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Research Paper

Optimization of an automatic counting system for the quantification of *Staphylococcus epidermidis* cells in biofilms

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Biofilm formation is recognized as the main virulence factor in a variety of chronic infections. *In vitro* evaluation of biofilm formation is often achieved by quantification of viable or total cells. However, these methods depend on biofilm disruption, which is often achieved by vortexing or sonication. In this study, we investigated the effects of sonication on the elimination of *Staphylococcus epidermidis* cell clusters from biofilms grown over time, and quantification was performed by three distinct analytical techniques. Even when a higher number of sonication cycles was used, some stable cell clusters remained in the samples obtained from 48- and 72-h-old biofilms, interfering with the quantification of sessile bacteria by plate counting. On the other hand, the fluorescence microscopy automatic counting system allowed proper quantification of biofilm samples that had undergone any of the described sonication cycles, suggesting that this is a more accurate method for assessing the cell concentration in *S. epidermidis* biofilms, especially in mature biofilms.

Keywords: Biofilm / *Staphylococcus epidermidis* / Cell clusters / Sonication / Automatic cell counting

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Introduction

Staphylococcus epidermidis, a member of the coagulase-negative staphylococci, is now recognized as one of the most prevalent pathogens in nosocomial infections, which frequently originate from biofilms formed on the surface of synthetic medical devices [1]. A biofilm is generally described as a microbial community attached to a surface that develops by accumulation of multilayered cell clusters and is embedded in an extracellular matrix [2]. Biofilm formation is recognized as the main virulence factor in a variety of chronic infections [1, 3], representing a major problem in public healthcare. Biofilm bacteria usually present higher resistance to antibiotics [2, 4, 5], higher tolerance to the immune system [5, 6], and better adaptation to environmental stress factors [7, 8].

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Except when more fundamental and detailed studies are being pursued, colorimetric methods such as those using crystal violet or safranin staining or optical density (OD) evaluation of bacterial cultures are common, easy, and straightforward biofilm cellular quantification techniques [9–13]. While very useful for screening purposes [14, 15], these methods do not provide information regarding the number of total or viable bacteria. Due to this limitation, biofilm quantification is often made by colony-forming unit (CFU) counting. However, CFU evaluation has been described as suffering from a lack of reproducibility [16, 17] and can lead to significant errors due to the presence of cell clusters promoted by the biofilm matrix. Furthermore, as it has been shown by flow cytometry, *S. epidermidis* biofilm CFU counting only allows the quantification of cultivable bacteria, but does give an indication of total and live bacteria [18]. Despite these limitations, this is a widespread method. An important aspect of CFU quantification concerns the requirement of preparing a homogeneous cell suspension, derived from the biofilm. This is often achieved by vortexing or sonication. Some studies focusing on the removal of biofilms from infected

medical devices have clearly demonstrated that vortexing and scraping do not offer sufficient biofilm disaggregation [19, 20]. On the other hand, sonication contributes to a better dispersion of the cells, making the culture after sonication easier to quantify [20–22]. However, the issue of biofilm maturation was not taken into account, and often these studies were performed using young biofilms (24 h of growth). As we have shown before, biofilm formation is a dynamic process [23], and mature *S. epidermidis* biofilms are often associated with higher biomass and higher expression levels of adhesins [24, 25] and are consequently more complex to evaluate.

In this study, we addressed the effect of sonication on the elimination of *S. epidermidis* cell clusters present in cell suspensions derived from biofilms molded for 24, 48, and 72 h. The bacterial cells from the biofilms were quantified by three analytical techniques to test the accuracy of each method. We show that older biofilms formed more cell clusters which remain present after sonication, significantly affecting bacterial quantification by CFU counting, while fluorescence microscopy automatic counting proved to be a more accurate method.

Material and methods

Bacterial strains and culture conditions

Three well-known biofilm-forming strains were used in this study: *S. epidermidis* RP62A (PubMed accession number: PRJNA57663, ID: 57663), *S. epidermidis* 9142 [26] and *S. epidermidis* 1457 [27]. Biofilm cultures of each strain were performed in fed-batch mode as previously described [23]. Briefly, a starter culture was grown overnight in Tryptic Soy Broth (TSB) (Oxoid) at 37 °C with agitation (120 rpm). Of the starter culture, 5 μl was inoculated into 1 ml TSB supplemented with 1% (w/v) glucose (TSBG) to induce biofilm formation in a 24-well plate (Orange Scientific). The cultures were grown for 24, 48, and 72 h at 37 °C on an orbital shaker at 120 rpm. The growth medium was completely removed and replaced using an equal volume of fresh TSBG every 24 h. Each experiment was repeated at least three times.

Biofilm disruption

After the respective incubation times, the biofilms were washed twice with saline solution before being detached from the culture plate surface. Each biofilm was resuspended in 1 ml of a physiological saline solution (NaCl 0.9%) and dislodged by scraping, followed by sonication (Cole-Parmer® 750-Watt Ultrasonic Homogenizer, 230 VAC, employing a 13-mm microtip) using three different cycles differing in time (s) and amplitude (%): cycle A – 10 s at 30%; cycle B – 30 s at 30% plus 40 s at 40%, and cycle C – cycle B plus 120 s at 40%. The tubes containing the samples were kept in ice during sonication. The scraping procedure removed more than 98% of the biomass, determined by the reduction in crystal violet staining, as previously shown by us [28]. A cell suspension vortexed for 1 min (WS) was used as the no-sonication control. Vortexing for 1 min was previously used by Olson *et al.* [29] to dislodge bacteria from intravascular catheters.

Biofilm quantification

Biofilms were quantified using three different methods. To determine cell viability, the biofilms were resuspended in 0.9% NaCl, followed by sonication or vortexing as described above. Several serial 10-fold dilutions were made in saline solution and plated on Tryptic Soy Agar (TSA). The plates were incubated at 37 °C for 24 h before counting the number of CFU. Biofilm biomass quantification was done by measuring the OD at 595 nm of each sonicated cell suspension, a method used to evaluate the bacterial growth rate [12, 30]. For this, biofilm suspensions were diluted until the measured OD was below 0.8; then, the determination of the OD was performed by multiplying the dilution factor by the measured OD. Finally, total and dead cells were quantified using a Neubauer chamber coupled with an Olympus BX51 epifluorescence microscope equipped with a CCD color camera DP71 (Olympus). Cell suspensions were stained with the commercially available LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) following the manufacturer's instructions. A negative control was used to determine the baseline threshold for dead cells, killed by treating the cells for 15 min at 100 °C. Cells were counted using the automated enumeration software SigmaScan Pro 5.0 (Systat Software Inc.), as described before [28], using a magnification of 200×. Briefly, 20 TIFF images (1360 × 1024) per condition were acquired and converted to eight bit 256 grayscale, to be analyzed by differences in the gray intensity of each pixel, by using an appropriate intensity threshold determined experimentally. Under these conditions, 18420 ± 1575 pixels were equivalent to 0.0025 cm^2 at 200× magnification.

Quantification of bacterial cell clusters

Biofilms of *S. epidermidis strain* 9142 grown for 24, 48, and 72 h as described above were sonicated for 10 s at 30% (cycle A) and then adjusted to the same OD (OD_{595nm} ≈ 0.8). OD readings at 595 nm (Spectronic 20 Genesys,

Sigma–Aldrich) were carried out every 2 min for a total of 30 min. Before being placed in a 2.5-ml polystyrene spectrophotometer cuvette (Labbox), the biofilm cell suspension was vortexed to assure homogeneity. Planktonic bacteria grown to the early exponential phase were used as a control. This experiment was performed three times.

Statistical analysis

All the assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and Tukey's multiple comparisons test, and also the paired sample *t*-test, using SPSS. All tests were performed with a confidence level of 95%.

Results

Automatic image counting validation

To validate the SigmaScan Pro 5.0 software for automatic counting of adhered *S. epidermidis* cells using the fluorescence-based Live/Dead staining, we tested several different parameters and compared the results with manual counting. As can be seen in Fig. 1A, no significant differences were found using any of the three software thresholds and the manual counting ($\rho > 0.05$), indicating that the Live/Dead staining was strongly discriminative between bacteria and background and that there was no significant fluorophore bleach effect that could impair the automatic counts. Furthermore, in Fig. 1B, no significant differences were found when using two different appropriate optical magnifications ($\rho > 0.05$). Finally, to discriminate between the two different fluorophores present in Live/Dead staining, the total number of live and dead bacteria was determined either with manual or automatic counting, using 200× magnification and a medium intensity threshold (Fig. 1C). Both fluorophores were correctly discriminated by the software ($\rho > 0.05$), validating our automatic counting system.

Effect of cell agglomeration on bacteria quantification

In order to address the impact of sonication in biofilm quantification, we tested three different sonication cycles. The optimization of the sonication conditions in biofilm quantification following 24, 48, and 72 h of bacterial growth was done on the *S. epidermidis* 9142 strain, using three different methods broadly employed in research laboratories: biomass determination by OD, quantification of viable cells by plate counting, and quantification of total cells by fluorescence microscopy analysis. A no-sonication control was used.

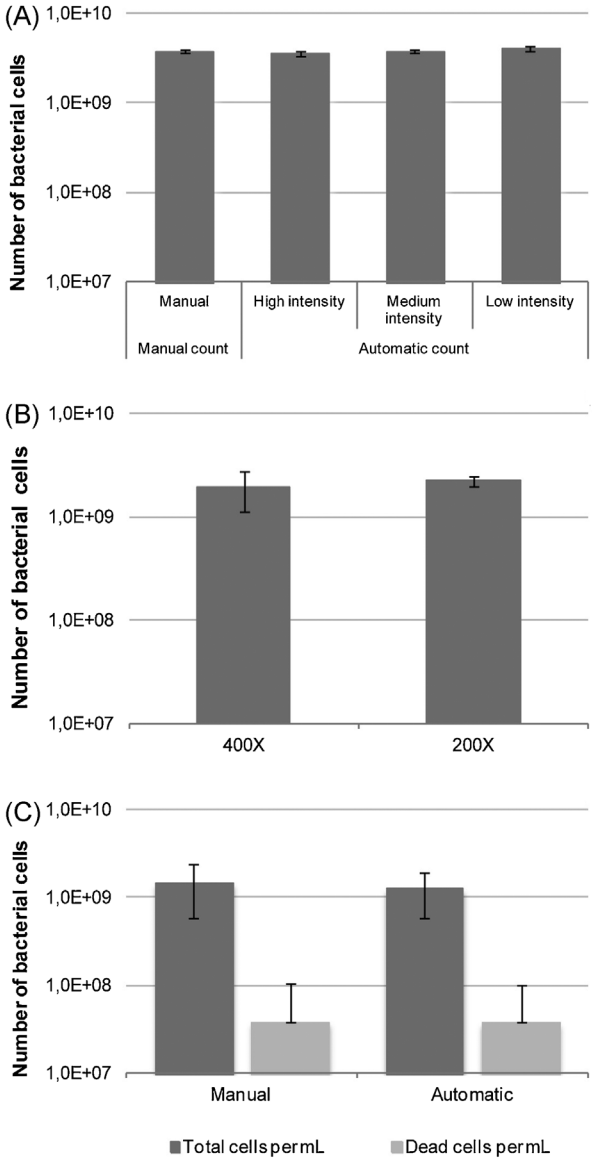

Figure 1. Validation of the SigmaScan Pro 5.0 software. (A) Effect of the intensity threshold range in bacteria quantification, as compared with manual counting. (B) Manual versus automatic counting of total and dead cells obtained by using Live/Dead staining. (C) Bacterial quantification by automatic counting using 400× or 200× magnification. The values represent the means ± standard deviation of three independent experiments. No significant changes were found ($\rho < 0.05$, paired *t*-test).

As expected, biofilm biomass quantification by OD determination showed a progressive accumulation during the analyzed time course of biofilm formation. Noticeably, the different sonication cycles resulted in significant changes in the OD quantification in more mature biofilms (Fig. 2), while in 24-h-old biofilms differences were only found between vortexing and the

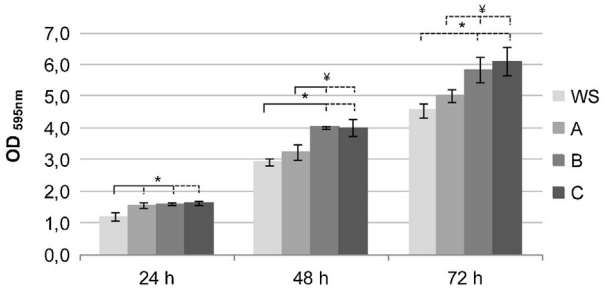

Figure 2. OD_{595nm} measurements in cell suspensions of 24-, 48- and 72-h-old biofilms, following vortexing (WS) and the different sonication cycles (A, B, and C). The values represent the means ± standard deviation of three independent experiments. Statistical differences ($\rho < 0.05$) between the no-sonication control (WS) and any other sonication cycle (*) and between sonication cycle A and other sonication cycles (¥) are indicated (ANOVA Tukey's test).

three sonication cycles ($\rho < 0.05$, ANOVA). Similar to the OD measurements, significant differences ($\rho < 0.05$, ANOVA) in the cell quantification of samples that had undergone different sonication cycles were only observed for older biofilms (48 and 72 h). Interestingly, only in these two growth periods significant differences between samples prepared by either sonication or vortexing ($\rho < 0.05$, ANOVA) were detected, as shown in Fig. 3B and C. Not surprisingly, microscopic quantification allowed the detection of higher numbers of bacteria in biofilms after 48 and 72 h of growth (Fig. 3B and C). Moreover, microscopic quantification was the only approach that provided constant amounts of quantified bacteria, under all conditions tested, as further discussed below. Also, to determine if our observations could be extrapolated to other *S. epidermidis* strains, we selected two other known biofilm-forming strains to validate our findings: 1457 and RP62A. As shown in Table 1, similar results were found in comparison to the strain 9142, validating the model strain used in this study.

As can be seen in Fig. 4A, the microscopic observations detected small cell clusters in mature biofilms, despite the sonication cycles used. As expected, the size of the microscopic cell aggregates was reduced by increasing the sonication period; however, they were nonetheless present. Since bigger cell aggregates would sediment more quickly than individual bacteria, we devised a simple experiment based on the sedimentation velocity of particles of different size, to quantify the presence of the microscopic cell aggregates through detecting the corresponding reduction in the OD. As can be seen in Fig. 4B, older biofilms had a higher content of cell clusters, as determined by the faster reduction in the OD.

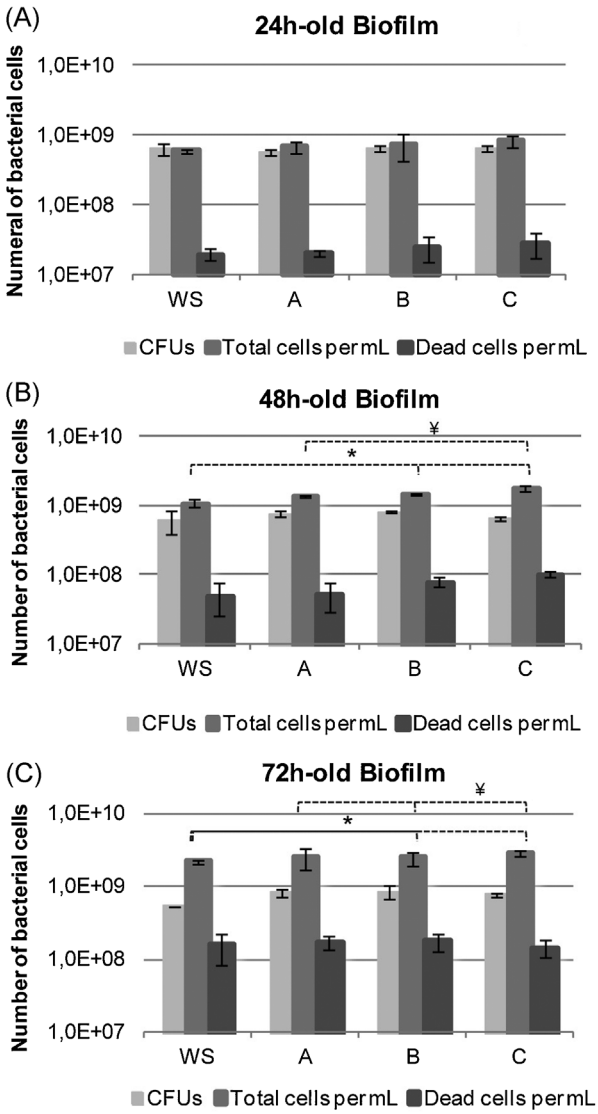

Figure 3. Quantitative results obtained by CFU counting and by fluorescence microscopy in 24- (A), 48- (B), and 72-h-old (C) biofilms, after each treatment. Bars represent the means of the number of bacterial cells within a biofilm ± standard deviation evaluated in samples obtained upon the different treatments tested, as indicated. Results are representative of three independent experiments. Statistical differences ($\rho < 0.05$) between the no-sonication control (WS) and any other sonication cycle (*) and between sonication cycle A and other sonication cycles (¥) are indicated (ANOVA Tukey's test).

Discussion

Automatic image counting validation

An automatic image counting software is a useful tool in research laboratories, but care should be taken to guarantee that the selected software accurately quantifies the desired study object [31]. We previously used the

Table 1. Biomass and viable and total cell quantification from biofilms of the *S. epidermidis* strains 9142, 1457, and RP62A grown for 24, 48, and 72 h.

	24-h-old biofilm	48-h-old biofilm	72-h-old biofilm
9142			
OD	1.61 ± 0.05*	4.02 ± 0.03*	5.84 ± 0.41
CFU[a]	6.48 ± 0.68E + 08	8.18 ± 0.44E + 08¥	8.43 ± 1.76E + 08
Total cells[a]	7.40 ± 3.05E + 08	1.44 ± 0.06E + 09	2.45 ± 0.49E + 09
1457			
OD	1.92 ± 0.07*	5.72 ± 0.33*	6.79 ± 0.23
CFU[a]	6.15 ± 0.35E + 08	6.11 ± 0.12E + 08¥	6.63 ± 1.03E + 08
Total cells[a]	8.13 ± 0.30E + 08	1.96 ± 0.24E + 09	3.37 ± 0.53E + 09
RP62A			
OD	0.41 ± 0.01*	1.72 ± 0.04*	2.30 ± 0.03*
CFU[a]	1.97 ± 0.12E + 08¥	4.38 ± 0.16E + 08¥	4.45 ± 0.07E + 08
Total cells[a]	2.21 ± 0.41E + 08	5.84 ± 0.95E + 08θ	7.78 ± 0.99E + 08θ

The values represent the means ± standard deviation of two to three independent experiments. Statistical differences ($\rho < 0.05$) between OD (*), CFU (¥), and total cells (θ) in the different strains are indicated (ANOVA Tukey's test).
[a]Values are expressed in the logarithmic scale.

SigmaScan Pro 5.0 software for automatic counting of adhered *S. epidermidis* cells stained with safranin [28]. To validate this approach using the fluorescence-based Live/Dead staining, we first tested the intensity threshold settings of the software (Fig. 1A) since it has been shown before that different fluorophores can yield different quantification results of bacteria [32]. We also tested bacteria quantification by using a magnification of either 200× or 400× (Fig. 1B). A high intensity threshold has the potential to exclude bacteria that either have low fluorescence or are slightly out of focus. A low intensity threshold can include pixels that do not represent bacteria but result from overstaining of the fluorophore. The automatic counting system proved to be robust as all tested settings resulted in numbers of cells equivalent to the manual counting. A big advantage was the ability to use a lower magnification. While using 200× magnification would not be appropriate for manual counting of *S. epidermidis* cells, as it would be nearly impossible to discriminate between the background and individual cells or even between individual cells and small cell aggregates, this was not the case with the automatic counting software since it is possible to differentiate individual pixels. A further advantage of using 200× images is that, with the same amount of images, double the actual area (as compared with 400× magnification) can be analyzed. While it clearly demands some optimization, automatic counting has important advantages [16, 33, 34]: It is fast (and the speed of processing is of upmost importance), whereas manual counting is very time consuming. Furthermore, by applying the same settings, automatic counting is more reliable since it is not affected by user-to-user interpretation variability.

Effect of cell agglomeration in bacterial quantification

S. epidermidis is known to adhere to multiple surfaces, and subsequent cell–cell aggregation and matrix production allows the establishment of biofilms [16, 35]. Since biofilm formation is considered a major virulence factor of *S. epidermidis* [1], many studies addressed the optimization of methodologies to detach bacteria from infected medical implants [20, 36, 37]. An *in vitro* implant infection model employed by Kobayashi *et al.* [37] showed that a sonication time between 1 and 5 min (frequency of 40 kHz) is ideal for dislodging biofilm bacteria from a metal substrate; however, the authors also remark that the use of short periods of sonication may be beneficial since the cell morphology and viability are less perturbed. Our results confirmed that, under certain conditions, intense vortexing can be used instead of sonication. As a vortex mixer is more affordable than a sonicator, some researchers might choose this option. However, it was clear that 1 min of vortexing was unable to reduce the cell cluster size formed in older biofilms, showing that sonication is a more effective treatment, even at reduced duration and lower intensity. Of note, the longest duration and sonication intensity used here (cycle C) did not influence the viability of *S. epidermidis* bacteria within the biofilm, as determined by the live/dead microscopic observations (Fig. 3). While a strong sonication cycle can easily kill gram-negative bacteria, gram-positive ones withstand higher sonication rates [38]. Moreover, our results are in agreement with the study performed by Joyce *et al.* [36].

Interestingly, microscopic quantification was the only approach that provided a constant amount of quantified bacteria, under all conditions tested. This can be explained by the ability to accurately differentiate

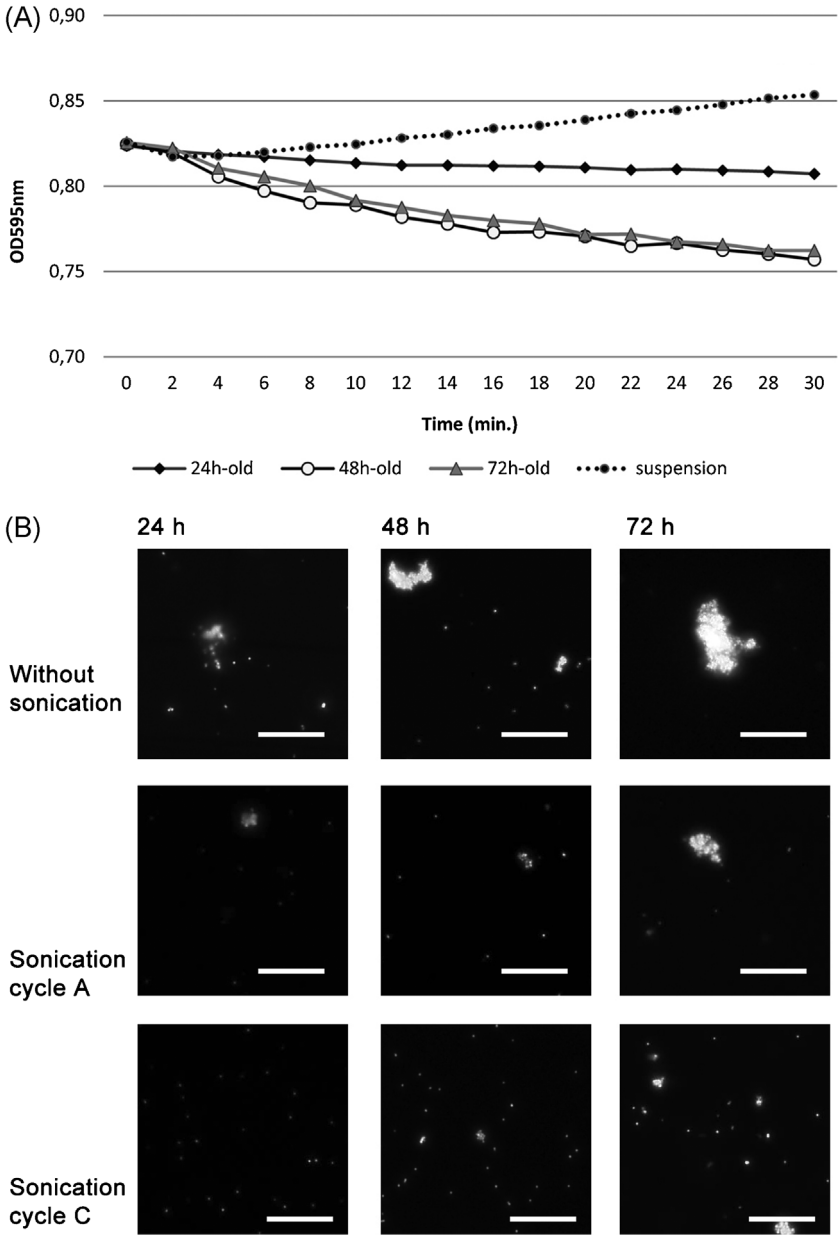

Figure 4. Effect of bacterial cell clusters during the time course of biofilm formation: (A) OD_{595nm} measurements over time. (B) Typical examples of cell clusters observed by microscopy in samples under the indicated conditions. The results are representative examples of three independent experiments.

between a cell cluster and an individual cell, which would otherwise be considered indistinguishable by CFU determination. This reasoning is based on the fact that one bacterium or a cluster of bacteria will both result in only one CFU, despite the obvious fact that they represent different amounts of bacteria, as has been shown previously [17].

Although most of our study was performed with *S. epidermidis* 9142, our findings were also confirmed by repeating key experiments with the biofilm-forming strains 1457 and RP62A. These strains were selected since they show a distinct ability to produce biofilms: Strains 9142 and 1457 produce a more dense and crusty biofilm whereas the biofilm produced by RP62A is smoother [39]. Of note, the *S. epidermidis* strain RP62A used in the present study did not produce a significant amount of biofilm, in contrast to studies performed by Christensen *et al.* [40]. This is, however, in agreement with the findings of Handke *et al.* [39]. Indeed, it is documented that phenotypic variations in *S. epidermidis* RP62A biofim

production occur in 30% of the variants [41]. Of note, while strains 9142 and 1457 showed a significantly higher biomass than RP62A, these differences were not correlated with the CFU counting at 72 h of growth. While this can be easily explained by the fact that a biofilm is the sum of the bacteria and the matrix, suggesting only that 1457 and 9142 would accumulate a denser matrix, it is nevertheless peculiar that microscopic counting was better able to discriminate between the three tested strains.

Our study pointed out that older biofilms will have more microscopic clusters that can interfere with the quantification of biofilm bacteria. Taken together, our results show that fluorescence-based microscopy, in association with an automatic image counting software, appears to be the most promising and more precise method among the conventional techniques to assess the amount of bacteria in *S. epidermidis* biofilms at different incubation times. While it clearly demands some initial optimization, automatic counting has important advantages [16]. Furthermore, by applying the same settings, automatic counting is more reliable since it is not affected by the user-to-user interpretation variability.

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Conflict of interest

The authors declare no competing interests.

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