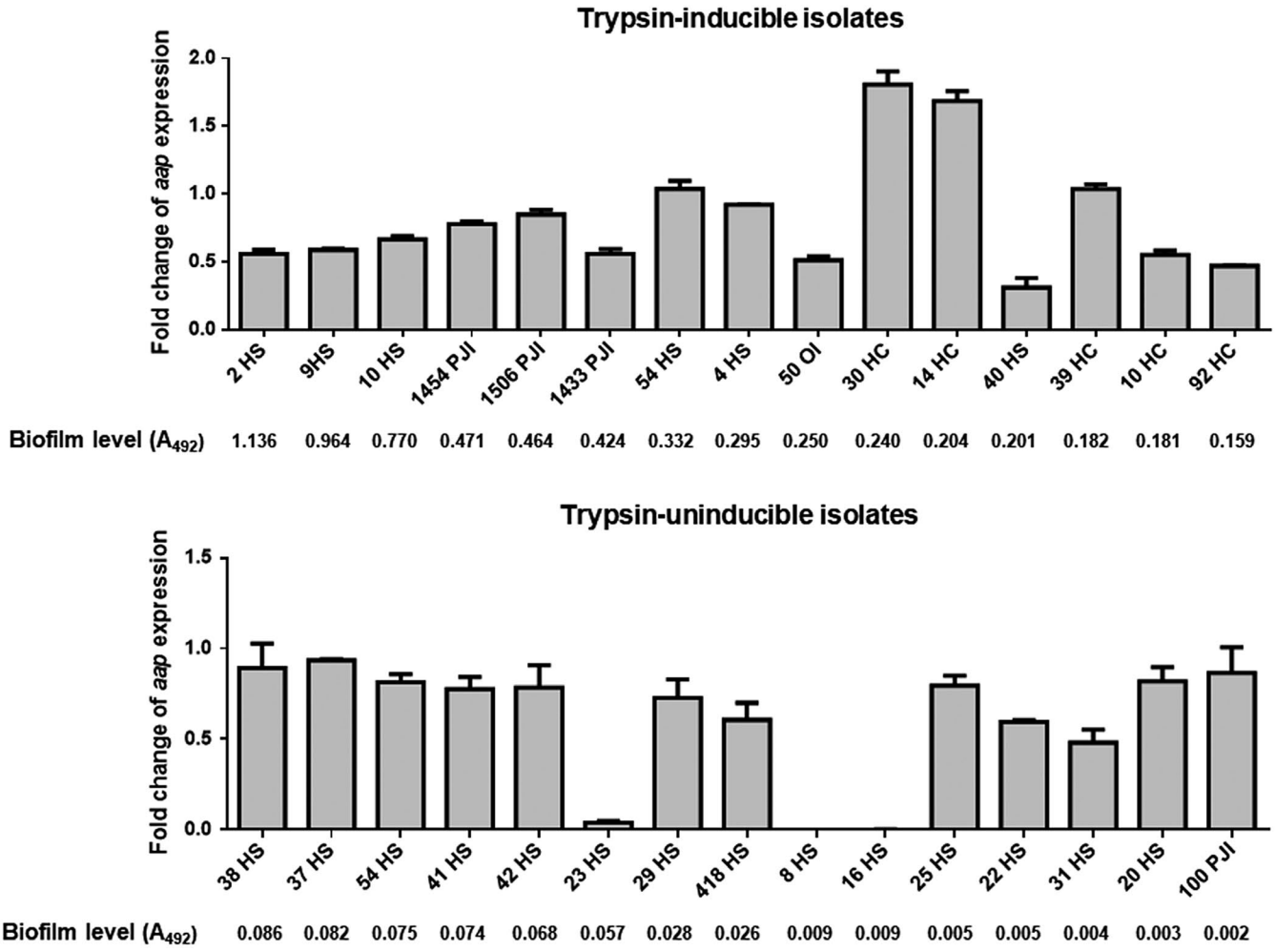

FIGURE 5 Relative *aap* expression in trypsin-inducible and uninducible isolates. The isolates were grown statically with and without trypsin. Relative expression was determined by the $2^{-\Delta\Delta Ct}$ method. The results shown are expressed as the average of triplicates, and the standard deviation is represented by error bars

TABLE 7 Trypsin-induced biofilm in non-epidermidis CoNS isolates

	Staphylococcus hominis		*Staphylococcus warneri*		*Staphylococcus capitis*		Others[a]	
Isolates *n* = 23	Inducible *n* = 2 (18.2%)	Uninducible *n* = 9 (81.8%)	Inducible *n* = 2 (40%)	Uninducible *n* = 3 (60%)	Inducible *n* = 1 (50%)	Uninducible *n* = 1 (50%)	Inducible *n* = 0 (0)	Uninducible *n* = 5 (100%)
HC (*n* = 5)	2	1	0	1	1	0	0	0
HS-1 (*n* = 6)	0	3	1	2	0	0	0	0
OI (*n* = 1)	0	0	1	0	0	0	0	0
PJI (*n* = 11)	0	5	0	0	0	1	0	5

[a]*Staphylococcus lentus*, *Staphylococcus caprae*, *Staphylococcus sciuri*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis*.

However, this procedure is not applicable in the cases of non-epidermidis CoNS.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

SMG and SOP performed the experiments. MJHC and MGAA performed the identification of isolates by MALDI-TOF. GBC and SMPT designed and performed the experiments of scanning electron microscopy (SEM). MDAC performed PFGE analysis of isolates. JJR, SRM, and MECD helped with the conceiving of the study and the manuscript draft. JCCD conceived the study, purchased materials, and participated in the study's design and coordination. All authors read and approved the final manuscript.

ETHICS STATEMENT

This study was carried out following the recommendations of the ethics board committee of the "Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional." The protocol was approved by the ethics board committee of the "Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional." All subjects gave written informed consent under the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

All data associated with the article have been included in this manuscript.

REFERENCES

Brambilla, L. Z. S., Endo, E. H., Cortez, D. A. G., & Filho, B. P. D. (2017). Anti-biofilm activity against *Staphylococcus aureus* MRSA and MSSA of neolignans and extract of *Piper regnellii*. *Brazilian Journal of Pharmacognosia*, *27*(2017), 112–117.

Büttner, H., Mack, D., & Rohde, H. (2015). Structural basis of *Staphylococcus epidermidis* biofilm formation: mechanisms and molecular interactions. *Frontiers in Cellular and Infection Microbiology*, *5*, 14. https://doi.org.10.3389/fcimb.2015.00014.

Catalanotti, P., Lanza, M., Del Prete, A., Lucido, M., Catania, M. R., Gallè, F., … Rossano, F. (2005). Slime-producing *Staphylococcus epidermidis* and *S. aureus* in acute bacterial conjunctivitis in soft contact lens wearers. *New Microbiology*, *28*(4), 345–354.

Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, M. D., & Beachey, E. H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology*, *22*(6), 996–1006.

Christner, M., Franke, G. C., Schommer, N. N., Wendt, U., Wegert, K., Kroll, G., … Rohde, H. (2010). The giant extracellular matrix binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. *Molecular Microbiology*, *75*(1), 187–207.

Conlan, S., Mijares, L. A., Becker, J., Blakesley, R. W., Bouffard, G. G., Brooks, S., … Segre, J. A. (2012). *Staphylococcus epidermidis* pan-genome sequence analysis reveals diversity of skin commensal and hospital infection-associated isolates. *Genome Biology*, *13*, R64. https://doi.org/10.1186/gb-2012-13-7-r64

Darouiche, R. O. (2004). Treatment of infections associated with surgical implants. *New England Journal of Medicine*, *350*(4), 1422–1429. https://doi.org/10.1056/NEJMra035415

Dice, B., Stoodley, P., Buchinsky, F., Metha, N., Ehrlich, G. D., & Hu, F. Z. (2009). Biofilm formation by *ica*-positive and *ica*-negative strains of *Staphylococcus epidermidis in vitro*. *Biofouling*, *25*(4), 367–375.

Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology*, *26*(3), 297–302. https://doi.org/10.2307/1932409

Dimick, J. B., Pelz, R. K., Consunji, R., Swoboda, S. M., Hendrix, C. W., & Lipsett, P. A. (2001). Increased resource use associated with catheter-related bloodstream infection in the surgical intensive care unit. *Archives of Surgery*, *136*(2), 229–234. https://doi.org/10.1001/archsurg.136.2.229

Duggirala, A., Kenchappa, P., Sharma, S., Peeters, J. K., Ahmed, N., Garg, P., … Hasnain, S. E. (2007). High-resolution genome profiling differentiated *Staphylococcus epidermidis* isolated from patients with ocular infections and normal individuals. *Investigative. Ophthalmology and Visual Science*, *48*(7), 3239–3245.

Flores-Páez, L. A., Zenteno, J. C., Alcántar-Curiel, M. D., Vargas-Mendoza, C. F., Rodríguez-Martínez, S., Cancino-Diaz, M. E., … Cancino-Diaz, J. C. (2015). Molecular and phenotypic characterization of *Staphylococcus epidermidis* isolates from healthy conjunctiva and a comparative analysis with isolates from ocular infection. *PLoS ONE*, *10*(8), e0135964. https://doi.org/10.1371/journal.pone.0135964

Frank, K. L., & Patel, R. (2007). Poly-*N*-acetylglucosamine is not a major component of the extracellular matrix in biofilms formed by *icaADBC*-positive *Staphylococcus lugdunensis* isolates. *Infection and Immunity*, *75*(10), 4728–4742. https://doi.org/10.1128/IAI.00640-07

Fredheim, E. G. A., Klingenberg, C., Rohde, H., Frankenberger, S., Gaustad, P., Flægstad, T., & Sollid, J. E. (2009). Biofilm formation by *Staphylococcus haemolyticus*. *Journal of Clinical Microbiology*, *47*(4), 1172–1180. https://doi.org/10.1128/JCM.01891-08

Harris, L. G., Dudley, E., Rohde, H., Frommelt, L., Siemssen, N., Wilkinson, T. S., & Mack, D. (2017). Limitations in the use of PSMγ, *agr*, RNAIII and biofilm formation as biomarkers to define invasive *Staphylococcus epidermidis* from chronic biomedical device-associated infections. *International Journal of Medical Microbiology*, *307*(7), 382–387. https://doi.org/10.1016/j.ijmm.2017.08.003

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., & Götz, F. (1996). Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Molecular Microbiology*, *20*(5), 1083–1091. https://doi.org/10.1111/j.1365-2958.1996.tb02548.x

Hellmark, B., Söderquist, B., Unemo, M., & Nilsdotter-Augustinsson, Å. (2013). Comparison of *Staphylococcus epidermidis* isolated from prosthetic joint infections and commensal isolates in regard to antibiotic susceptibility, *agr* type, biofilm production, and epidemiology. *International Journal of Medical Microbiology*, *303*(2013), 32–39. https://doi.org/10.1016/j.ijmm.2012.11.001

Hennig, S., Nyunt Wai, S., & Ziebuhr, W. (2007). Spontaneous switch to PIA-independent biofilm formation in an *ica*-positive *Staphylococcus epidermidis* isolate. *International Journal of Medical Microbiology*, *297*(2), 117–122. https://doi.org/10.1016/j.ijmm.2006.12.001

Hussain, M., Herrmann, M., Von Eiff, C., Perdreau-Remington, F., & Peters, G. (1997). A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infection and Immunity*, *65*(2), 519–524.

Jain, A., & Agarwal, A. (2009). Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. *Journal of Microbiological Methods*, *76*(2009), 88–92.

Juárez-Verdayes, M. A., Ramón-Peréz, M. L., Flores-Páez, L. A., Camarillo-Márquez, O., Zenteno, J. C., Jan-Roblero, J., … Cancino-Diaz, J. C. (2013). *Staphylococcus epidermidis* with the *icaA*$^{-}$/*icaD*$^{-}$/IS*256*$^{-}$ genotype and protein or protein/extracellular-DNA biofilm is frequent in ocular infections. *Journal of Medical Microbiology*, *62*(Pt 10), 1579–1587. https://doi.org/10.1099/jmm.0.055210-0

Klug, D., Wallet, F., Kacet, S., & Courcol, R. J. (2003). Involvement of adherence and adhesion *Staphylococcus epidermidis* genes in pacemaker lead-associated infections. *Journal of Clinical Microbiology*, *41*(7), 3348–3350. https://doi.org/10.1128/JCM.41.7.3348-3350.2003

Knobloch, J.-K.-M., Bartscht, K., Sabottke, A., Rohde, H., Feucht, H.-H., & Mack, D. (2001). Biofilm formation by *Staphylococcus epidermidis* depends on functional RsbU, an activator of the *sigB* operon: Differential activation mechanisms due to ethanol and salt stress. *Journal of Bacteriology*, *183*(8), 2624–2633. https://doi.org/10.1128/JB.183.8.2624-2633.2001

Macintosh, R. L., Brittan, J. L., Bhattacharya, R., Jenkinson, H. F., Derrick, J., Upton, M., & Handley, P. S. (2009). The terminal A domain of the fibrillar accumulation-associated protein (Aap) of *Staphylococcus epidermidis* mediates adhesion to human corneocytes. *Journal of Bacteriology*, *191*(22), 7007–7016. https://doi.org.10.1128/JB.00764-09.

Magill, S., Edwards, J., Bamberg, W., Beldavs, Z., Dumyati, G., Kainer, M., … Fridkin, S. K. (2014). Multistate point-prevalence survey of health care–associated infections. *New England Journal of Medicine*, *370*(13), 1198–1208. https://doi.org/10.1056/NEJMoa1306801

Martínez-García, S., Ortiz-García, C. I., Cruz-Aguilar, M., Zenteno, J. C., Murrieta-Coxca, J. M., Pérez-Tapia, S. M., … Cancino-Diaz, J. C. (2019). Competition/antagonism associations of biofilm formation among *Staphylococcus epidermidis* Agr groups I, II, and III. *Journal of Microbiology*, *57*(2), 143–153. https://doi.org/10.1007/s12275-019-8322-5

McCann, M. T., Gilmore, B. F., & Gorman, S. P. (2008). *Staphylococcus epidermidis* device-related infections: Pathogenesis and clinical management. *Journal of Pharmacy and Pharmacology*, *60*(12), 1551–1571.

Mertens, A., & Ghebremedhin, B. (2013). Genetic determinants and biofilm formation of clinical *Staphylococcus epidermidis* isolates from blood cultures and indwelling devises. *European Journal of Microbioogy and Immunology*, *3*(2), 111–119.

Okee, M. S., Joloba, M. L., Okello, M., Najjuka, F. C., Katabazi, F. A., Bwanga, F., … Kateete, D. P. (2012). Prevalence of virulence determinants in *Staphylococcus epidermidis* from ICU patients in Kampala, Uganda. *Journal of Infection in Developing Countries*, *6*(3), 242–250.

Ortega-Peña, S., Vargas-Mendoza, C. F., Franco-Candejas, R., Aquino-Andrade, A., Vázquez-Rosas, G. J., Betanzos-Cabrera, G., … Cancino-Diaz, J. C. (2019). *sesA*, *sesB*, *sesC*, *sesD*, *sesE*, *sesG*, *sesH*, and *embp* genes are genetic markers that differentiate commensal isolates of *Staphylococcus epidermidis* from isolates that cause prosthetic joint infection. *Infectious Diseases*, *51*(6), 435–445.

Otto, M. (2009). *Staphylococcus epidermidis*: The "accidental" pathogen. *Nature Reviews Microbiology*, *7*(8), 555–567. https://doi.org/10.1038/nrmicro2182

Paharik, A., Kotasinska, M., Both, A., Hoang, T., Büttner, H., Roy, P., … Rohde, H. (2017). The metalloprotease SepA governs processing of accumulation-associated protein and shapes intercellular adhesive surface properties in *Staphylococcus epidermidis*. *Molecular Microbiology*, *103*(5), 860–874.

Rohde, H., Burandt, E. C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., … Mack, D. (2007). Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials*, *28*(9), 1711–1720. https://doi.org/10.1016/j.biomaterials.2006.11.046

Rohde, H., Kalitzky, M., Kröger, N., Scherpe, S., Horstkotte, M. A., Knobloch, J. K., … Mack, D. (2004). Detection of virulence-associated genes not useful for discriminating between invasive and commensal *Staphylococcus epidermidis* strains from a bone marrow transplant unit. *Journal of Clinical Microbiology*, *42*(12), 5614–5619. https://doi.org.10.1128/JCM.42.12.5614-5619.2004

Schoenfelder, S. M. K., Lange, C., Eckart, M., Hennig, S., Kozytska, S., & Ziebuhr, W. (2010). Success through diversity – How *Staphylococcus epidermidis* establishes as a nosocomial pathogen. *International Journal of Medical Microbiology*, *300*(6), 380–386. https://doi.org/10.1016/j.ijmm.2010.04.011

Schommer, N. N., Christner, M., Hentschke, M., Ruckdeschel, K., Aepfelbacher, M., & Rohde, H. (2011). *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. *Infection and Immunity*, *79*(6), 2267–2276. https://doi.org/10.1128/IAI.01142-10

Suzuki, T., Kawamura, Y., Uno, T., Ohashi, Y., & Ezaki, T. (2005). Prevalence of *Staphylococcus epidermidis* strains with biofilm-forming ability in isolates from conjunctiva and facial skin. *American Journal of Ophthalmology*, *140*(5), 844–850. https://doi.org/10.1016/j.ajo.2005.05.050

Szczuka, E., Telega, K., & Kaznowski, A. (2014). Biofilm formation by *Staphylococcus hominis* strains isolated from human clinical specimens. *Folia Microbiologica*, *60*(1), 1–5. https://doi.org/10.1007/s12223-014-0332-4

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed- field gel electrophoresis: Criteria for bacterial strain typing. *Journal of Clinical Microbiology*, *33*(9), 2233–2239.

Weiner, L. M., Webb, A. K., Limbago, B., Dudeck, M. A., Patel, J., Kallen, A. J., … Sievert, D. M. (2016). Antimicrobial-resistant pathogens associated with healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. *Infection Control and Hospital Epidemiology*, *37*(11), 1288–1301. https://doi.org/10.1017/ice.2016.174

Yokoi, K. J., Kuzuwa, S., Iwasaki, S. I., Yamakawa, A., Taketo, A., & Kodaira, K. I. (2016). Aureolysin of *Staphylococcus warneri* M accelerates its proteolytic cascade, and participates in biofilm formation. *Bioscience, Biotechnology and Biochemistry*, *80*(6), 1238–1242.

Ziebuhr, W., Heilmann, C., Götz, F., Meyer, P., Wilms, K., Straube, E., & Hacker, J. (1997). Detection of the intercellular adhesion gene cluster (*ica*) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. *Infection and Immunity*, *65*(3), 890–896.

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APPENDIX 1

We suspected that Aap is processed by trypsin to induce biofilm formation, which is supported by the work of Rohde et al. (2004). It has been demonstrated that Aap is a polymorphic protein of variable molecular weight, mainly due to variations in the number of repeats on the B domain (Büttner et al., 2015) and that clinical isolates can bear variations in their B domains (Rohde et al., 2004). It has been also found that the number of B repeats affects adhesion properties of *Staphylococcus epidermidis* (Macintosh et al., 2009). We performed SDS-PAGE of cell wall proteins of trypsin-inducible and uninducible isolates. However, of the subset of isolates tested, we detected, in one isolate, a protein of molecular weight of approximately 220 kDa which disappears when the isolate is treated with trypsin. However, this is not observed in other tested isolates (Figure A1). This result can be explained for the Aap features mentioned above, mainly due to Aap polymorphism.

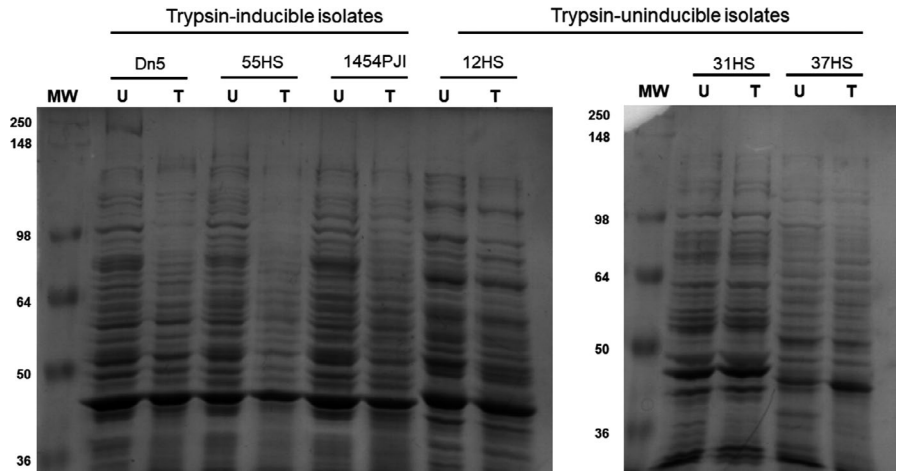

FIGURE A1 SDS-PAGE 7.5% of cell wall proteins of trypsin-inducible and uninducible *Staphylococcus epidermidis* isolates. Cells were grown in 24-well tissue plates with TSB, then recovered (trypsin-induced biofilms were scraped from the bottoms of wells), washed with 1× PBS, treated with lysostaphin and lysozyme, centrifuged, and the proteins in the supernatant were recovered. Protein concentration was determined, and the same quantity was charged in wells. MW, molecular weight ladder (kDa); T, treated; U, untreated with 20 μg/ml trypsin