Biofilm released cells can easily be obtained in a fed-batch system using *ica+* but not with *ica-* isolates (#48028)

First submission

Guidance from your Editor

Please submit by **17 May 2020** for the benefit of the authors (and your $200 publishing discount) .

Structure and Criteria
Please read the 'Structure and Criteria' page for general guidance.

Author notes
Have you read the author notes on the guidance page?

Raw data check
Review the raw data.

Image check
Check that figures and images have not been inappropriately manipulated.

Privacy reminder: If uploading an annotated PDF, remove identifiable information to remain anonymous.

Files
Download and review all files from the materials page.

4 Figure file(s)
2 Table file(s)
4 Raw data file(s)

For assistance email peer.review@peerj.com

Structure and Criteria

2

Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Speculation is welcome, but should be identified as such.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

3

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Biofilm released cells can easily be obtained in a fed-batch system using *ica*+ but not with *ica*- isolates

Vânia Gaio [1], **Nuno Cerca** [Corresp. 1]

[1] Centre of Biological Engineering (CEB), Laboratory of Research in Biofilms Rosário Oliveira (LIBRO), University of Minho, Braga, Portugal

Corresponding Author: Nuno Cerca
Email address: nunocerca@ceb.uminho.pt

S. epidermidis is one of the major opportunistic bacterial pathogens in healthcare facilities, mainly due to its strong ability to form biofilms in the surface of indwelling medical devices. To study biofilms under *in vitro* conditions, both fed-batch and flow systems are widely used, with the first being the most frequent due to their low cost and ease of use. **Aim.** To asses if a fed-batch system previously developed to obtain biofilm released cells (Brc) from strong biofilm producing *S. epidermidis* isolates could also be used to obtain and characterize Brc from isolates with lower abilities to form biofilms. **Methodology.** The applicability of a fed-batch system to obtain Brc from biofilms of 3 *ica*$^+$ and 3 *ica*$^-$ isolates was assessed by quantifying the biofilm and Brc biomass by optical density (OD) and colony forming units (CFU) measurements. The effect of media replacement procedures of fed-batch systems on the amount of biofilm was determined by quantifying the biofilm and biofilm bulk fluid, by CFU, after consecutive washing steps. **Results.** The fed-batch model was appropriate to obtain Brc from *ica*$^+$ isolates, that presented a greater ability to form biofilms and release cells. However, the same was not true for *ica*$^-$ isolates, mainly because the washing procedure would physically remove a significant number of cells from the biofilm. **Conclusions.** This study demonstrates that a fed-batch system is only feasible to be used to obtain Brc from *S. epidermidis*, when studying strong and cohesive biofilm forming isolates.

Comment [S1]: At the first appearance in the abstract and the text, abbreviations should be preceded by words for which they stand

Stapylococcus epidermidis

Biofilm released cells can easily be obtained in a fed-batch system using *ica*+ but not with *ica*- isolates

Vânia Gaio[1], Nuno Cerca[1]

[1] Laboratory of Research in Biofilms Rosário Oliveira - Centre of Biological Engineering(CEB), University of Minho, Braga, Portugal

Corresponding Author:
Nuno Cerca[1]
Centre of Biological Engineering – University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
Email address: nunocerca@ceb.uminho.pt

Abstract

S. epidermidis is one of the major opportunistic bacterial pathogens in healthcare facilities, mainly due to its strong ability to form biofilms in the surface of indwelling medical devices. To study biofilms under *in vitro* conditions, both fed-batch and flow systems are widely used, with the first being the most frequent due to their low cost and ease of use.

Aim. To asses if a fed-batch system previously developed to obtain biofilm released cells (Brc) from strong biofilm producing *S. epidermidis* isolates could also be used to obtain and characterize Brc from isolates with lower abilities to form biofilms.

Methodology. The applicability of a fed-batch system to obtain Brc from biofilms of 3 ica^+ and 3 ica^- isolates was assessed by quantifying the biofilm and Brc biomass by optical density (OD) and colony forming units (CFU) measurements. The effect of media replacement procedures of fed-batch systems on the amount of biofilm was determined by quantifying the biofilm and biofilm bulk fluid, by CFU, after consecutive washing steps.

Results. The fed-batch model was appropriate to obtain Brc from ica^+ isolates, that presented a greater ability to form biofilms and release cells. However, the same was not true for ica^- isolates, mainly because the washing procedure would physically remove a significant number of cells from the biofilm.

Conclusions. This study demonstrates that a fed-batch system is only feasible to be used to obtain Brc from *S. epidermidis* when studying strong and cohesive biofilm forming isolates.

Keywords. Fed-batch systems; Biofilm-released cells; Biofilm disassembly; *S. epidermidis*

Introduction

Staphylococcus epidermidis is a well-known nosocomial pathogenic associated with recurrent biofilm-infections, acknowledged as the major agent involved in biofilm-associated medical devices infections (Becker et al., 2014). Importantly, this bacterium, which was previously seen as a commensal microorganism due to its benign relationship with the host (Cogen et al., 2008; Gardiner et al., 2017), is nowadays accepted as an important opportunistic pathogen, of particular concern in ill and immunocompromised patients (Otto, 2009). *S. epidermidis* infections are more likely to happen upon invasive procedures involving indwelling medical devices, in which the physiological barriers are compromised, since this bacterium is a ubiquitous inhabitant of the skin and mucosae in humans (Ziebuhr et al., 2006) and has a strong ability to form biofilms on the surface of medical devices (Cerca et al., 2005; Laverty et al., 2013). Bacteria within biofilms are undoubtedly more resistant to antibiotics (Albano et al., 2019; Cerca et al., 2005a; Dias et al., 2018) and to the host immune defense (Cerca et al., 2006; Gray et al., 1984; Yao et al., 2005), contributing to the persistence and recurrence of infections (Mah, 2012; Schommer et al., 2011; Singh and Ray, 2014). For all the reasons, biofilms have been a major research target and extensive studies allowed to characterize the biofilm lifecycle and divide it into three main stages: attachment, maturation and disassembly (as reviewed in (Boles and Horswill, 2011; Otto, 2013)). The importance of a better characterization of the disassembly process in biofilms has been pointed out, since cells released from the biofilm can enter systemic circulation and contribute to the spreading of the infection (Boles and Horswill, 2011; Kaplan, 2010) and cause severe systemic diseases, as bacteraemia (Cervera et al., 2009; Wang et al., 2011) which are associated with high levels of morbidity and mortality among immunocompromised patients (Kleinschmidt et al., 2015; Rogers et al., 2009).

Both fed-batch and dynamic systems have been used to study and characterize initial adhesion (Cerca et al., 2005b; Isberg and Barnes, 2002) and maturation of the biofilm (Moormeier and Bayles, 2014; Periasamy et al., 2012). However, both present advantages and drawbacks, depending on the main focus of the study (Bahamondez-Canas et al., 2019). The few studies addressing disassembly rely almost entirely in dynamic systems, which is not surprising, as these systems present key advantages such as a controlled flow, allowing a continuous diffusion of oxygen, nutrients and waste, and are thought to be a more accurate representation of the

conditions in which biofilms are formed in various diseases, as previously reviewed (Azeredo et al., 2017; Bahamondez-Canas et al., 2019). However, these systems are significantly more expensive and are often more difficult to assemble, being essential to have a good background knowledge on hydrodynamics to study biofilms in such conditions (Yawata et al., 2016). Hence, it is no wonder that fed-batch systems are more frequently used on biofilm research, since they are easier to implement and already widely used *in vitro* condition (Azeredo et al., 2017; Bahamondez-Canas et al., 2019). Thus, the ability to implement fed-batch systems to high-throughput research in biofilms disassembly would be beneficial, as it would allow more studies to be undertaken on this research topic.

Earlier, we demonstrated the feasibility to use a fed-batch system to obtain *S. epidermidis* cells released from biofilms (Brc) (França et al., 2016a; Gaio and Cerca, 2019). However, we failed to include low biofilm forming isolates on those studies and, as a consequence, the applicability of this model on such isolates could be questioned. Hence, the aim of the current study ~~is~~ was to better understand the limitations of a fed-batch system to obtain Brc from *S. epidermidis* biofilms, by testing its potential to characterize Brc from *ica*$^{+}$ and *ica*$^{-}$ isolates with distinct abilities to form biofilms.

Materials & Methods

Bacterial isolates and growth conditions

Six isolates of *S. epidermidis*, with different abilities to form biofilms and characterized by the presence (+) or absence (-) of the intercellular adhesion gene (*ica*), generally involved in *S. epidermidis* biofilm formation (Cafiso et al., 2004) were selected to conduct this study (Table 1). Growth conditions followed the fed-batch model previously described to obtain Brc from *S. epidermidis* (França et al., 2016a). First, a colony of *S. epidermidis* was inoculated into 2 mL of Tryptic Soy Broth (TSB) (Liofilchem, Teramo, Italy) and incubated overnight at 37°C with shaking at 120 rpm in an orbital shaker. The overnight inoculum was then diluted in the same growth medium to reach an optical density (OD) of 0.250±0.05, measured at 640 nm, which corresponds to a concentration of approximately 2×10^{8} CFU/mL (Freitas et al., 2014). To form biofilms, 15 µL of the previously adjusted suspension were added to 1 mL of TSB supplemented with 0.4 % (v/v) glucose (TSBG) to induce biofilm formation, into a 24-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), that was incubated in an orbital incubator at 37

Comment [S2]: No need to provide space

Comment [S3]: No need to provide space

°C with agitation at 120 rpm, for as long as 72 (±1) hours. Spent medium was carefully removed after each 24 (± 1) hours of incubation, followed by washing twice the biofilm with a 0.9 % (m/v) NaCl solution to remove unattached cells. Next, 1 mL of fresh TSBG was carefully added to the biofilms and the plate was incubated in the same temperature and agitation conditions. Then, at either 28, 48 or 72-hours of growth, the supernatant was removed and biofilms were washed twice with saline solution. Remaining biofilms cells were scrapped from the microtiter plate and resuspended in 1 mL of the NaCl solution. Cells were pooled together from at least 4 distinct disrupted biofilms to decrease biofilm formation variability (Sousa et al., 2014). Planktonic cultures were grown in an orbital shaker for 24 (±1) hours at 37 °C with shaking at 120 rpm. Finally, Brc were carefully aspirated from the biofilm bulk fluid after 28, 48 and 72 hours of growth.

Homogenization and quantification of the populations

Before quantification, all 3 populations (disrupted biofilm cells, Brc and planktonic cells) were homogenized by sonication through a pulse of 5 seconds at 40 % amplitude (Ultrasonic Processor Model CP-750, Cole-Parmer, Illinois, U.S.A.). As shown before, this sonication cycle did not affect cell viability (Cerca et al., 2005a). The total biomass of all bacterial populations was quantified by OD measurement at 640 nm (OD_{640}), as previously optimized (Freitas et al., 2014). At least three independent experiments, with technical duplicates, were performed.

Effect of consecutive biofilm washing on cell detachment from the biofilms

Biofilms were formed for 24 hours, as described above. Then, the supernatant was carefully removed and the total number of cells on the supernatant was quantified by CFU. Biofilms were then washed with a saline solution, up to 6 consecutive times. Between each wash, bacteria in the supernatant were quantified by CFU. Simultaneously, the quantification of CFU of the remaining biofilm was done after 1, 2 and 6 washes. Four independent assays were performed for each strain and technical duplicates were used.

Quantification of active dispersion of cells from 24h biofilms

After discarding the spent medium and washing twice the preformed 24 hours biofilms, 1 mL of TSB was carefully added to the wells. In half of the biofilm wells, the newly added TSB was

immediately transferred into empty sterile wells, as described in Figure 1. This medium contained cells released from the pre-established biofilm (Brc), due to the shear forces exerted by medium addition, as determined before (França et al., 2016a). The plates were incubated at 37°C with shaking at 120 rpm. At different time points, a 20 µL aliquot was collected from both conditions. The number of cultivable cells was determined by CFU. Four independent experiments with three technical replicates were performed.

Comparison of the antibiotic susceptibility of Brc collected at distinct timepoints

Brc were collected after 28h, 48h and 72h of biofilm formation. The 28h timepoint was included to assess the effect of Brc physiology 4h after the first medium removal. The bacterial cell concentration was adjusted by OD to a final concentration of around 2×10^8 CFU/mL and bacterial suspensions were incubated with peak serum concentrations (PSC) of vancomycin (40 mg/L), rifampicin (10 mg/L) or tetracycline (16 mg/L) (National Committee for Clinical Laboratory Standards, 1997) for 2 hours at 37 °C with agitation at 120 rpm. Controls were performed in simultaneous by incubating the suspensions in the same conditions, without the addition of the antibiotics. The effect of the antibiotics was assessed by CFU counting upon 10-fold serial dilutions and plating into Tryptic Soy Agar (TSA) plates. This assay was performed with technical duplicates and at least three independent times.

Statistical analysis

Statistical significance between consecutive washes performed on biofilms (Figure 2) and between control and antibiotic treated samples (Supplementary Figure 1) was determined with one-way ANOVA multiple comparisons ($p < 0.05$). Statistical difference regarding growth kinetics in the presence or absence of the originating biofilm (Figure 3) was determined using multiple T-tests ($p < 0.01$). All analysis was performed using GraphPad Prism version 6 (Trial version, CA, USA). At least three replicates (independent experiments) were performed for all assays.

Results

Characterization of biofilm formation and Brc collection by multiple *S. epidermidis* isolates

A total of 6 distinct *S. epidermidis* isolates, previously characterized regarding their biofilm formation ability and the presence of biofilm associated genes, namely the *ica* operon, were selected for this study (Cerca et al., 2013; Freitas et al., 2017). Initially, biofilms were grown up to 72h. The ability to produce Brc using the implemented fed-batch system was evaluated by calculating the ratio between the number of cells within the biofilm and the number of cells existing in the biofilm supernatant at different time points (Table 2). From the 6 isolates used in this study, all 3 *ica*$^{+}$ isolates produced remarkably more biofilm than isolates without the *ica* gene, especially after 72 hours of incubation. This was not surprising since several studies showed a relation between the presence of the *ica* locus and the increased ability to form biofilms (Heilmann et al., 1996; Mack et al., 1994; Qin et al., 2007a), despite also being known that some *ica*$^{-}$ isolates are also able to produce biofilms (Dice et al., 2009; O'Gara, 2007; Qin et al., 2007b; Tormo, 2005). Interestingly, it was observed that the thickest biofilms produced had a lower ratio between Brc and biofilm cells.

Comment [S4]: Use only "h" uniform in whole paper

Comment [S5]: h

Washing biofilm and replacing the growth medium in fed-batch systems triggers the physical detachment of biofilm cells

It was previously shown that the typical medium replacement procedures needed for fed-batch systems trigger the detachment of cells from the biofilm due to shear forces (França et al., 2016a). Using the selected isolates, a total of 6 consecutive washing steps were performed on each biofilm, followed by quantification of the number of cells released immediately after each wash, as well as cells remaining in the biofilm (Figure 2). Interestingly, all strains used each successive wash kept detaching cells from the biofilm, ranging from ~10^{7} to ~10^{6} CFU/mL per wash, independently of their biofilm formation capacity. Remarkably, the total biomass of stronger biofilms producers (9142, DEN69 and PT13032) was only moderately affected after the 6 washes, with circa 40% decrease of total biofilm biomass from the first to the last washing step. Conversely, *ica*$^{-}$ isolates (ICE102, DEN185 and PT12004) biofilm structure was relatively more affected by shear forces, with more than 70% of the biofilm being removed after the 6 washes ($p = 0.03$).

Higher number of cells are released to the supernatant when growing in the presence of strong biofilms

To differentiate between physical detachment and active dispersion of cells, a second experiment was performed. After washing a preformed 24-hours biofilm and replacing the growth medium, the total number of cells in the bulk fluid was quantified right after medium replacement and after 2 hours of incubation. In half of the wells, the bulk fluid containing Brc was transferred to new sterile wells. As shown before for strain 9142 (França et al., 2016a), the presence of the originating biofilm significantly increased the number of cells in the bulk fluid of *ica*$^{+}$ strains, especially at 120 minutes (Figure 3), when compared to the inoculum transferred to new sterile wells (in the absence of the preformed biofilm). Conversely, the same was not true for the *ica*$^{-}$ isolates, since the effect of growing in the presence of the biofilm was significantly less pronounced and no statistical differences between growing in the presence or absence of the originating biofilm were found, growing up to 120 minutes. This is not surprising, since we know from the previous experiment that a significant number of cells was removed from the weakest biofilms, leaving a low amount of cells available to be released.

Brc obtained at different timepoints present the same antimicrobial susceptibility to vancomycin, tetracycline and rifampicin

Previously, we observed that Brc collected from 24h preformed biofilm, up to 4 hours after medium replacement, presented enhanced tolerance to vancomycin, tetracycline and rifampicin (França et al., 2016a). As a complementary analysis to be included in this study, we decided to test if this enhanced antimicrobial tolerance was somewhat influenced if the cells were collected from more mature biofilms, namely from 48 and 72h biofilms. To assess this, the antimicrobial susceptibility of Brc obtained at 28, 48 and 72 hours to peak serum concentrations of vancomycin, tetracycline and rifampicin was determined. Interestingly, no significant differences in the tolerance to vancomycin, tetracycline or rifampicin were found between the different Brc populations, obtained 4h (28H biofilms) or 24h (48H/72H biofilms) after medium replacement (Supplementary Figure 1). This data is an indirect evidence suggesting that the overall phenomena of Brc is not affected by the maturity of the biofilm.

Discussion

It has been point out that during biofilm disassembly phase, cells are released from the biofilm to the surrounding environment, spreading the infection and increasing the risk of systemic diseases, as bacteraemia (Cervera et al., 2009; Boles and Horswill, 2011). Recently, we demonstrated the feasibility of using a fed-batch system to obtain Brc from high biofilm forming *S. epidermidis* strains (França et al., 2016a), and showed that Brc had enhanced tolerance to antibiotics (Gaio and Cerca, 2019) and also induced a more inflammatory response in the host (França et al., 2016b). An important limitation of the previous studies was the fact that we only tested *ica*$^{+}$ *S. epidermidis* isolates, with considerable abilities to form thick and multi-layered biofilms (Christensen et al., 1985; Mack et al., 1994, 1992). As inter- and intra-species variability has been observed regarding *Staphylococcal ~~spp.~~* sp. biofilm formation (Handke et al., 2004; Oliveira et al., 2015; Tremblay et al., 2013), it was important to determine if the previous findings were reproducible when using strains with lower ability to form biofilms. Since it is well known that strains without a functional *ica* operon form biofilms with lower biomass (Handke et al., 2004), we compared 3 *ica*$^{+}$ and 3 *ica*$^{-}$ isolates.

Not surprisingly, significant differences were found between *ica*$^{+}$ and *ica*$^{-}$ isolates, regarding the effect of shear forces on the biofilm biomass reduction. A higher proportion of cells was found to be detached from weaker biofilms, as well as the decrease on total biofilm biomass was significantly more pronounced on *ica*$^{-}$ isolates, while a similar number of cells was being removed from the second to the last washing step, suggesting an almost inversely proportional ability to physically detach cells from the biofilms as related to the biofilm cohesiveness (Mack et al., 1996). The opposite was found for stronger biofilms, as it seems that a higher number of cells was recovered from initial washes (W1 and W2), but remarkably lower amounts of cells were detached in the last stages of washing, presumably because deeper layers of the biofilm are more cohesive and resistant to shear forces.

We also assessed if the enhanced antimicrobial tolerance described before (França et al., 2016a) was dependent on biofilm maturation stage. By obtaining Brc from ~1, 2 and 3 day-old biofilms, we were able to determine that the effect observed in early stage biofilms also occurred in older biofilms.

As noted before, a key limitation of using a fed-batch model to originate Brc is the difficulty to differentiate between physically detached cells, resulting from the washing procedures, from

actively dispersed cells (Boles and Horswill, 2011; França et al., 2016a; Kaplan, 2010). As shown with the multiple washing steps experiment, our data confirms that shear forces exerted during washing and medium replacement trigger the detachment of cells, independently of the ability of the isolates to produce thicker or thinner biofilms or the number of washes involved. However, active dispersion could only be determined in the *ica*$^{+}$ strains tested. Conversely, for the strains without a functional *ica* operon, the amount of cells on the biofilm bulk fluid incubated in the presence or absence off the biofilm was generally the same. This phenomenon in *ica*$^{-}$ isolates may be a consequence of the large proportion of cells removed upon washing their weak biofilms, which led to a higher proportion of cells in the supernatant immediately upon the addition of fresh media and, consequently, to a lower availability of cells in the biofilm to be continuously released. On the other hand, stronger biofilms were less affected by the washing steps used to remove non-adherent cells, leading to a lower proportion of cells detached from shear forces, comparing to the originating biofilm, and, consequently, to a higher concentration of cells actively released from the biofilm to the supernatant.

Conclusions

The results obtained herein demonstrated that a fed-batch system is only reliable in obtaining Brc from *S. epidermidis* biofilms for some isolates, especially from those who can form thick and strong biofilms. While all *ica*$^{+}$ isolates used herein were found to be high biofilm producing strains, it should be noted that some *ica*$^{+}$ isolates lack a functional intact operon (Cafiso et al., 2004; Cue et al., 2012), and the mere presence of the gene might not be related to its expression (Freitas et al., 2017; Lerch et al., 2019). As such, to assess the feasibility of this method in more strains, it is important not only to determine the presence of *ica*, but to assess if the operon is functional, as mutations in major biofilm regulators may influence the dynamics of Brc production (Cue et al., 2012).

References

Albano, M., Karau, M.J., Greenwood-Quaintance, K.E., Osmon, D.R., Oravec, C.P., Berry, D.J., Abdel, M.P., Patel, R., 2019. In vitro activity of rifampin, rifabutin, rifapentine, and rifaximin against planktonic and biofilm states of staphylococci isolated from periprosthetic joint infection. Antimicrob. Agents Chemother. doi:10.1128/AAC.00959-19

309 Azeredo, J., Azevedo, N.F., Briandet, R., Cerca, N., Coenye, T., Costa, A.R., Desvaux, M., Di
310 Bonaventura, G., Hébraud, M., Jaglic, Z., Kačániová, M., Knøchel, S., Lourenço, A.,
311 Mergulhão, F., Meyer, R.L., Nychas, G., Simões, M., Tresse, O., Sternberg, C., 2017.
312 Critical review on biofilm methods. Crit. Rev. Microbiol.
313 doi:10.1080/1040841X.2016.1208146
314 Bahamondez-Canas, T.F., Heersema, L.A., Smyth, H.D.C., 2019. Current status of in vitro
315 models and assays for susceptibility testing for wound biofilm infections. Biomedicines.
316 doi:10.3390/biomedicines7020034
317 Becker, K., Heilmann, C., Peters, G., 2014. Coagulase-negative staphylococci. Clin. Microbiol.
318 Rev. 27, 870–926. doi:10.1128/CMR.00109-13
319 Boles, B.R., Horswill, A.R., 2011. Staphylococcal biofilm disassembly. Trends Microbiol. 19,
320 449–55. doi:10.1016/j.tim.2011.06.004
321 Cafiso, V., Bertuccio, T., Santagati, M., Campanile, F., Amicosante, G., Perilli, M.G., Selan, L.,
322 Artini, M., Nicoletti, G., Stefani, S., 2004. Presence of the *ica* operon in clinical isolates of
323 *Staphylococcus epidermidis* and its role in biofilm production. Clin. Microbiol. Infect.
324 doi:10.1111/j.1469-0691.2004.01024.x
325 Cerca, N., Gomes, F., Bento, J.C., França, A., Rolo, J., Miragaia, M., Teixeira, P., Oliveira, R.,
326 2013. Farnesol induces cell detachment from established *S. epidermidis* biofilms. J.
327 Antibiot. (Tokyo). 66, 255–258. doi:10.1038/ja.2013.11
328 Cerca, N., Jefferson, K.K., Oliveira, R., Pier, G.B., Azeredo, J., 2006. Comparative antibody-
329 mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the
330 planktonic state. Infect. Immun. 74, 4849–4855. doi:10.1128/IAI.00230-06
331 Cerca, N, Martins, S., Cerca, F., Jefferson, K.K., Pier, G.B., Oliveira, R., Azeredo, J., 2005a.
332 Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in
333 biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT
334 colorimetry. J. Antimicrob. Chemother. 56, 331–336. doi:10.1093/jac/dki217
335 Cerca, N, Martins, S., Sillankorva, S., Jefferson, K.K., Pier, G.B., Oliveira, R., Azeredo, J., 2005.
336 Effects of growth in the presence of subinhibitory concentrations of dicloxacillin on
337 *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* biofilms. Appl. Environ.
338 Microbiol. 71, 8677–8682. doi:10.1128/AEM.71.12.8677-8682.2005
339 Cerca, N, Pier, G.B., Vilanova, M., Oliveira, R., Azeredo, J., 2005b. Quantitative analysis of

adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. Res. Microbiol. 156, 506–14. doi:10.1016/j.resmic.2005.01.007

Cervera, C., Almela, M., Martínez-Martínez, J.A., Moreno, A., Miró, J.M., 2009. Risk factors and management of Gram-positive bacteraemia. Int. J. Antimicrob. Agents. doi:10.1016/S0924-8579(09)70562-X

Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M., 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. J. Clin. Microbiol. 22, 996–1006.

Cogen, A.L., Nizet, V., Gallo, R.L., 2008. Skin microbiota: A source of disease or defence? Br. J. Dermatol. 158, 442–455. doi:10.1111/j.1365-2133.2008.08437.x

Cue, D., Lei, M.G., Lee, C.Y., 2012. Genetic regulation of the intercellular adhesion locus in staphylococci. Front. Cell. Infect. Microbiol. doi:10.3389/fcimb.2012.00038

Dias, C., Borges, A., Oliveira, D., Martinez-Murcia, A., Saavedra, M.J., Simões, M., 2018. Biofilms and antibiotic susceptibility of multidrug-resistant bacteria from wild animals. PeerJ. doi:10.7717/peerj.4974

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. doi:10.1080/08927010902803297

França, A., Carvalhais, V., Vilanova, M., Pier, G.B., Cerca, N., 2016a. Characterization of an in vitro fed-batch model to obtain cells released from *S. epidermidis* biofilms. AMB Express 6, 23. doi:10.1186/s13568-016-0197-9

França, A., Pérez-Cabezas, B., Correia, A., Pier, G.B., Cerca, N., Vilanova, M., 2016b. *Staphylococcus epidermidis* biofilm-released cells induce a prompt and more marked in vivo inflammatory-type response than planktonic or biofilm cells. Front. Microbiol. 7, 1–12. doi:10.3389/fmicb.2016.01530

Freitas, AI; Lopes, N; Oliveira, F; Brás, S; França, A; Vasconcelos, C; Vilanova, M; Cerca, N., 2017. Comparative analysis between biofilm formation and gene expression in commensal and clinical *Staphylococcus epidermidis*. Future Microbiol. doi: 10.2217/fmb-2017-0140

Freitas, A.I., Vasconcelos, C., Vilanova, M., Cerca, N., 2014. Optimization of an automatic

counting system for the quantification of *Staphylococcus epidermidis* cells in biofilms. J. Basic Microbiol. 54, 750–757. doi:10.1002/jobm.201200603

Gaio, V., Cerca, N., 2019. Cells released from *S. epidermidis* biofilms present increased antibiotic tolerance to multiple antibiotics. PeerJ 2019. doi:10.7717/peerj.6884

Gardiner, M., Vicaretti, M., Sparks, J., Bansal, S., Bush, S., Liu, M., Darling, A., Harry, E., Burke, C.M., 2017. A longitudinal study of the diabetic skin and wound microbiome. PeerJ. doi:10.7717/peerj.3543

Gray, E.D., Verstegen, M., Peters, G., Regelmann, W.E., 1984. Effect of extracellular slime substance from *Staphylococcus epidermidis* on the human cellular immune response. Lancet. doi:10.1016/S0140-6736(84)90413-6

Handke, L.D., Conlon, K.M., Slater, S.R., Elbaruni, S., Fitzpatrick, F., Humphreys, H., Giles, W.P., Rupp, M.E., Fey, P.D., O'Gara, J.P., 2004. Genetic and phenotypic analysis of biofilm phenotypic variation in multiple *Staphylococcus epidermidis* isolates. J. Med. Microbiol. 53, 367–374. doi:10.1099/jmm.0.05372-0

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., Götz, F., 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol. Microbiol. doi:10.1111/j.1365-2958.1996.tb02548.x

Isberg, R.R., Barnes, P., 2002. Dancing with the host: Flow-dependent bacterial adhesion. Cell Press 110, 1–4. doi:10.1016/S0092-8674(02)00821-8

Kaplan, J.B., 2010. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. J. Dent. Res. 89, 205–218. doi:10.1177/0022034509359403

Kleinschmidt, S., Huygens, F., Faoagali, J., Rathnayake, I.U., Hafner, L.M., 2015. *Staphylococcus epidermidis* as a cause of bacteremia. Future Microbiol. doi:10.2217/fmb.15.98

Laverty, G., Gorman, S.P., Gilmore, B.F., 2013. Biomolecular mechanisms of staphylococcal biofilm formation. Future Microbiol. doi:10.2217/fmb.13.7

Lerch, M.F., Schoenfelder, S.M.K., Marincola, G., Wencker, F.D.R., Eckart, M., Förstner, K.U., Sharma, C.M., Thormann, K.M., Kucklick, M., Engelmann, S., Ziebuhr, W., 2019. A non-coding RNA from the intercellular adhesion (*ica*) locus of *Staphylococcus epidermidis* controls polysaccharide intercellular adhesion (PIA)-mediated biofilm formation. Mol. Microbiol. doi:10.1111/mmi.14238

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., Laufs, R., 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear β-1,6-linked glucosaminoglycan: Purification and structural analysis. J. Bacteriol. doi:10.1128/jb.178.1.175-183.1996

Mack, D., Nedelmann, M., Krokotsch, A., Schwarzkopf, A., Heesemann, J., Laufs, R., 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: Genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect. Immun. 62, 3244–3253.

Mack, D., Siemssen, N., Laufs, R., 1992. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: Evidence for functional relation to intercellular adhesion. Infect. Immun. 60, 2048–2057.

Mah, T., 2012. Biofilm-specific antibiotic resistance. Future Microbiol. 1061–1072. doi:10.2217/fmb.12.76

Moormeier, D.E., Bayles, K.W., 2014. Examination of *Staphylococcus epidermidis* biofilms using flow-cell technology. Methods Mol. Biol. doi:10.1007/978-1-62703-736-5_13

National Committee for Clinical Laboratory Standards, 1997. Methods for dilution: antimicrobial susceptibility tests for bacteria that grow aerobically - Fifth Edition: Approved Standard M7-A5. NCCLS, Wayne, PA, USA.

O'Gara, J.P., 2007. *ica* and beyond: Biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol. Lett. doi:10.1111/j.1574-6968.2007.00688.x

Oliveira, F., Lima, C.A., Bras, S., França, A., Cerca, N., 2015. Evidence for inter- and intraspecies biofilm formation variability among a small group of coagulase-negative staphylococci. FEMS Microbiol. Lett. 362, 1–20. doi:10.1093/femsle/fnv175

Otto, M., 2009. *Staphylococcus epidermidis*—the'accidental'pathogen. Nat. Rev. Microbiol. 7, 555–567. doi:10.1038/nrmicro2182.Staphylococcus

Otto, M., 2013. Staphylococcal infections : mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Ann. Rev. Microbiol. doi:10.1146/annurev-med-042711-140023

Periasamy, S., Joo, H., Duong, A.C., Bach, T.L., Tan, V.Y., Chatterjee, S.S., Cheung, G.Y.C., Otto, M., 2012. How *Staphylococcus aureus* biofilms develop their characteristic structure.

Proc. Natl. Acad. Sci. U. S. A. 109, 1281–6. doi:10.1073/pnas.1115006109

Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S., Qu, D., 2007a. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology. doi:10.1099/mic.0.2007/006031-0

Qin, Z., Yang, X., Yang, L., Jiang, J., Ou, Y., Molin, S., Qu, D., 2007b. Formation and properties of in vitro biofilms of *ica*-negative *Staphylococcus epidermidis* clinical isolates. J. Med. Microbiol. doi:10.1099/jmm.0.46799-0

Rogers, K.L., Fey, P.D., Rupp, M.E., 2009. Coagulase-negative staphylococcal infections. Infect. Dis. Clin. North Am. 23, 73–98. doi:10.1016/j.idc.2008.10.001

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. Infect. Immun. 79, 2267–2276. doi:10.1128/IAI.01142-10

Singh, R., Ray, P., 2014. Quorum sensing-mediated regulation of staphylococcal virulence and antibiotic resistance. Future Microbiol. 9, 669–81. doi:10.2217/fmb.14.31

Sousa, C., França, A., Cerca, N., 2014. Assessing and reducing sources of gene expression variability in *Staphylococcus epidermidis* biofilms. Biotechniques 57, 295–301. doi:10.2144/000114238

Tormo, M.A., 2005. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? Microbiology 151, 2465–2475. doi:10.1099/mic.0.27865-0

Tremblay, Y.D.N., Lamarche, D., Chever, P., Haine, D., Messier, S., Jacques, M., 2013. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. J. Dairy Sci. 96, 234–246. doi:10.3168/jds.2012-5795

Wang, R., Khan, B.A., Cheung, G.Y.C., Bach, T.H.L., Jameson-Lee, M., Kong, K.F., Queck, S.Y., Otto, M., 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J. Clin. Invest. 121, 238–248. doi:10.1172/JCI42520

Yao, Y., Sturdevant, D.E., Otto, M., 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. J. Infect. Dis.

191, 289–298. doi:10.1086/426945

Yawata, Y., Nguyen, J., Stocker, R., Rusconi, R., 2016. Microfluidic studies of biofilm formation in dynamic environments. J. Bacteriol. doi:10.1128/JB.00118-16

Ziebuhr, W., Hennig, S., Eckart, M., Kränzler, H., Batzilla, C., Kozitskaya, S., 2006. Nosocomial infections by *Staphylococcus epidermidis*: how a commensal bacterium turns into a pathogen. Int. J. Antimicrob. Agents. doi:10.1016/j.ijantimicag.2006.05.012

Table 1 (on next page)

Origin of the 11 *Staphylococcus epidermidis* isolates used in this study.

Table 1. Origin of the 11 *Staphylococcus epidermidis* isolates used in this study

S. *epidermidis* isolate	Description	Country of origin	Ica operon
9142 (42)	Clinical isolate from blood culture	Germany	Detected (36)
DEN69 (36)	Unknown	Denmark	Detected (36)
PT13032 [1]	Clinical isolate from a patient with chronic renal failure	Portugal	Detected [1]
ICE102 (36)	Clinical isolate from a patient with infective endocarditis	United States of America	Undetected (36)
DEN185 (36)	Unknown	Denmark	Undetected (36)
PT12004 (37)	Clinical isolate from a patient with chronic renal failure	Portugal	Undetected (37)

[1] Unpublished isolate obtained from a previous epidemiological study in Portugal. Isolates were obtained after patient informed consent with the approval of the Ethical Committee of the Hospital Geral de Santo António, Porto, Portugal. Each isolate was first identified at the species level using the commercially available VITEK® two identification system using the gram-positive ID card (BioMérieux, Marcy l'Etoile, France) and molecular identification was confirmed by sequencing of the rpoB gene (50).

Table 2(on next page)

Characterization of biofilm biomass

Characterization of biofilm biomass (by OD measurements) and the ratios between the biofilm bulk fluid, containing Brc, and the biofilm suspension (Brc/Bio).

Table 2. Characterization of biofilm biomass (by OD measurements) and the ratios between the biofilm bulk fluid, containing Brc, and the biofilm suspension (Brc/Bio).

Classification	*S. epidermidis* isolates	OD Biofilm			Ratio Brc/Bio	
		28H	**48H**	**72H**	**28H**	**48H**
ica$^+$	**9142**	1.65	2.54	3.05	0.64	0.47
	DEN69	1.92	1.92	2.26	0.51	0.79
	PT13032	0.48	0.88	1.29	1.10	1.29
ica$^-$	**ICE102**	0.61	0.81	0.89	2.12	3.65
	DEN185	0.43	0.78	0.77	2.85	3.63
	PT12004	0.57	0.69	0.72	1.81	2.77

Figure 1

Scheme illustrating the process of obtaining biofilm released cells using a fed-batch growth system.

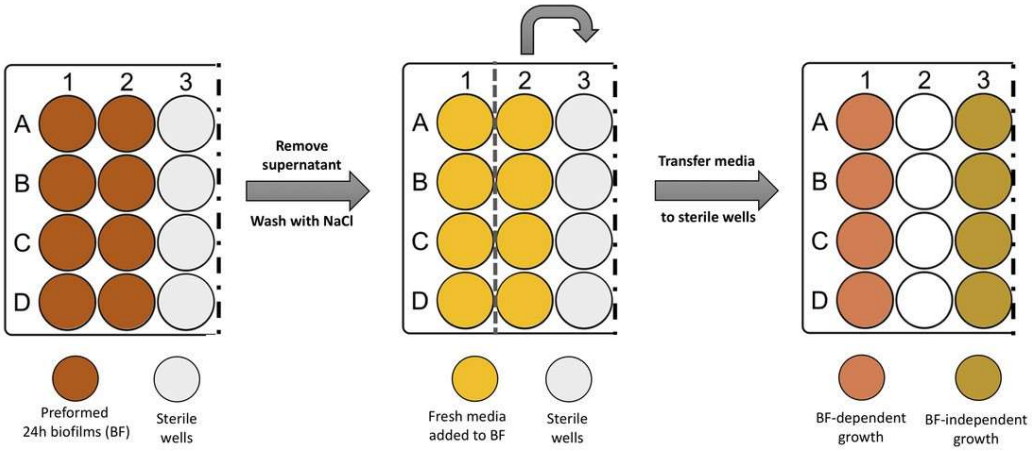


Figure 2

Log_{10} number of CFU in the biofilm or in the wash liquid after continuous washing steps in (A) *S. epidermidis ica*$^{+}$ isolates or (B) *S. epidermidis ica*$^{-}$ isolates.

CFU were determined from biofilms after 1 (BF1), 2 (BF2) or 6 washes (BF6) and from the NaCl solution obtained after each washing step (W1 to W6). The columns represent the mean plus or minus standard error deviation of at least three independent experiments, with duplicates. Statistical differences were analysed with one-way ANOVA multiple comparisons, with a representing statistically significant differences (p <0.05) between the first condition (BF1 or W1) and all remaining conditions, while ß represents significant differences (p <0.05) between each consecutive washing step.

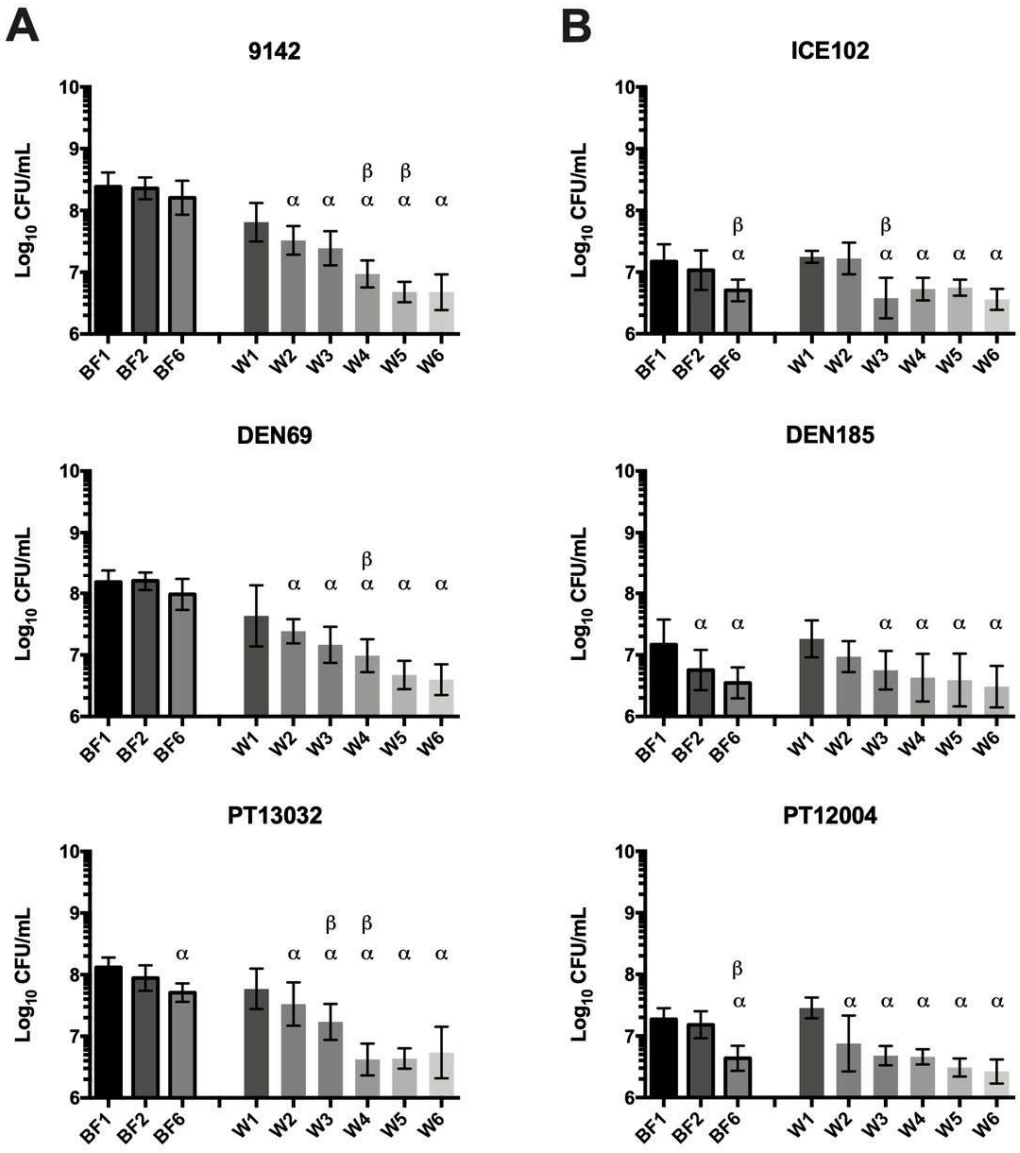
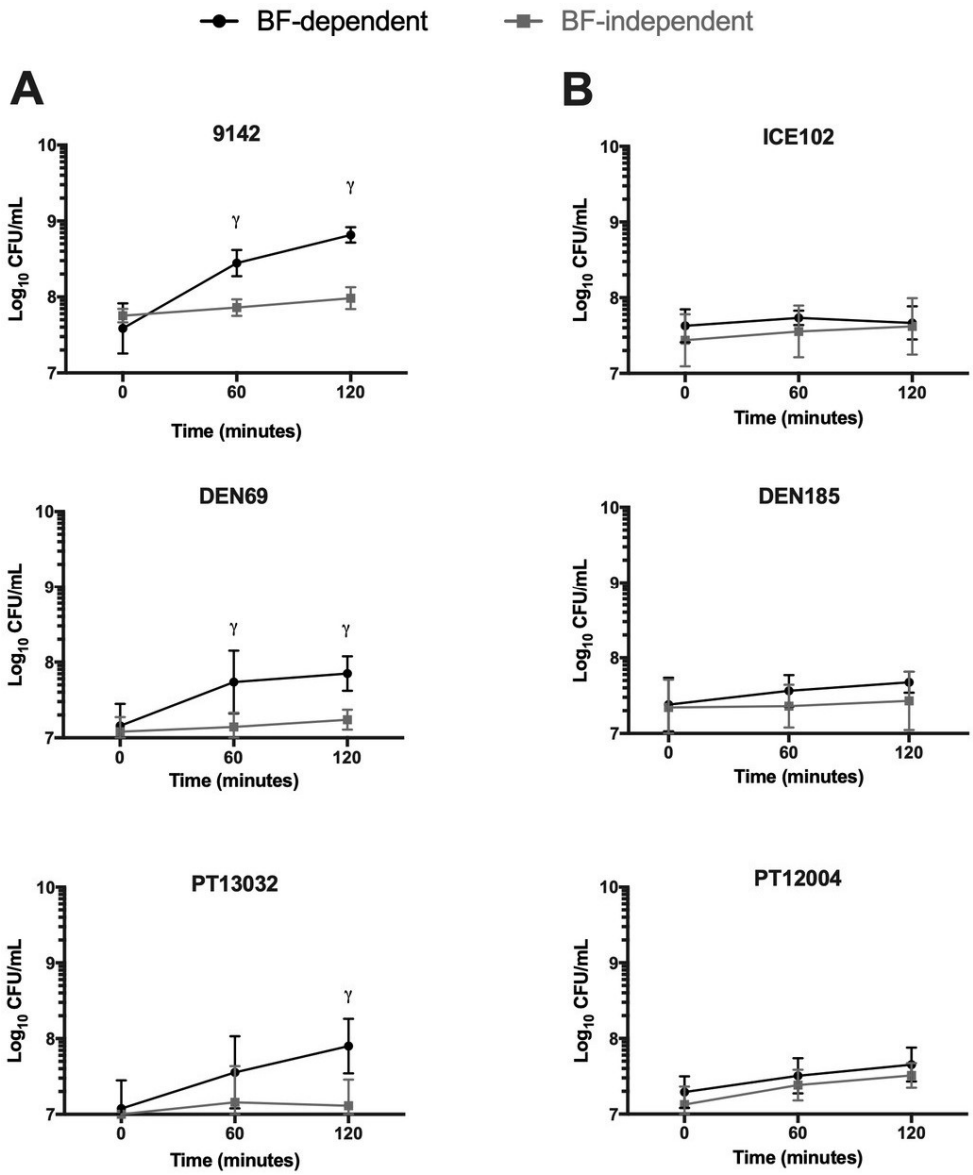

A
9142
DEN69
PT13032
B
ICE102
DEN185
PT12004
Log10 CFU/mL
BF1
BF2
BF6
W1
W2
W3
W4
W5
W6
α
β

Figure 3

Growth kinetics (Log_{10} CFU/mL) of Brc in the presence or absence of the originating biofilms, under agitation (120 rpm) in (A) *S. epidermidis ica*$^{+}$ isolates or (B) *S. epidermidis ica*$^{-}$ isolates.

The number of CFU ~~were~~ was determined at 0, 60 and 120 minutes after media change in the presence (BF-dependent) or absence (BF-independent) of the originating biofilm. The columns represent the mean plus or minus standard error deviation of at least three independent experiments. Statistical differences were analyzed with multiple T-tests, with γ (p <0.01) representing statistically significant differences between the number of cells recovered at each time point between growth in both conditions (BF- dependent and -independent).


BF-dependent
BF-independent
A
B
9142
ICE102
DEN69
DEN185
PT13032
PT12004
Log10 CFU/mL
Time (minutes)
0
60
120
7
8
9
10
γ