

Fernando Oliveira, Claudia Afonso Lima, Susana Brás, Ângela França, Nuno Cerca***Evidence for inter- and intra-species biofilm formation variability among a small group of coagulase-negative staphylococci**

Centre of Biological Engineering, LIBRO – Laboratory of Research in Biofilms Rosário Oliveira, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.

* Corresponding author: Nuno Cerca, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal. Phone: (+351) 253604423. Fax: (+351) 253504400. E-mail: nunocerca@ceb.uminho.pt

Abstract

Coagulase-negative staphylococci (CoNS) are common bacterial colonisers of the human skin. They are often involved in nosocomial infections due to biofilm formation in indwelling medical devices. While biofilm formation has been extensively studied in *Staphylococcus epidermidis*, little is known regarding other CoNS species. Here, biofilms from six different CoNS species were characterised in terms of biofilm composition and architecture. Interestingly, the ability to form a thick biofilm was not associated with any particular species, and high variability on biofilm accumulation was found within the same species. Cell viability assays also revealed different proportions of live and dead cells within biofilms formed by different species, although this parameter was particularly similar at the intra-species level. On the other hand, biofilm disruption assays demonstrated important inter- and intra-species differences regarding extracellular matrix composition. Lastly, confocal laser scanning microscopy (CLSM) experiments confirmed this variability, highlighting important differences and common features of CoNS biofilms. We hypothesised that the biofilm formation heterogeneity observed was rather associated with biofilm matrix composition than with cells themselves. Additionally, our results indicate that polysaccharides, DNA and proteins are fundamental pieces in the process of CoNS biofilm formation.

Keywords: coagulase negative staphylococci; biofilm formation; flow cytometry; biofilm disruption assays; CLSM

Introduction

Coagulase-negative staphylococci (CoNS) are widely disseminated in the environment, colonizing specific ecological niches, including the human skin, where they usually develop a benign relationship with their hosts (Otto, 2009). However, they are also opportunistic pathogens, being the causative agents of a wide range of infections, especially hospital-acquired infections (Vadyvaloo & Otto, 2005). This shift from a commensal to a pathogenic lifestyle is frequently achieved when external barriers, such as the skin, are damaged (e.g. implantation of indwelling medical devices) (Longauerova, 2006). Persistence in the host and evasion of the immune machinery are the pathogenic strategies employed by these bacteria, which are mainly achieved by their striking ability to form thick, multi-layered biofilms (Otto, 2008). Biofilms are commonly referred as structured communities of microorganisms attached to a surface and embedded in a self-produced matrix of extracellular polymeric components, mainly polysaccharides, proteins and nucleic acids (Donlan & Costerton, 2002). It is well established that bacteria exhibiting a biofilm phenotype are recalcitrant to antimicrobial therapy (Cerca *et al.*, 2005) and can evade the mechanisms of innate host defence (Cerca *et al.*, 2006).

Interestingly, it has been proposed that biofilm production is also essential in an environment like the human skin, where CoNS are exposed to extensive mechanical stress (Otto, 2009).

Most of the knowledge about CoNS biofilm formation is essentially derived from studies on *Staphylococcus epidermidis*. So far, this process is known to comprise three major steps: (i) bacterial adhesion to artificial or natural surfaces, (ii) accumulation and formation of multi-layered bacterial consortia, and (iii) dispersal of biofilm cells (Rohde *et al.*, 2006). A key event during the accumulative phase is the production of the extracellular matrix, which is crucial in the stabilisation of the biofilm architecture (Otto, 2008). One of the first described factors mediating biofilm accumulation in *S. epidermidis* was the polysaccharide intercellular adhesin (PIA), a poly-N-acetylglucosamine (PNAG) synthesised by the *icaADBC* operon-encoded enzymes (Ziebuhr *et al.*, 1997). Besides PIA, other factors mediating biofilm accumulation have been identified, especially the accumulation-associated protein (Aap) (Rohde *et al.*, 2005), a homologue to the *S. aureus* biofilm-associated protein (Bap), called Bhp (Tormo *et al.*, 2005), and the extracellular matrix binding protein (Embp) (Christner *et al.*, 2010). It is therefore clear that different molecules play important roles in the accumulative phase of *S. epidermidis* biofilm formation (Schommer *et al.*, 2011).

However, and notwithstanding all the knowledge achieved during the last decade in this field, CoNS biofilm formation needs to be addressed in species other than *S. epidermidis*, since most of them have been recognised as important nosocomial pathogens (Piette & Verschraegen, 2009). This work aimed to evaluate the biofilm formation ability displayed by six different CoNS species as well as to characterise the extracellular matrix produced by those biofilms.

Materials and methods

Bacteria and growth conditions

Ten CoNS isolates were selected out of one collection of 61 CoNS recently isolated from healthy individuals (Oliveira & Cerca, 2013), comprising six different species (Table 1). *S. epidermidis* ATCC 35984 (RP62A) was used as reference strain.

Table 1. List of microorganisms used in this study

Species	Origin
<i>S. epidermidis</i> RP62A	ATCC 35984
<i>S. epidermidis</i> SECOM010B	Healthy 52-years old male
<i>S. epidermidis</i> SECOM020A.1	Healthy 15-years old male
<i>S. equorum</i> SECOM021B	Healthy 15-years old male
<i>S. equorum</i> SECOM060A	Healthy 79-years old female
<i>S. haemolyticus</i> SECOM047A	Healthy 51-years old female
<i>S. haemolyticus</i> SECOM065A.1	Healthy 20-years old male
<i>S. hominis</i> SECOM057B	Healthy 40-years old male
<i>S. hominis</i> SECOMM11	Healthy 22-years old male
<i>S. capitis</i> SECOM052A	Healthy 28-years old male
<i>S. warneri</i> SECOMF16	Healthy 21-years old female

Bacterial identification at the species level was achieved by partially sequencing *rpoB* gene, as previously described {Mellmann, 2006 #72}. Partial *rpoB* sequences were then analysed using BLAST (*Basic Local Alignment Search Tool*: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and BiBi (*Bio-informatic Bacterial identification*: <http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi>).

For each experiment, isolated colonies were picked from Tryptic Soy Agar (TSA, Liofilchem, Teramo, Italy) plates, inoculated in Tryptic Soy Broth (TSB) (Liofilchem) and incubated overnight at 37°C with shaking at 120 rpm (ES-20 Shaker-Incubator, BioSan, Riga, Latvia).

Quantitative biofilm assays

Biofilm formation ability of each isolate was determined using a modified microtiter-plate assay (Stepanovic *et al.*, 2007), during 24 or 48 h of growth. Briefly, overnight cultures were adjusted to $\sim 2 \times 10^8$ CFU/ml and diluted 1:100 in TSB supplemented with 0.4 % (w/v) glucose (TSBg, Fisher Scientific, Waltham, MA, US). Subsequently, 200 μ l of each bacterial suspension were placed into a 96-well microtiter-plate (Orange Scientific, Braine-l'Alleud, Belgium) and incubated at 37°C with shaking at 120 rpm for 24/ 48 h, with medium replacement at 24 h (Cerca *et al.*, 2004). Then, bacterial suspensions were removed carefully and biofilms were washed twice with 200 μ l of 0.9 % NaCl. The plates were then air-dried and biofilms were stained with crystal violet (Stepanovic *et al.*, 2000). Absorbance was measured at 570 nm using a microplate reader (Synergy HT, BioTek Instruments, USA). All isolates were tested in eight wells in two parallel runs, with at least three biological replicates.

Cell viability assessment by flow cytometry

Twenty-four and 48 h-old biofilms were formed as mentioned above. Flow cytometry experiments were carried out using SYBR Green and propidium iodide staining as optimized before (Cerca *et al.*, 2011), with minor modifications. In brief, biofilms were suspended in 1 ml of 0.9 % NaCl and then sonicated, using a 13 mm probe tip (Cole-Parmer 750-Watt Ultrasonic Homogenizer 230 VAC, IL, USA), for 5 plus 5 seconds at 40 % amplitude in order to disrupt cell clusters. Importantly, the viability of biofilm cells was not impaired by this process (Freitas *et al.*, 2014). Thereafter, 10 μ l of suspended biofilm cells were mixed with 90 μ l of phosphate buffered saline containing 1:80000 of SYBR Green (Invitrogen, CA, USA) and 20 μ g/ml of propidium iodide (Sigma, MO, USA), and the number of cells assessed using an EC800™ flow cytometer (Sony Biotech, CA, USA). A total of 45000 events were acquired with a sample flow rate of 10 μ l/min. Data analysis was performed using EC800™ 1.3.6 analysis software (Sony Biotech). This experiment was performed twice.

Biofilm disruption assays

Biofilm disruption assays were carried out following previously published protocols (Kogan *et al.*, 2006, Fredheim *et al.*, 2009). Pre-formed biofilms (24 or 48 h) were treated, for 2 h at 37°C, with (i) 40 mM NaIO₄ (Sigma-Aldrich, MO, USA) dissolved in water (Kalman & Cruickshank, 1970), (ii) 0.1 mg/ml proteinase K (Sigma-Aldrich) in 20 mM Tris-HCl (pH 7.5) and 1 mM CaCl₂ (Ebeling *et al.*, 1974), or (iii) 0.1 mg/ml DNase I (Sigma-Aldrich) in 20 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂ and 0.5 mM CaCl₂ (Liao *et al.*, 1973). Control wells were filled with water or appropriate buffer. After treatment, biofilms were quantified as described above. Each treatment condition was assayed in quadruplicate with at least three biological replicates.

Confocal laser scanning microscopy (CLSM) analysis

Biofilms were grown for 48 h as described above but using 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium), and then stained with: (i) 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for nucleic acids visualization, (ii) wheat germ agglutinin (WGA) conjugated with Oregon Green (Invitrogen, Paisley, UK), which stains *N*-acetyl-D-glucosamine residues (Wright, 1984); and (iii) FilmTracer SYPRO Ruby Biofilm Matrix stain (Invitrogen), which labels most classes of proteins (Berggren *et al.*, 2000). All staining procedures were performed according to the manufacturer instructions. The stained biofilms were visualized under an Olympus™ FluoView FV1000 confocal laser scanning microscope (Olympus™ FluoView FV1000, Olympus, Lisboa, Portugal). Biofilms were observed using a 60 \times water-immersion objective (60 \times /1.2 W), in at least ten different regions of each surface analysed and images were acquired with 512 \times 512 resolution. Two surfaces of two independent biological replicates were observed in each CLSM experiment, and representative images were selected.

Statistical analysis

Data were analysed using GraphPad Prism version 6.02 (La Jolla, CA, USA). Outliers were identified and excluded using Grubbs' test. Differences among groups were detected using two-way ANOVA with Tukey's multiple comparisons test. A $p < 0.05$ was considered statistically significant. Biofilm disruption data was classified into three groups: no disruption ($\leq 5\%$), moderate disruption, and strong disruption ($\geq 50\%$).

Results

Quantification of biofilm formation

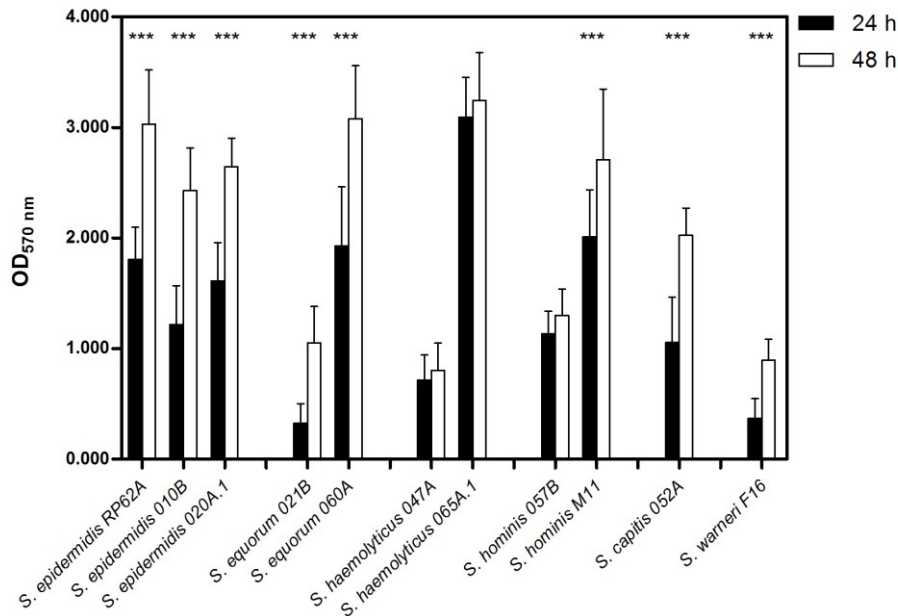


Figure 1. Biofilm formation by *S. epidermidis* RP62A and 10 CoNS isolates on 96-well microtiter plate when grown in TSBg for 24 and 48 h. Biofilms were stained with crystal violet and quantified at OD₅₇₀. Bars represent the average of eight duplicate wells. Error bars represent the standard deviation of at least three independent assays.

Our first assay revealed a high degree of variability regarding the amount of biomass produced among the group of isolates tested, both at 24 and 48 h of growth (Figure 1). Some isolates produced higher amounts of biofilm than *S. epidermidis* RP62A, considered a strong biofilm producer (Mack *et al.*, 1996). This variability was observed not only among different species, but also within the same species, which was particularly evident for *S. equorum*, *S. haemolyticus* and *S. hominis*. Not surprisingly, the amount of biofilm biomass at 48 h was significantly higher than the observed at 24 h, for all tested species, except for *S. haemolyticus*.

Flow cytometry

Live/dead staining was used to assess the number of total, live, damaged and dead cells within 24 and 48 h-old biofilms. The number of total cells increased from 24 to 48 h of incubation in most isolates, although such increment was statistically significant only for *S. epidermidis* RP62A and *S. capitis* 052A (Figure 2A). On the other hand, and although a slight variation was observed in the number of total cells among different species, this parameter was particularly similar within isolates of the same species. Regarding cell viability (Figure 2B), all isolates produced biofilms with $\geq 80\%$ live cells at 24 h of growth. After 48 h, it was observed an overall increment on the proportion of dead cells, although this phenomenon was more relevant in *S. haemolyticus*, *S. hominis* and *S. warneri* biofilms (40-50 % of dead cells, in some cases). Conversely, biofilms formed by *S. epidermidis*, *S. equorum*, and *S. capitis* isolates exhibited a much lower proportion of dead cells. The proportion of damaged cells did not exceeded 10 % of

the number of total cells for most biofilms. Curiously, no fluorescence was detected for *S. equorum* 060A, even after increasing the staining procedure for 1 hour.

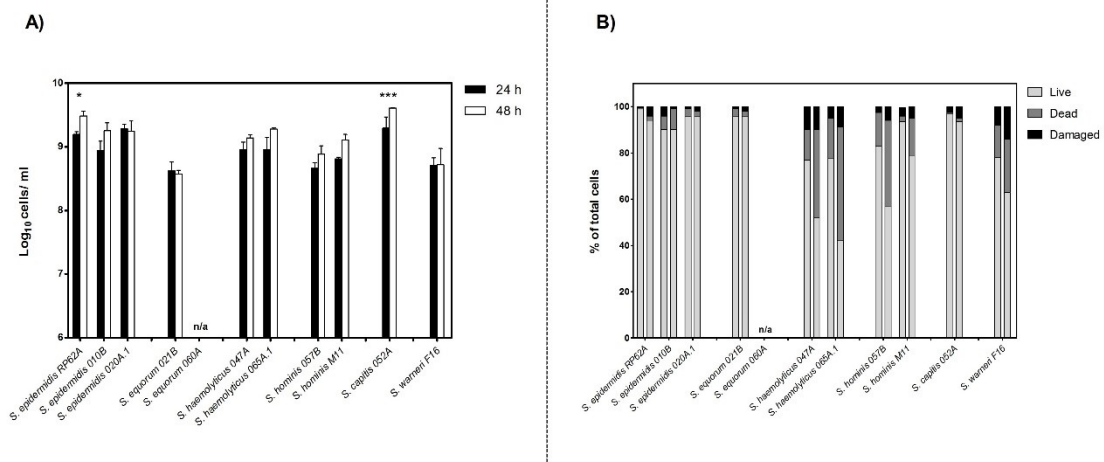


Figure 2. Number of total cells (A), and proportion of live, dead, and damaged cells (B) within 24- (left) and 48-h (right) biofilms as determined by flow cytometry analysis. Bars represent the average \pm standard deviation of two independent assays. n/a, not applicable.

Disruptive effect of NaIO₄, proteinase K, and DNase on CoNS biofilms

In order to unravel the molecules behind biofilm accumulation in the CoNS species tested, the composition of their biofilm matrices was assessed by using a biofilm disruption assay. According to the disruption pattern exhibited by each isolate it was possible to partially infer their biofilm matrix composition (Figure 3). From a general point of view, significant variations in the biofilm disruption pattern were observed among isolates. Additionally, the disruption effects observed in 24 and 48 h-old biofilms were similar, with some minor exceptions. *S. epidermidis* RP62A biofilms, known to contain high amount of polysaccharides (Mack *et al.*, 1996), were partially disrupted by NaIO₄ and highly resistant to proteinase K and DNase I treatments. Proteinase K was the most effective compound, disrupting at varying extents almost all 24 and

Isolate	Time (h)	NaIO ₄	DNase I	Proteinase K
<i>S. epidermidis</i> RP62A	24	22%	3%	1%
	48	48%	3%	3%
<i>S. epidermidis</i> SECOM010B	24	0%	0%	10%
	48	11%	2%	20%
<i>S. epidermidis</i> SECOM020A.1	24	44%	11%	15%
	48	52%	0%	26%
<i>S. equorum</i> SECOM021B	24	3%	27%	39%
	48	48%	40%	31%
<i>S. equorum</i> SECOM060A	24	12%	52%	79%
	48	38%	33%	54%
<i>S. haemolyticus</i> SECOM047A	24	0%	22%	37%
	48	25%	29%	30%
<i>S. haemolyticus</i> SECOM065A.1	24	18%	19%	3%
	48	40%	4%	11%
<i>S. hominis</i> SECOM057B	24	1%	26%	28%
	48	0%	0%	19%
<i>S. hominis</i> SECOMM11	24	26%	20%	1%
	48	22%	31%	13%
<i>S. capitis</i> SECOM052A	24	1%	26%	57%
	48	5%	34%	32%
<i>S. warneri</i> SECOMF16	24	0%	24%	22%
	48	22%	16%	27%

Figure 3. Biofilm disruption pattern of *S. epidermidis* RP62A and 10 biofilm-producing CoNS isolates after treatment of 24- and 48-h pre-formed biofilms with NaIO₄, DNase I and proteinase K [\leq 5 % disruption (white); moderate disruption (grey); \geq 50 % disruption (black)].

48 h-old biofilms. DNase I treatment was also effective at disrupting biofilms formed by most species tested, although at lower extents than those observed with proteinase K. All *S. epidermidis* biofilms were resistant to DNase I treatment.

CLSM

After inferring the composition of the different CoNS biofilms, we used CLSM in conjugation with three different fluorescent stains to differentiate bacterial cells from polysaccharides and proteins within the biofilm matrix. Figure 4 shows representative images of all CLSM experiments (the respective z-stacks can be found in Supplementary Figure 1). In accordance with the results obtained in the previous experiments, it was not possible to identify a pattern of biofilm composition and structure within each species. While some biofilms exhibited a compact structure, with bacterial cells evenly distributed, others exhibited a mushroom-like structure

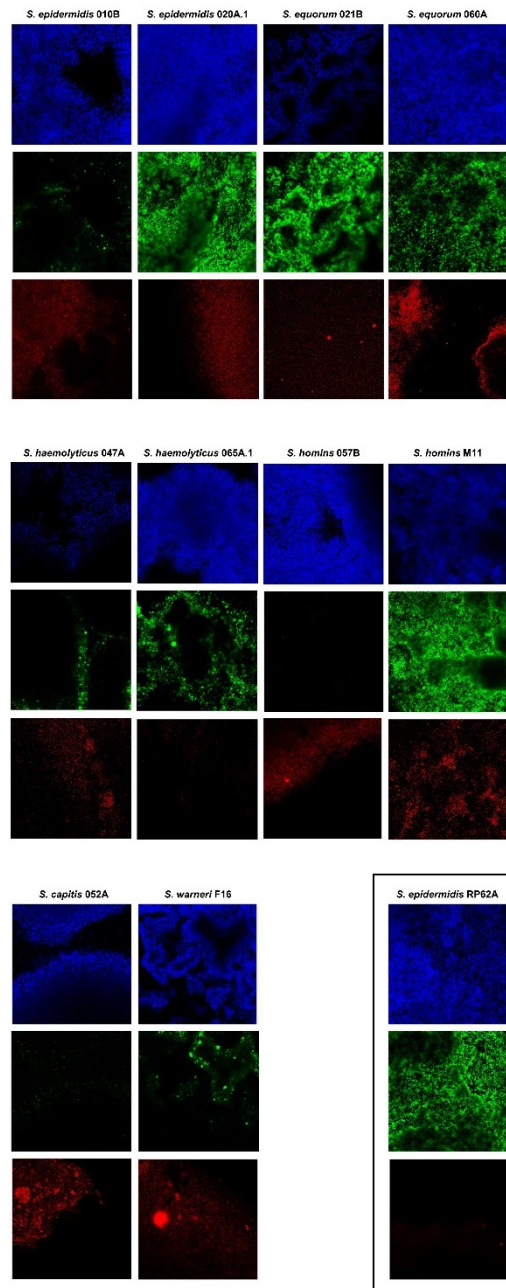


Figure 4. Confocal laser scanning microscopy of CoNS biofilms stained with DAPI (depicted in blue), WGA-Oregon green (depicted in green) and SYPRO Ruby (depicted in red) (more details can be found in Supplementary Figure 1).

surrounded by hollow regions, which may be channels. Moreover, it was evident that PIA presented two main distribution patterns: often it can be widely distributed throughout biofilms or organised into clusters. Proteins were found equally distributed throughout the biofilms tested.

Discussion

A common approach for the characterisation of biofilms is the determination of the total biomass produced throughout a specific period. Our initial results highlighted a remarkable inter- and intra-species variability. Strain-to-strain biofilm formation variability within some CoNS species have already been reported (Handke *et al.*, 2004, Frank & Patel, 2007, Fredheim *et al.*, 2009, Tremblay *et al.*, 2013). However, unlike these earlier studies, the present work aimed to evaluate biofilm formation at a later maturation stage (48 h), which allowed us to observe that such variability was present even after an extended period of growth. Overall, no correlation between the bacterial species and the degree of biofilm formation was found.

More than just characterizing the total biomass, we were also interested in determining the changes in bacterial populations observed between 24 and 48 h-old biofilms. Interestingly, biofilms with higher number of cells were not necessarily those exhibiting higher biomass amounts (e.g. *S. capitis* 052A). Furthermore, the high intra-species variability observed in the biomass production was not reflected in the number of cells. This was particularly evident for *S. haemolyticus* and *S. hominis* biofilms. Also, biofilms produced by those species exhibited the highest amounts of dead cells, which in the case of *S. haemolyticus* isolates dramatically increased from 24 to 48 h. These were exactly the cases where no further biomass accumulation was observed from 24 to 48 h, suggesting that dead cells did not contribute significantly to biomass production. Nevertheless, it seems clear that the biofilm formation heterogeneity initially observed was rather associated with biofilm matrix production and composition than with the number of cells itself. This hypothesis is further supported by the variability observed in biofilm disruption assays. By using reagents that selectively react with well-known staphylococcal biofilm matrix components (polysaccharides, proteins and extracellular DNA (eDNA)) (Otto, 2008), we observed significant inter- and intra-species variations in the biofilm disruption pattern, which are likely to be in the origin of the variability observed in the biofilm quantification assays. Also, NaIO₄ had a significant disruptive effect in all isolates producing the highest amounts of biofilm biomass, suggesting that polysaccharides are a major component of those biofilms. Regarding eDNA, there has been growing evidence that this molecule plays an important role in the structural integrity and stabilisation of biofilms formed by different bacteria (Das *et al.*, 2013). In the present study, DNase I was able to disrupt a reasonable number of biofilms formed by different species. Interestingly, all *S. epidermidis* biofilms were resistant to DNase I treatment. Qin *et al.* (2007) had already investigated the role of eDNA on *S. epidermidis* biofilm formation, having concluded that this molecule is an important matrix component at the initial stage of biofilm formation, whereas mature biofilms (after 12 h of growth) were fairly resistant to DNase I treatment. Together with the results from the present study, it seems that eDNA does not have a significant role on *S. epidermidis* maturation stage. Nevertheless, our results point out a potential role of eDNA in the matrix of mature biofilms formed by the other species, which is a completely novel concept in *S. warneri*, *S. capitis*, *S. hominis* and *S. equorum*.

Lastly, CLSM experiments were performed in order to inspect the spatial distribution of cells and matrix components within the biofilms formed by each isolate under study. These experiments also allowed validating most of the results drawn from biofilm disruption assays. Despite these assays have revealed a moderate disruption of *S. equorum* 021B biofilms with proteinase K, CLSM has shown that these biofilms were weakly bound by SYPRO Ruby. Taking into account the good correlation between proteinase K-mediated disruption and staining with SYPRO Ruby observed for the other isolates, it is likely that an impaired penetration of the stain towards the deepest biofilm layers may have occurred in this discrepant case.

Despite the reduced number of isolates tested, our results show that CoNS biofilm formation is unlikely to occur due to a single component and/ or process. Instead, this work suggests a multifactorial biofilm formation process, as already described for *S. epidermidis* (Schommer *et al.*, 2011), *S. haemolyticus* (Fredheim *et al.*, 2009), and *S. lugdunensis* (Frank & Patel, 2007), and which seems to occur in other less studied CoNS species.

This study also shows that i) proteins and eDNA are important components of the extracellular matrix, and ii) polysaccharides play a pivotal role in the consolidation and stabilisation of the architecture of thick biofilms. Furthermore, and besides functioning together, apparently these molecules are also able to build up biofilms in an independent manner. PIA-independent biofilm formation has been reported in some staphylococcal species (Fitzpatrick *et al.*, 2005, Frank & Patel, 2007, Qin *et al.*, 2007, Fredheim *et al.*, 2009, Tremblay *et al.*, 2013). To the best of our knowledge, we are the first to report a similar process in other species, namely *S. hominis* and *S. capitis*. Interestingly, a study on *S. hominis* biofilms recently published by Szczuka *et al.* (2015) found that polysaccharides were present in a considerable amount in the biofilm matrix of all strains tested. Therefore, and most importantly, our results indicate that inter- and intra-species variability in the composition of CoNS biofilms is a common phenomenon among these species. Therefore, and taking into account the results drawn from this restricted group of isolates, one has to seriously consider the possibility that each strain produces a unique biofilm, with specific amounts of cells, extracellular matrix composition and architecture.

In the future, it would be important to perform these experiments with a larger number of isolates and from different sources. In our view, since a high degree of biofilm formation heterogeneity seems to be a common feature among this bacterial group, it would be interesting to assess which kind of biofilm matrix composition (e.g. polysaccharide-based or proteinaceous biofilms) and architecture (e.g. compact or mushroom-like biofilms) is more prevalent within these bacteria. This approach could potentially open the door to alternative therapeutic schemes for biofilm-associated CoNS infections, for instance by targeting different molecules involved in this highly complex process.

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Conflict of interests

The authors declare that they have no conflict of interest.

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