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ABSTRACT
The microbial biofilm is a structure often developed by microorganisms when performing their harmful effects, both in the medical and industrial fields. Therefore, methods allowing identification and analysis of the biofilm play a fundamental role in determining the kind of intervention needed to avoid these effects. The microtiter spectrophotometric assay is rec-ognized as the gold standard method to quantify a biofilm and to analyze the anti-biofilm activity of various substances. The aim of the present work is to validate this method through an uncertainty evaluation, covering eight different microbial species.
The results show that the microtiter spectrophotometric assay is adequate to perform the biofilm analysis, with a good reproducibility and a reasonable uncertainty. However, the method requires a thorough knowledge of bio-dynamics concerning microbial species tested, in order to perform some protocol improvements catering in turn for better results.

Keywords:
Biofilm
Microtiter spectrophotometric assay
Uncertainty evaluation

1. Introduction

Microorganisms can live in one of two possible states: sessile or planktonic. The sessile phenotype results from attachment and usually develops into a biofilm that has unique characteristics [1]. The biofilm is commonly defined as "*an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material.*" [2]. This defini-tion is not entirely satisfactory, since a biofilm may be not only an aggregation of bacteria but also, as recently defined, "*a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phe-notype with respect to growth rate and gene transcription*" [3]. A biofilm is characterized by the adhesion of the cells to a non exfoliative surface, immersed in an aqueous medium and/or on other bacteria cells. The mechanism of attachment may be explained in terms of several factors, namely substratum effects, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, character-istics of the medium, and various properties of the cell surface [2]. The association between biofilms and diseases is not always easy, because the biofilm infection cannot be proved according to Koch's postulates [1]. Infections strongly linked to a biofilm development, such as periodon-tal disease, endodontic infections, candidiasis, valve endo-carditis, cystic fibrosis, urinary catheter cystitis, have all in common the resistance to non-invasive therapies (as drug therapy). The study of this microbial state is today indis-pensable to obtain a diagnosis and to decide an appropriate therapy [1,4–8]. Biofilm infections are often originated by nosocomial infections linked to poorly sterilized surfaces of medical devices, entailing critical consequences for in-volved patients [1,9,10]. Among the microbial species in-volved in biofilm infections are some microbes having a

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primary role or considered model organisms for in vitro analysis, and therefore are among the most studied microbial species (spp) both in vivo and in vitro. Eight of these microbes, namely *Escherichia coli* (*Ec*), *Pseudomonas aeruginosa* (*Pa*), *Klebsiella pneumonia* (*Kp*), *Bacillus subtilis* (*Bs*), *Enterococcus faecalis* (*Ef*), *Staphylococcus aureus* (*Sa*), *Candida albicans* (*Ca*) and *Aggregatibacter actynomicetemcom-itans* (*Aa*) [1,4,9–15] (Table 1), were considered in this study. The development of a reproducible, specific and sensi-tive biofilm measurement method is today necessary in both medical and industrial fields. Among the various methods, indirect and direct applications may be distin-guished. Indirect applications, such as standard plate counts, roll techniques, and sonication, allow the operator to obtain a quantification analysis of the biofilm after a detaching action. Other indirect techniques, such as radio-labeled bacteria, enzyme-linked immunosorbent assay,

Table 1
Main diseases related to bacterial spp examined; nosocomial infections are identified.

Biofilm microbial species	Infection or disease	Nosocomial
Escherichia coli (*Ec*)	Biliary tract infection	NO
	Bacterial prostatitis	NO
	Orthopedic devices infection	YES
Pseudomonas aeruginosa (*Pa*)	Cystic fibrosis	NO
	Contact lens infection	YES
	Central venous catheters infection	YES
	Orthopedic devices infection	YES
Klebsiella pneumonia (*Kp*)	Urinary catheter cystitis	YES
	Central venous catheters infection	YES
Bacillus subtilis (*Bs*)	Model organism	–
Enterococcus faecalis (*Ef*)	Endodontic infection	NO
	Urinary catheter cystitis	YES
	Mechanical heart valves infection	YES
	Orthopedic devices infection	YES
	Intra-Uterin devices infection	YES
Staphylococcus aureus (*Sa*)	Arteriovenous shunts infection	YES
	Intra-Uterin devices infection	YES
	Pentile prostheses infection	YES
Candida albicans (*Ca*)	Candidiasis	NO
	Vaginitis	NO
	Peritoneal dialysis peritonitis	YES
	Vascular catheters infection	YES
	Joint prostheses infection	YES
	Central venous catheters infection	YES
Aggregatibacter actynomicetemcomitans (*Aa*)	Periodontal disease	NO
	Meningitis	NO
	Heart disease	NO

biologic assays, stained bacterial films, and microtiter plate procedures, enable the observer to obtain a quantification evaluation of the biofilm by measuring some attribute for the attached organism [16]. While many works in literature found limits often linked to the indirect methods [16–18], the direct methods show a better performance in terms of biofilm assessment, offset however by greater difficulties associated with techniques and equipment which may not be readily available (laser-scanning confocal, transmission electron and scanning electron microscopes) [16].

An indirect method which showed a good level of reproducibility, specificity and sensitivity, along with substantial simplicity, is the microtiter or microplate spectrophotomet-ric assay [16,19–22]. This method, first described in 1977 [19] and modified and improved in 1998 and in 2002 [20,16], is highly adaptable to the type of organisms to be studied in various and different growing conditions, is used routinely [23,24], and is nowadays considered as the gold standard for the indirect evaluation of biofilm [22].

Spectrophotometry, a technique based on the interaction of light and matter, investigates the absorption of different substances within the wavelength range 190–780 nm. In this range the absorption of the electromagnetic radiation is caused by the excitation of the bonding and non-bonding electrons of the ions or molecules. Spectrophotometry is used for both qualitative and quantitative investigations of samples. The wavelength at the maximum of the absorption band is related to the amount of the species absorbing the light.

This work is aimed at validation of the microtiter spectrophotometric biofilm production assay as a measurement tool using a metrological approach, exploiting statistical methods in order to perform an uncertainty evaluation [25].

2. Materials and methods

2.1. Culture preparation

The following species were used for this study:

1. *Gram positive bacteria*: *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212 [26], *Bacillus subtilis* (clinical strain) [8].
2. *Gram negative bacteria*: *Escherichia coli* ATCC 7075, *Pseudomonas aeruginosa* ATCC 27853, *Aggregatibacter actinomycetemcomitans* DSM 11123 (genotype JP2) (Deutsche Sammlung von Mikrorganismen und Zellkulturen GmbH, Braunschweig, Germany) [26], *Klebsiella pneumoniae* (clinical strain) [27].
3. *Fungi*: *Candida albicans* from oral clinical isolates. These specimens were plated in Sabouraud glucose agar for 48 h at 35 °C (Microbiol, UTA, Cagliari, Italy). The colonies were identified with an API ID32C system (Biomerieux, St Louis, MO) and maintained at −20 °C in skimmed milk (Oxoid, Basingstoke, UK) [26].

Before the application of the spectrophotometric assay method, the selected microorganisms were divided into three groups:

(i) Strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were incubated in Müller Hinton agar, (MH Microbiol, UTA, Cagliari Italy) at 37 °C for 24 h.
(ii) One strain of Candida albicans was cultured in Sabouraud glucose agar at 37 °C for 48 h.
(iii) One strain of *Aggregatibacter actinomycetemcomitans* was incubated in Anaerobic difficile agar (Microbiol, UTA, Cagliari, Italy) at 37 °C for 24 h with a CO_2 concentration of 5% [28].

After the incubation, group (i) was suspended in Müller Hinton broth (MH), group (ii) in Sabouraud glucose broth (SAB) and group (iii) in vials containing Schaedler Broth (SH) [28]. Bacterial suspensions were performed to obtain a concentration with a turbidity equivalent to the No. 3 McFarland standard (about 10^8 CFU/ml), then diluted to 1/100 (obtaining a 10^6 CFU/ml) using a spectrophotometer at 620 nm (DMS100s, Varian, New Hampshire, USA) [6].

2.2. Microtiter plate biofilm production assay

The protocol described in 2007 [22] was applied to perform the biofilm analysis. During the application of the method, each step was numbered and analyzed, reading at 620 nm with a microtiter plate reader (Microplate Reader TECAN SPECTRA II) for the metrological evaluation. To realize the colorimetric assay, 200 µl of each suspended strain, were added to six wells of a 96-well plate and incubated for 24 h. After the incubation (STEP 1: initial condition) the medium was removed (STEP 2: pre-washing) and the microtiter plate wells were washed three times with 200 µl of PBS (0.1 M, pH 7.4) buffer using a multichannel pipette, and allowed to dry for 15 min (STEP 3: post-washing). The microtiter wells were stained with 200 µl of 0.4% crystal violet for 15 min at room temperature. The unbound crystal violet stain was removed and the wells were washed three times with 200 µl of PBS buffer (STEP 4: post-crystal violet). The wells were air-dried for 15 min and the crystal violet in each well was solubilized by adding 200 µl of 33% acetic acid (STEP 5: post-acetic acid). The biofilm value was represented by the analysis of the absorbance carried out with a spectrophotometer at 620 nm (SLT-Spectra II™, SLT Instruments, Germany).

2.3. Step by step spectrophotometric measurement

During the colorimetric assay, the five critical steps previously described, i.e. STEP 1 to STEP 5, were identified. Each phase was analyzed through a spectrophotometric reading at 620 nm obtaining 240 measurements of absorbance. In STEP 1 the absorbance value of the bacteria planktonic growth after the incubation was obtained; it could be considered as the value representing the development of each microorganism in an aqueous medium before any manipulation. By STEP 2 the value that represents the turbidity of each well after the broth removal was obtained. STEP 3 determined the absorbance after the first washing phase. In STEP 4 the turbidity of the dried colored biofilm after washing and before suspension was analyzed. STEP 5 was the final phase of the microtiter plate biofilm production assay method; by this phase the turbidity of the colored biofilm suspended by acetic acid may be determined. Table 2 shows some descriptive statistics (mean and standard deviation) and values for each specie at each step, corrected subtracting a relevant *C*-value different for each broth (MH, SAB or SH). Since negative values of absorbance are physically meaningless, such values were arbitrarily set equal to zero, enabling the evaluation of metrological characteristics using statistical methods.

2.4. Statistical analysis

An uncertainty evaluation of the whole process has been performed according to the GUM [25]. This may be properly organized in a tabular format (Table 3), referring to EA-4/02:1999 [29]. A minor modification was adopted by substituting the standard deviations with variances, in order to show the individual contribution to the variance of output quantity *y* [30,31].

The considered mathematical model is:

$$y = x - b \tag{1}$$

where x is the general mean of the means of six replications of absorbance values considering all the

Table 2
Descriptive statistics for each specie at each step. Means corrected by subtracting the relevant *C*- value are also shown (negative values were arbitrarily set equal to zero).

	STEP 1 Initial condition			STEP 2 Pre-washing			STEP 3 Post-washing			STEP 4 Post-crystal violet			STEP 5 Post-acetic acid		
	Mean	St. Dev.	Corrected	Mean	St. Dev.	Corrected	Mean	St. Dev.	Corrected	Mean	St. Dev.	Corrected	Mean	St. Dev.	Corrected
Kp (*MH*)	1.381	0.07	1.282	0.134	0.04	0.102	0.093	0.07	0.000	0.087	0.02	0.022	0.273	0.05	0.174
Bs (*MH*)	0.739	0.07	0.639	0.339	0.12	0.307	0.093	0.06	0.000	0.073	0.07	0.009	0.123	0.03	0.025
Sa (*MH*)	0.549	0.08	0.449	0.136	0.02	0.104	0.103	0.07	0.008	0.094	0.05	0.030	0.158	0.05	0.060
Pa (*MH*)	0.933	0.06	0.833	0.113	0.02	0.082	0.113	0.05	0.018	0.109	0.07	0.045	0.138	0.03	0.039
Ec (*MH*)	0.991	0.04	0.892	0.100	0.02	0.068	0.122	0.04	0.026	0.103	0.08	0.039	0.108	0.03	0.009
Ef (*MH*)	0.149	0.05	0.049	0.073	0.01	0.041	0.105	0.07	0.009	0.070	0.02	0.006	0.127	0.03	0.029
Ca (*SAB*)	1.232	0.04	1.145	0.324	0.05	0.289	0.107	0.04	0.000	0.104	0.08	0.028	0.147	0.04	0.051
Aa (*SH*)	0.359	0.04	0.258	0.247	0.02	0.196	0.270	0.06	0.151	0.274	0.09	0.136	0.380	0.05	0.259
C-(*MH*)	0.100	0.01		0.032	0.00		0.095	0.04		0.064	0.03		0.099	0.01	
C-(*SAB*)	0.088	0.01		0.035	0.01		0.114	0.01		0.076	0.04		0.096	0.01	
C-(*SH*)	0.101	0.01		0.051	0.04		0.119	0.03		0.138	0.02		0.120	0.01	

Table 3
Uncertainty table, showing main contributions and resulting expanded uncertainty.

x_j			s_j	a_j	k_{aj}	$u^2(x_j)$	c_j	$u_j^2(y)$	v_j	$u_j^4(y)/v_j$
Symbol	Value	Note								
x	0.279	Res		5.0E−04	3	8.3E−08	1.0E + 00	8.3E−08	100	6.9E−17
		Repr.	4.4E−02			2.0E−03	1.0E + 00	2.0E−03	100	3.9E−08
b	0.089	Res		5.0E−04	3	8.3E−08	−1.0E + 00	8.3E−08	100	6.9E−17
		Repr.	4.4E−02			2.0E−03	−1.0E + 00	2.0E−03	100	3.9E−08
y	0.191		Variance of y, $u^2(y)$					3.9E−03	Σ	7.7E−08
			Standard deviation of y, $u(y)$					6.3E−02	v_y	200
			Confidence level					95%		
			Coverage factor (Student's t)					2.0		
			Expanded uncertainty, $U(y)$					1.2E-01		

microorganisms at each step, while b is the overall mean of the means of six replications of absorbance values considering all the C- values at each step. The value of y is 1.9×10^{-1}.

The resolution of the spectrophotometer is equal to 1×10^{-3}. The reproducibility, calculated as the standard deviation of biases from the means of six replications considering all the absorbance values at each step, was found equal to 4.4×10^{-2}.

The resulting expanded uncertainty at 95% confidence level is 1.2×10^{-1}, i.e. relative expanded uncertainty of about 65%.

This value concerns the whole process, namely the five steps referred to above. To detect some possible criticalities of the method, linked to biological behavior or to practical implementation, a statistical comparison among the five steps was performed in terms of variance. In particular, a F-test [32] was exploited to check, for each microbe, whether there are significant differences in terms of variability among the different phases (Table 4). Only STEP 4 for microbe *Ec* exhibits a variance significantly greater than the variance of the mean of the five steps, at a 5% level.

3. Results

3.1. Preliminary results

Given the different strains of microorganisms, they are best considered individually. Since the turbidity of the suspended biofilm is the focus of the method, STEP 5 was examined in detail. In this step, considering absorbance values corrected with respect to *C*-values, *Ec*, *Ef*, *Bs* show some negative values, making the evaluation of the uncertainty meaningless. Furthermore, *Pa*, *Sa*, *Ca* exhibited a very large variability, entailing excessively large values of uncertainty. On the other hand, results related to *Kp* and *Aa* showed an acceptable variability. The method described in Table 3 was applied, obtaining values of the relative expanded uncertainty, respectively, equal to 28% for *Kp* and to 22% for *Aa* (Table 5). The experiments were repeated for *Ec*, *Kp* and *Aa* in order to improve the method.

3.2. Method improvement

The preliminary analysis enabled identification of some critical phases with step-by-step spectrophotomet-

Table 4
F-test relative to the variability among different phases of all species. The *F* value at 95% confidence level is 2.60, since the degrees of freedom are 5 for the numerator and 25 for the denominator. Only STEP 4 exhibits a significant variance ratio for *Ec* (bold number), the significance level is barely approached for *Aa* and *Ca*.

	Var m	STEP 1 Initial condition		STEP 2 Pre-washing		STEP 3 Post-washing		STEP 4 Post-crystal violet		STEP 5 Post-acetic acid	
		Var	Var ratio	Var	Var ratio	Var	Var ratio	Var	Var ratio	Var	Var ratio
Kp	0.003	0.005	1.82	0.002	0.64	0.005	1.68	<0.001	0.10	0.002	0.76
Bs	0.006	0.005	0.83	0.014	2.50	0.004	0.65	0.005	0.90	0.001	0.12
Sa	0.003	0.006	1.92	<0.001	0.12	0.005	1.53	0.002	0.73	0.002	0.70
Pa	0.003	0.004	1.58	<0.001	0.09	0.002	0.88	0.006	2.12	0.001	0.33
Ec	0.002	0.002	0.75	<0.001	0.12	0.002	0.82	0.006	**2.80**	0.001	0.52
Ef	0.002	0.003	1.58	<0.001	0.05	0.004	2.46	<0.001	0.28	0.001	0.63
Ca	0.003	0.002	0.56	0.003	1.03	0.002	0.58	0.007	2.33	0.001	0.50
Aa	0.003	0.002	0.52	<0.001	0.14	0.003	1.04	0.008	2.46	0.003	0.84

Table 5
Mean values, relevant absolute and relative expanded uncertainties (95% confidence level), before and after the method improvement.

	Before improvement			After improvement		
	m	U	U/m	m	U	U/m (%)
Ec	–	–	–	0.051	0.013	26
Kp	0.174	0.049	28%	0.546	0.124	22
Aa	0.259	0.057	22%	0.147	0.063	43

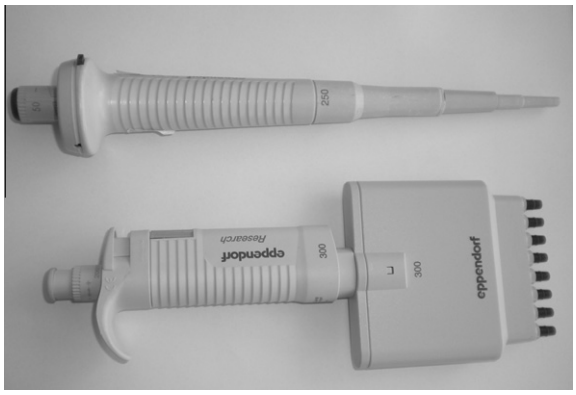

Fig. 1. The multichannel pipette (bottom) was substituted by the single pipette (top) to improve the method.

ric measurements. In particular, a strong criticality was highlighted in the values during STEP 3, exhibiting an increase of the dispersion of results (but for *Ca* and *Bs*). Another problem is due to the tendency shown by many microorganisms to have an absorbance value near zero at STEP 5, requiring remedial action. Since STEP 3 is the most critical phase, an improvement in the method of washing was devised, by a closer control of pipette tip during the insertion into the wells and a softer release of the washing liquid (PBS). To achieve these objectives, a single pipette tip was adopted, avoiding the use of the multichannel pipette (Fig. 1), thus enabling the operator to improve control during washing phases (STEP 3 and before STEP 4).

3.3. Final results

After improvement of the method, values of the relative expanded uncertainty equal to 26% for *Ec*, 22% for *Kp*, and 43% for *Aa* (Table 5), were obtained. On the other hand, comparing the values of absorbance before and after improvement of the method, *Kp* showed the highest values (Fig. 2).

4. Discussion

The microbial biofilm development process is strictly related to the presence or absence of a variety of influencing factors [21]. Bacterial metabolism, genotype presence or ab-sence of specific nutrients, level of O_2, pH, temperature are some examples of factors which could up- or down-regulate biofilm both quantitatively and qualitatively [21,24]. Bear-ing this in mind, since the biofilm is a dynamic structure, slight alterations among experiments may result into very different growth in vitro. Therefore comparative experi-ments with the method described above are best performed at the same time with the same conditions, since compari-son among absorbance values obtained for the same bacte-ria grown in different times and/or under different conditions might yield inconsistent results.

Observing STEP 1 before and after improvement of the method, significant differences at the 95% confidence level for *Aa* and *Kp* planktonic growth may be observed, with a marked reduction of dispersion. In fact, *Aa* shows in STEP 1 of the first test a mean value of absorbance equal to 0.36 with an upper confidence limit of 0.40 and a lower confidence limit of 0.32, while in the second test the mean decreases to 0.29 with limits of 0.30 and 0.27. Accordingly,

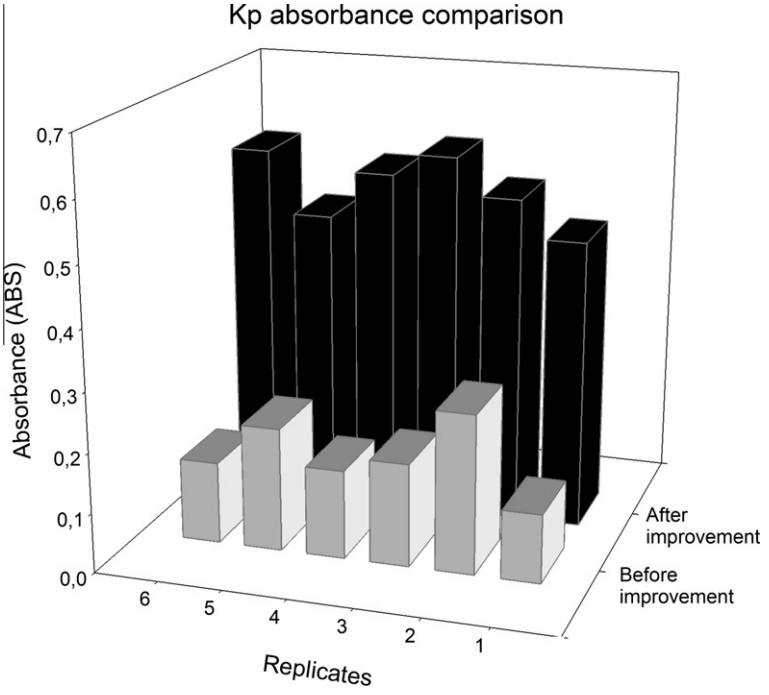

Fig. 2. Bar diagram pertaining to replicated spectrophotometric measurements for *Kp* (absorbance values before and after the method improvement).

Kp shows in STEP 1 of the first test a mean of 1.38 with limits equal to 1.46 and 1.31, while in the second test these values change to a mean of 1.22 and limits of 1.24 and 1.21. *Ec* showed a similar planktonic growth in both tests obtaining a mean of 0.99 before the method improvement and of 1.00 after the method improvement, with limits equal to 1.03 and 0.95 against 1.06 and 0.95. These results highlight the growth stability of this bacteria, supporting the choice of *Ec* as a standard for studies in the microbiological field. The different growth rates showed by *Aa* and *Kp* may not be easily explained, since in both tests the same protocol performing the cultural phase was applied; some uncontrolled environmental factor may somehow influenced the bacterial growth.

Further considerations are necessary to understand why the method showed substantially different results between different microbial species. In the first analysis, for some microbial species biofilm analysis was made impossible by negative results and/or excessive variability. These problems may be related to a too light and thin biofilm; indeed, the biofilm is a microbial structure linked to both virulence and preservation, as well as the result of an intra-species cooperation [5,6,24]. Experiments in vitro may not readily replicate the conditions necessary to determine the development of virulence factors, as the analysis of each microorganism alone (necessary to under-stand the biodynamic linked to the biofilm development) implies the formation of a much thinner biofilm than when many bacteria grow together. The use of antibacterial sub-stances at low concentrations might determine the forma-tion of a thicker biofilm, however this could negate comparison among different microorganisms.

The improvement of the method enabled to determine *Kp* and *Ec* biofilms with a reasonable uncertainty thanks to a major compliance with the biofilm light base-structure, on the contrary *Aa* showed the worst results with respect to the first analysis (see Table 5). This problem may be explained by the bio-dynamic of *Aa*; during the planktonic growth, this bacteria forms micelle with consequent precipitation on the bottom of the walls, so that these structures, while not participating in the formation of the biofilm, develop a weak adhesion to the biofilm surface [23,24]. Previous studies show that removal of loosely adherent or non-adherent cells requires many washings, up to 15, i.e. 6 more than the base protocol [23].

In conclusion, the microtiter spectrophotometric assay proved to be a valid method to perform biofilm analysis and measurements. Key factors for proper use are knowledge of the dynamics of the biofilm formation of the species to be tested, and application of the method on all samples simultaneously when performing comparative studies.

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