An improved and versatile methodology to quantify biofilms formed on solid surfaces and exposed to the air-liquid interphase

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Abstract

To study pellicle formation, a new method has been developed to quantify biofilm formed on solid surfaces and exposed to air-liquid interphase. It is a versatile system since different adherent material surfaces might be tested. The methodology is a robust and reproducible approach to quantify biofilm.

In many natural habitats, bacteria grow in form of biofilms, which are complex structures where bacteria are embedded within a self-produced extracellular matrix. These microbial communities are highly resistant to environmental stresses and antimicrobial substances such as disinfectants and antibiotics (Donlan and Costerton, 2002); (Steenackers et al., 2012). During the last decades, important efforts to develop new anti-biofilm strategies have been done. These approaches focused in developing new surfaces resistant to bacteria adherence and finding compounds that might prevent biofilm formation or dissolve existing biofilms (Simões et al., 2010). To assess the efficiency of new products, reliable methodologies to quantify biofilm production are required. In general, different bacteria might form two types of biofilm, air-liquid interphase biofilms (pellicles) and solid-liquid biofilms. Although relevant pathogens form pellicle biofilm, not many quantitative methodologies are currently available. Most of the studies are based in visual inspection of the pellicle formed on the surface of the cultures and qualitative comparison among samples. In this report, we have developed a simple and reliable procedure to quantify biofilm formed on solid surfaces and exposed to the air-liquid interphase based on the use of a Hellendahl type staining jar as a culture flask to grow bacteria. The surfaces used to assess the biofilm formation were provided as slides that are partially submerged in the bacterial culture during the incubation. Several slides can be used within a staining jar allowing multiple technical replicas for each culture. Customized slides (76 x 26 mm) of different material might be used, making this a versatile system to assess biofilm growth (Fig. 1A).

To set up this methodology, *Salmonella enterica* serovar Enteritidis strain 3934, a clinical isolate capable of forming pellicle biofilms in standing culture, was selected as a model organism (Solano et al., 1998). Its ability to form biofilm on a glass surface in contact with the air-liquid interphase was monitored. Staining jars containing the glass slides were autoclave-sterilized. Up to four slides per jar were successfully tested. Cultures of the strain *S*. Enteritidis 3934, grown in LB at 37ºC for 16 hours, were used to inoculate fresh CFA medium (Suzuki et al., 2002) at a 1/100 dilution. Then, 80 ml of the inoculated media was added to the staining jars. Cultures were grown statically at 25ºC, optimal conditions for the formation of biofilm by *Salmonella* (Hamilton et al., 2009). Biofilm was monitored after 24, 48 and 72 hours incubation. Upon gentle removal of the pellicle by vacuum aspiration, the media was discarded. The glass slides were soaked three times in PBS for 10 seconds using a staining rack to remove no adherent bacterial cells. Next, the biofilm adhered to the glass slides was heat fixed by 30 minutes incubation of the slides at 80ºC. The fixed biofilm could be visualized as a white band on the glass slide. As shown in figure 1B, after 24 h incubation a white band is detected on the glass surface indicating the existence of biofilm. The band thickness increases with the incubation time, suggesting a progressive increase in biofilm mass. Therefore, the visual observation of the white band thickness provides a qualitative methodology to study biofilm formation. For quantitative measurement of the biofilm biomass, crystal violet (CV) staining was performed. Slides were submerged for 15 minutes in a CV solution (1% w/v), rinsed with water and air-dried (Fig. 1C). Each slide was then placed in a 50 ml tube containing 5 ml of 30% acetic acid solution and vigorously shaken to ensure the complete solubilisation of the CV.

The OD_{545nm} of the resulting solution was determined. As expected, the results obtained (Fig. 2A) allow a more precise quantification of the biofilm than the visual observation. The biomass values when comparing 24 and 48 h show a very striking difference which could not be easily predicted by visual inspection. Interestingly, although at 24 h a clear white band was observed (Fig. 1B), the quantitative data clearly indicate that does not represent a mature biofilm. The amount of biomass at 24 h is more than 20-fold lower than at 48 h. These results highlight the convenience of using quantitative methodologies to study biofilm formation.

To compare the developed method with a common used methodology, quantification of biofilm formed using borosilicate glass tubes (16 x 100 mm, round bottom) was performed. In this case, 2 ml of bacterial culture was used under the same experimental conditions (static incubation at 25ºC for 24, 48 and 72 hours). The staining protocol was followed as above with minor differences. To solubilise the CV, 3 ml of acetic acid 30 % solution was added and the tube was vigorously shaken. The result of the quantification of the biofilm formed is shown in figure 2B. A time-course increase in the biofilm biomass is also observed. However, when using this methodology an astonishing variability between technical replicas was detected, as shown by the relative standard deviation values obtained.

When both methodologies are compared (Fig. 2C), is evident that the slide-band methodology is more statistically reliable than the tube based methodology. When considering samples showing an apparent biofilm (cultures incubated for 48 and 72 h), the relative standard deviation with the slide-band

method is much lower than with the tube-based method. In the latest, the relative standard deviation is approximately 70% of the mean value.

Similar experiments were performed with another pellicle forming bacteria, *Pseudomonas aeruginosa* ATCC 15692. When this strain was grown in TSB at 37ºC for 24, 48 and 72 hours, a clear biofilm was observed, both in Hellendahl type staining jars (Fig. 2D) and borosilicate glass tubes (Fig. 2E). The quantification of the biofilm formed on the solid surface exposed to the air-liquid interphase corroborates the high reproducibility of the slide-band method described (Fig. 2F).

This methodology has also been successfully used to detect and quantify biofilm formed on glass slides by a pellicle-forming Gram positive strain as *Bacillus subtilis* ATCC 6051a grown in minimal media MSgg (Connelly et al., 2004) at 25ºC (data not shown).

The current concern in evaluating the clinical and industrial impact of biofilm urges the development of reliable quantitative methodology to measure air-liquid interphase biofilm. In this work we describe a new method to quantify biofilms formed on solid surfaces and exposed to the air-liquid interphase using a Hellendahl type staining jar. This method i) is highly reproducible, ii) allows to test several technical replicas for the same culture, and iii) provides high versatility due to the wide range of materials that can be potentially used as attaching surfaces.

Acknowledgments

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Figure legends

Figure 1. Experimental setup of the slide-band method to detect and quantify air-liquid interphase biofilm. **A**. The left panel shows a Hellendahl jar with three glass slides containing CFA culture of *S. enterica* sv. Enteritidis 3934 strain. In the right panel, to illustrate the versatility of the described methodology, slides of glass (1), stainless-steel (2) and silicone (3) are shown. **B**. Biofilm formed by strain 3934 on glass slides after 24, 48 and 72 hours of static growth at 25ºC in CFA medium in Hellendahl jars containing three glass slides submerged. After removal of the culture and three washes in PBS, the biomass adhered to the glass surface is detected as a white band after fixation by incubating at 80ºC during 30 min. A negative control using non-inoculated CFA medium is shown. **C**. Same as in B after staining with crystal violet.

Figure 2. Quantification of biofilm (OD_{545nm}) formed on a glass surface after static growth of strain *S. enterica* sv. Enteritidis 3934 at 25ºC in CFA medium (A, B, C) and *P. aeruginosa* at 37ºC in TSB medium (D, E, F), either in Hellendahl jars containing three glass slides submerged (**A, D**) or on glass tubes (**B, E**). Biofilm biomass was quantified after 24, 48 and 72 hours of incubation. The data shown is the average and standard deviation of six samples. **C**, **F**. Summary of the data obtained in A, B and D, E, respectively. The relative standard deviation (percentage of the mean value that represents the standard deviation) is indicated.

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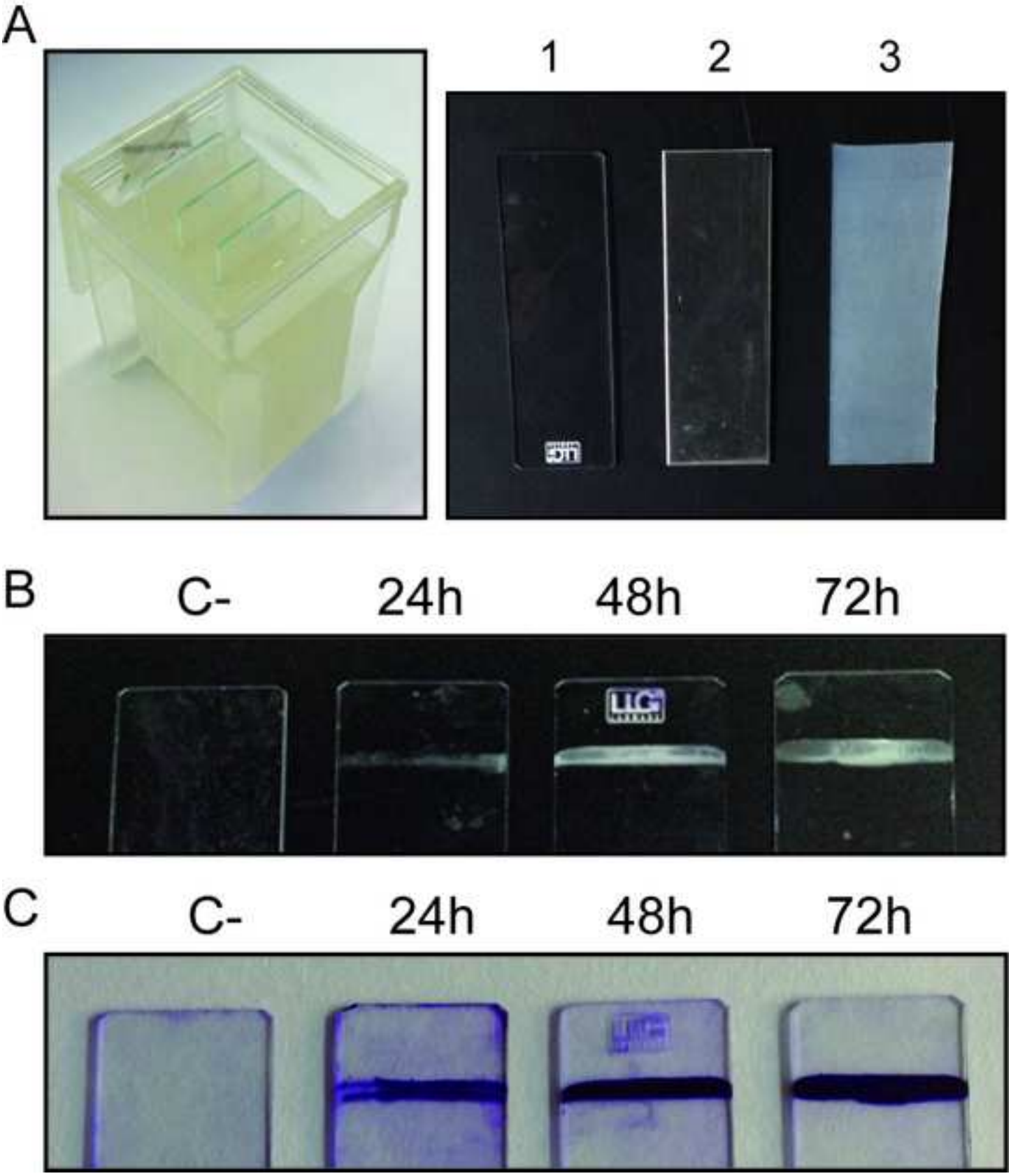

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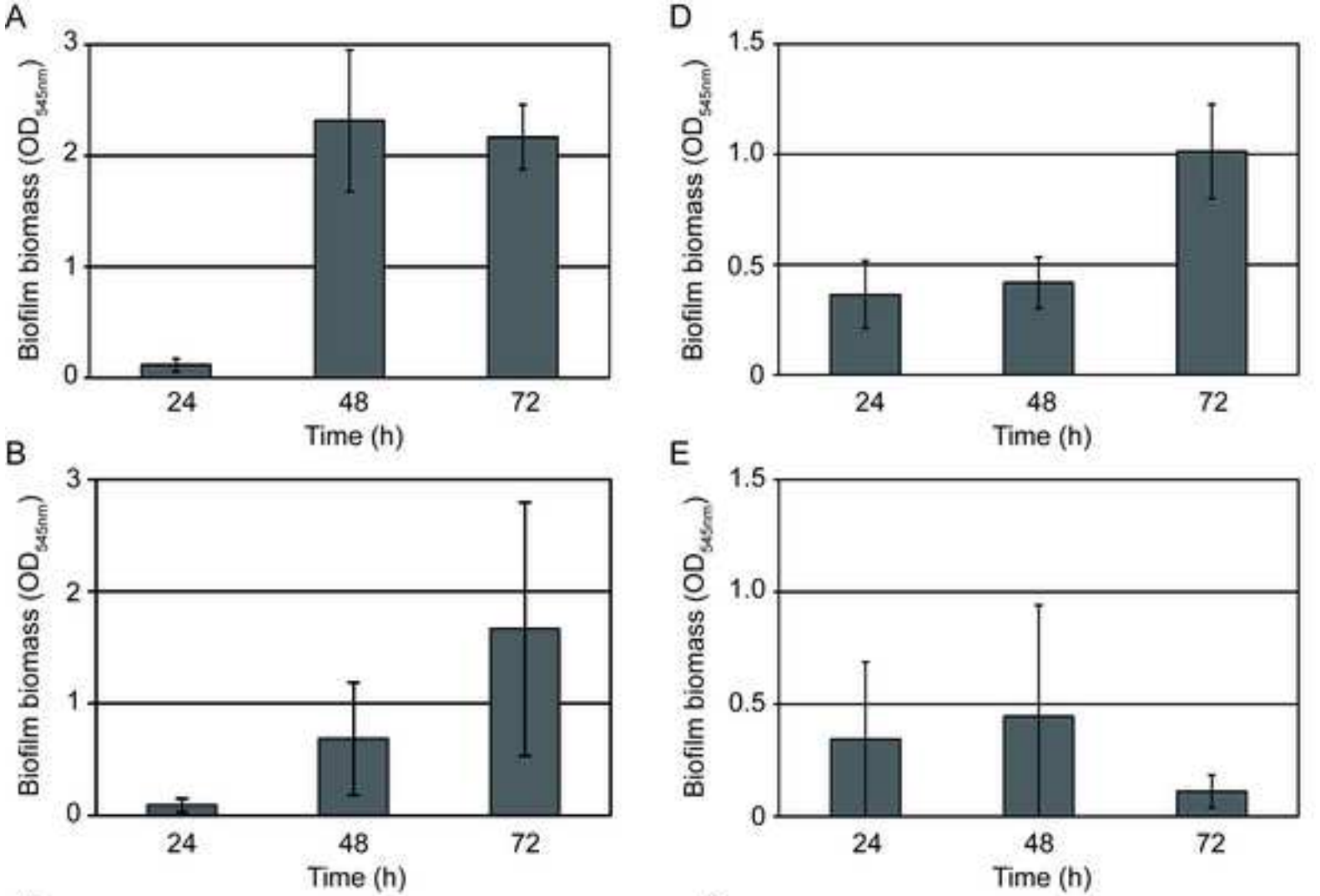

C

		24h	48h	72h
Staining jar	Average	0.11	2.31	2.17
	SD	0.05	0.63	0.29
	RSD	**49.3**	**27.6**	**13.5**
Tubes	Average	0.09	0.68	1.66
	SD	0.06	0.50	1.13
	RSD	**65**	**73**	**67**

F

		24h	48h	72h
Staining jar	Average	0.36	0.41	1.01
	SD	0.15	0.11	0.21
	RSD	**42**	**27.7**	**21.1**
Tubes	Average	0.34	0.44	0.11
	SD	0.34	0.49	0.07
	RSD	**100**	**111**	**64.2**