Use of Calgary and microfluidic BioFlux systems to test the activity of fosfomycin and tobramycin alone and in combination against cystic fibrosis *Pseudomonas aeruginosa* biofilms

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Running title: Fosfomycin-tobramycin activity on cystic fibrosis *P. aeruginosa* biofilms

Keywords *P. aeruginosa* biofilms, cystic fibrosis, Calgary, BioFlux, fosfomycin-tobramycin

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Abstract

Pseudomonas aeruginosa is a major cause of morbidity and mortality in chronically infected cystic fibrosis patients. Novel in *vitro* biofilm models, which reliably predict therapeutic success of antimicrobial therapies, should be implemented. The activity of fosfomycin, tobramycin and fosfomycin-tobramycin combination was tested against 6 susceptible *P. aeruginosa* strains isolated from respiratory samples of cystic fibrosis patients by using two *in vitro* biofilm models: a closed system (*Calgary* device) and an open model based on microfluidics (BioFlux). All but one of the isolates formed biofilm. The fosfomycin and tobramycin minimal biofilm inhibitory concentrations (MBIC) were 1,024->1,024 µg/ml and 8-32 µg/ml, respectively. According to fractional inhibitory concentration analysis, the combination behaved synergistically in all the isolates except in the *P. aeruginosa* ATCC 27853 strain.

The dynamic formation of the biofilm was also studied with the BioFlux system and the MIC and MBIC of each antibiotic were tested. For the combination, the lowest tobramycin concentration that was synergistic with fosfomycin was used. The captured images were analyzed measuring the intensity of colored pixels, which is proportional to the biofilm biomass. A statistically significant difference was found when comparing the intensity of the inoculum with the intensity in the microchannel where the MBIC of tobramycin or fosfomycin or their combination was used ($p<0.01$) but not when applying the MIC ($p>0.01$).

Fosfomycin-tobramycin demonstrated to be synergistic against cystic fibrosis *P. aeruginosa* strains in biofilm models, both when testing with the *Calgary* and the microfluidic BioFlux systems. These results support the clinical use of this combination.

Introduction

Biofilm mode of growth is directly involved in the pathogenesis of *Pseudomonas aeruginosa*, contributing to morbidity and mortality in chronically infected cystic fibrosis (CF) patients (1). The eradication of this biological structure is extremely difficult because of the increased tolerance to antimicrobials that microorganisms exhibit within its environment. Inhaled tobramycin (TOB) has been long used in CF treatments to control chronic colonization, but recently, antibiotic combination in CF has been suggested, not only to reduce and delay antimicrobial resistance but also to enhance antibacterial activity, particularly against bacteria growing in biofilm (2). Previously, a fosfomycin-tobramycin combination (FT) in a 4:1 ratio has shown to be synergistic *in vitro* against *P. aeruginosa*, especially in anaerobic environments and has proven effectiveness in Phase II clinical studies (3–5).

On the other hand, susceptibility testing results should predict therapeutic success, a situation hardly achieved when considering standard planktonic MIC values for biofilm-related infections. Consequently, susceptibility testing in biofilm has been claimed as a useful tool for this purpose (6). Currently, two types of assays are available to evaluate the *in vitro* antibiotic activity on biofilms, open and closed systems. The closed or static systems analyze biofilm formation in the wells of microtiter plates and are suitable for high-throughput analysis, while the open or dynamic systems better resemble the *in vivo* conditions (7).

The objectives of this work were to analyze the effect of fosfomycin (FOF), TOB and FT on CF *P. aeruginosa* growing in biofilms. With the *Calgary* closed system, pharmacodynamic (PD) parameters, as the minimal biofilm inhibitory concentration (MBIC) and the biofilm prevention concentration (BPC), were determined. Synergy was estimated by calculating the fractional inhibitory

concentration (∑FIC) adapted to the MBIC. To observe and describe the dynamics of CF *P. aeruginosa* biofilm formation, the BioFlux microfluidic open model (Fluxion Biosciences, South San Francisco, CA) was used. With this system, FOF, TOB and FT activities were determined by measuring their effect on biofilm biomass through image intensity colored pixels analysis.

Results

Susceptibility testing results. FOF and TOB MIC values are shown in **Table 1**, all of them were interpreted as susceptible.

Biofilm assays using the *Calgary* device. All the isolates except *Pab6* strain, which corresponded to a small colony variant, were able to form a biofilm. The OD_{450} difference between 0 and 6 hours (ΔOD) after incubation was ≥0.05 for *Pab1-Pab5* and the control ATCC 27853 *P. aeruginosa* strains. For *Pab6* strain, the ΔOD was 0.01 (**Figure 1**).

FOF, TOB, and FT MBIC results are shown in **Table 2**. The FOF MBICs ranged between 1,024->1,024 µg/ml and the range for TOB MBICs was 8-32 µg/ml. For the ATCC 27853 *P. aeruginosa* control strain, FOF MBIC and TOB MBIC were >1024 µg/ml and 2 µg/ml, respectively. For strains *Pab1-Pab5* the ∑FIC index was ≤0.5 for at least one of the tested concentrations, indicating synergy between FOF and TOB. However, for ATCC 27853, FT was not synergistic at any of the tested concentrations, probably due to low TOB MBIC values (**Table 3)**.

TOB BPC was ≤1 µg/ml for all the isolates, including those with higher TOB MICs (*Pab3*, TOB MIC =2 µg/ml, *Pab5*, TOB MIC= 4 µg/ml). Therefore, all the TOB BPC/MIC indexes were ≤1. However, although all the isolates presented the same FOF MIC (64 µg/ml), the FOF BPC/MIC indexes ranged from 1 to 32. This means that for *Pab1*, *Pab4* and *Pab5* and the ATCC 27853 strains, the BPC was close to the MIC,

while *Pab2* and *Pab3* strains presented a BPC 3-5 two-fold dilutions higher than the MIC (**Table 4**). The biofilm prevention TOB concentrations within the combination were ≤1 µg/ml, thus TOB alone was able to prevent the development of the biofilm at this concentration and the addition of fosfomycin did not increase the activity.

Biofilm assays using the BioFlux device. When grown in the positive control microchannels of the Bioflux device, *Pab1-Pab5* exhibited a biofilm mode of growth, while *Pab6* did not. This result is in agreement with that observed using the *Calgary* device. At 8-hour incubation, *Pab1-Pab4* and ATCC 27853 strains had formed a percentage of biofilm ranging from 37% to 59.7%. However, for the *Pab5* strain, it was only of 7.1%. The incubation for this strain was therefore prolonged up to 24 hours, a point at which the percentage of the formed biofilm was of 40.1. At this moment, the antibiotics were added, and the incubation was subsequently prolonged up to 48 hours. The captured images for each microchannel can be observed in **Figure S1.** The intensity of colored pixels and the transformed percentage of the remaining biofilm values after the antimicrobial challenge are shown in **Figure 2**. FT was not tested in the ATCC 27853 strain, as this combination did not exhibit synergy with the *Calgary* device.

For all biofilm-producing isolates, statistically significant differences were observed ($p<0.01$) between the biofilm formed in the inoculum control and those in the microchannels with FOF, TOB or FT when tested at concentrations equal to MBIC, indicating anti-biofilm and anti-microbial activity at those concentrations. However, no significant differences were found for these bacteria when incubating with FOF or TOB at concentrations identical to MIC ($p=0.0374$ and $p=0.0547$, respectively).

∑FIC results obtained by the *Calgary* device which indicated FT synergy were confirmed with the BioFlux device as, to achieve antibiofilm effect, TOB

concentrations within the FT combination were 2-4 twofold dilutions lower than when TOB was tested alone.

Discussion

Biofilms are involved in more than 80% of the infections (8). Penetration and activity of antibiotics are usually reduced in this type of growth, compromising their antimicrobial effect within these environments. Biofilms of *P. aeruginosa* are particularly relevant in chronic pulmonary infection of CF patients, where the eradication is very difficult. Around 54% of the patients under the age of 18 are colonized by this microorganism, while the percentage arises to 80% in adults (9).

Two types of *in vitro* biofilm models are currently being used to predict antimicrobial therapeutic success: closed and open systems. In closed systems, nutrients are limited and metabolic waste accumulates, which can create a bias in biofilm quantification. This technique, however, can be easily performed in high-throughput analysis. Moreover, PD parameters, which establish the antibiotic activity on biofilms, can be also determined. On the other hand, open systems better reproduce the *in vivo* conditions, as there is a permanent control of nutrient delivery, flow and temperature and anti-biofilm PK/PD of antibiotics can be determined. However, these systems are more expensive and are labour intensive. The BioFlux is a microfluidic system where multiple biofilms can be run in parallel covering all the advantages of open methods. In this work FOF, TOB, and FT were tested against *P. aeruginosa* biofilms using both, open and closed systems, in a complementary way. Through a mathematical formula, BioFlux image intensity results have been translated to a remaining biofilm percentage that enabled a graphic representation.

149 Studying the dynamics of biofilm formation, both the *Calgary* and BioFlux
150 devices showed that all isolates except *Pab6* were able to form a biofilm. The *Pab6*
151 strain was isolated from a CF patient with a prolonged chronic infection indicating, as
152 previously stated, that biofilm development is not essential for the ultimate survival of
153 *P. aeruginosa* in chronic lung infection (10).

154 Analyzing antibiotic activity against biofilms, the closed system showed high
155 MBIC values for FOF (1,024->1,024 µg/ml, 4 two-fold dilutions higher than the MIC)
156 and TOB (MBICs were 2-5 twofold dilutions higher than the MICs). According to the
157 FIC index based on MBICs, FT showed synergy against all tested biofilm-producing CF
158 strains.

159 BPC is a parameter that could be useful for treatment in the early stage of
160 colonization in CF patients. Our BPC results showed that TOB effectively prevents
161 biofilm development, while FOF has an erratic behavior depending on the strain tested.
162 These results match with those previously described, where fluoroquinolones,
163 tobramycin and colistin presented the lowest BPC values (11).

164 With the BioFlux device FOF, TOB and the FT MBICs exhibited a statistically
165 significant difference of biofilm intensity when comparing to the inoculum control, but
166 FT accounted less tobramycin and fosfomycin concentration.

167 These results reinforce the fact that antibiotic concentrations that inhibit
168 planktonic cells are not able to inhibit the same microorganism when growing in
169 biofilms. In fact, for most antibiotics, the MBICs are at least twofold dilutions higher
170 than the MICs (12). So, high antibiotic concentrations are needed into the biofilm
171 structure to exert their action. In CF patients, these concentrations can be achieved
172 through inhaled therapy. To evaluate antibiotics in these biofilm infection models,
173 clinical laboratories perform classical antibiotic susceptibility tests with planktonic

174 cells, as there is neither a feasible biofilm technique for routine testing, nor
175 standardization of procedures. Moreover, when an antibiotic is administrated by inhaled
176 therapy, susceptibility breakpoints should be based on
177 pharmacokinetic/pharmacodynamic (PK/PD) parameters adapted for this route of
178 administration, however, CLSI and EUCAST have not yet defined them. Therefore, *in*
179 *vitro* conventional MIC testing is not adequate to predict the possible *in vivo* therapeutic
180 effect of antibiotics in biofilm-mediated infections.

181 A high penetration of FOF into biofilms has been reported (13), but
182 monotherapy against *P. aeruginosa*, even in FOF susceptible strains, is not
183 recommended due to the high MICs of the wild-type population (ECOFF ≤128 µg/ml)
184 and the possibility of the rapid emergence of resistant mutants (14). The ATCC 27853
185 strain presents a much lower FOF MIC (4 µg/ml) than the modal MIC (64 µg/ml) of the
186 FOF MIC distribution for *P. aeruginosa* (15). The FOF hypersusceptibility of this strain
187 could be due to peptidoglycan recycling process inactivation (16), however, even in this
188 strain, a high FOF MBIC (1,024 µg/ml) was recorded. This fact reflects the frequent
189 emergence of high-level fosfomycin resistance mutants within the high bacterial
190 inoculum present in the biofilm that is due to the mutation of the glycerol 3-phosphate
191 permease (GlpT). Furthermore, although after administration of 120 mg of aerosolized
192 fosfomycin, a concentration of 2,500 µg/ml has been found in tracheal aspirates (17),
193 the high mutant prevention concentration values reported (>2,048 µg/ml) (14) prevent
194 again from its use in monotherapy.

195 On the other hand, TOB is less active on bacteria growing in biofilms than on
196 bacteria growing planktonically, as the anaerobic environments reduce its penetration
197 into bacterial cells (3). Peak concentrations of tobramycin measured in sputum after
198 aerosolized administration are approximately of 1,000 µg/ml (18). This Cmax of

tobramycin exceeds the MBIC, however, after the exposure to 1,000 µg/ml of tobramycin, there are still areas of living cells that remain within the inner part of biofilms (19). In this case, the association with fosfomycin could be advantageous as FT has increased activity under anaerobic condition because the expression of nitrate reductase genes, that are essential for the growth of *P. aeruginosa* , are downregulated (3).

Thus, within the FT association, FOF could behave as a TOB enhancer inducing its active uptake (20). The combination guarantees concentrations of both antibiotics above the MBIC, so the TOB levels reached inside the biofilm structure should warrant an adequate concentration thus ameliorating the negative side effects of tobramycin during treatment (4). In a previous study, prevention of the generation of resistant mutants and synergy between FT was observed in isolates which were susceptible to both antibiotics, while high-level tobramycin resistant isolates harboring aminoglycoside-modifying enzymes showed very weak or no synergy with high mutant prevention concentration values. So the possibility of using this combination is restricted to susceptible isolates. In TOB susceptible CF isolates but with the MIC close to the breakpoint (4 µg/ml) where the efflux system MexXY-OprM is altered, the FT synergy has been explained by the rapid accumulation inside the cell through the induction of the active uptake of TOB (14).

Also, this FT combination has proved to have disrupting activity on CF biofilms grown on cultured human CF-derived airway cells (4). FT was used as an inhaled treatment option in a multicenter study in CF patients with promising results (5). In addition, amikacin-FOF combination is currently undergoing a clinical trial in patients with mechanical ventilation associated pneumonia (https://clinicaltrials.gov/ct2/show/NCT02218359).

In conclusion, *P. aeruginosa* biofilms are implicated in numerous infections. In CF patients, the biofilm mode of growth makes the treatment a real challenge; therefore novel therapy interventions are needed. In *vitro* biofilm models should be implemented in clinical microbiology laboratories for routine susceptibility testing to predict therapeutic success when this mode of growth is present. FT has demonstrated to be a synergistic combination against CF *P. aeruginosa* isolates when using both the *Calgary* device and the BioFlux microfluidic open system. The latter system is a new tool that permits the study of biofilm formation resembling *in vivo* conditions.

Material and Methods

Bacterial strains and susceptibility testing.

Six *P. aeruginosa* clinical strains (*Pab1-Pab6*) obtained from respiratory samples of 6 CF patients (2 initial and 4 chronic infections, respectively) were collected. These strains represented different morphotypes (mucoid, small colony and smooth) and were nonhypermutable. *P. aeruginosa* ATCC 27853 was used as the control strain. MICs of FOF (Laboratorios Ern, S.A., Barcelona, Spain) and TOB (Sigma-Aldrich Chemical Co., St. Louis, MO) were determined by the agar dilution method (BBL Mueller-Hinton II cation-adjusted broth and agar; BD, Sparks, MD) as recommended for P. *aeruginosa* CF isolates (21). As previously stated, fosfomycin enters into *P. aeruginosa* cells only through the Glp-T transporter because this microorganism lacks the UhpT permease (15, 22), so glucose-6P (Uhp-T inducer) was not added to the medium when testing fosfomycin.

For the susceptibility categorization, EUCAST criteria were followed. As there are not a clinical breakpoints for fosfomycin, EUCAST ECOFF (128 µg/ml) was used. All the strains were susceptible to FOF and TOB (**Table 1**).

Biofilm assays using the *Calgary* static device.

This assay was performed as previously described with minimal variations (**Figure S2**) (23). Briefly, a 0.5 McFarland culture was transferred to a flat-bottom 96-well microtiter plate (Nunc International, Rochester, N.Y.). Bacterial biofilm was formed around the pegs of a modified polystyrene microtiter lid. This lid with pegs was immersed into a growth plate and incubated for 20 hours at 37ºC. After rinsing the pegs 3 times in sterile water they were placed into the antimicrobial Mueller-Hinton broth and incubated for 20 hours at 37ºC. Two-fold increasing FOF (2 to 1,024 µg/ml) and TOB (0.5 to 64 µg/ml) concentrations and a variable FOF concentration (1 to 512 µg/ml) with different fixed TOB concentrations (0.5 to 32 µg/ml) for the combinations were used. After this incubation, the biofilm was recovered centrifuging (800 RPM/10min) the peg lid in an antibiotic-free Muller Hinton microtiter plate. The MBIC was calculated after measuring the optical density (OD) (450 nm) before and after 6-hour incubation. Biofilm growth was defined as a mean difference of ≥0.05. MBIC was defined as the lowest antibiotic concentration that resulted in an OD difference at or below 10% of the OD positive control.

To determine synergy between FOF and TOB, the FIC value commonly used in checkerboard assays (24), was adapted to the MBIC and was calculated (25):

$$\sum \mathrm{FIC} = \frac{\mathrm{MBIC\ (TOB\mathbf{c})}}{\mathrm{MBIC\ (TOB)}} + \frac{\mathrm{MBIC\ (FOF\mathbf{c})}}{\mathrm{MBIC\ (FOF)}}$$

Being MBIC (TOBc) and MBIC (FOFc) the MBIC of TOB and FOF within the FT combination, respectively.

Synergy was defined when the ∑FIC was ≤0.5.

Using the *Calgary* static method, the PD BPC parameter was also estimated for FOF, TOB and FT following the protocol described by Fernández Olmos *et al*. (11). In

this protocol, the inoculum and the antimicrobials are simultaneously incubated in the microtiter plate with the pegs at the same time (**Figure S2**).

Biofilm formation and susceptibility determination were performed in duplicate for all the isolates.

Biofilm assays using the BioFlux microfluidic open system.

Using the BioFlux device, 24 biofilms were simultaneously developed in 48 wells, following an adapted protocol from Benoit *et al.* (**Figure S2**) (26). Microchannels were filled with 100 µl of pre-warmed Luria Broth media (LB) (Oxoid, LTD., Basingstoke, Hampshire, UK) through the input wells (5 min, 1 dyne/cm^2). For the cell attachment, 20 µl of a 10^8-10^9 CFU/ml bacterial suspension was inoculated into the output wells for 5 seconds at 2 dyne/cm^2 and incubated for 2 hours at 37ºC. For the positive control wells, fresh media was added to the input wells and biofilms were incubated for 24 hours at 37ºC (0.15 dyne/cm^2). In a first step, registration of the positive control microchannel of each isolate was made after 8 and 24 hours to see the dynamics of biofilm formation. Negative controls, for which the media were injected without bacterial suspension, were included in all assays. The antibiotic addition was performed after 8 hours of incubation, only if the percentage of the formed biofilm was equal or higher than approximately 40% of the total. The antibiotic concentrations tested corresponded to those of FOF and TOB MICs and MBICs obtained with the *Calgary* assays. For the FT, the lowest TOB concentration that resulted synergistic using the *Calgary* device was then applied in the BioFlux (**Table 3**). The results were analyzed by quantifying the image intensity of colored pixels in an 8-bits gray size, which was registered by the BioFlux software after microscopic observation of the selected area in the microchannel.

The percentage of the remaining biofilm after 24-hour incubation (48-hour incubation in case of *Pab5*) was estimated through the following equation and subsequently graphically represented (**Figure 2**).

$$\%\ \text{Biofilm} = \left[\frac{(Imax - \mathbf{X})}{(Imin - Imax)} \times 100\right] + 100$$

The image of the positive control was considered as the maximum intensity (*Imax*), the image of the negative control as the minimum intensity (*Imin*) and **X,** as the intensity of the evaluated sample. In order to reflect areas of congregation within the biofilm, the represented standard deviation in Figure 2 corresponded to the maximum intensity values recorded along the microchannel. In all cases, the results from at least two independent experiments were considered.

Statistical analysis.

Results from the image intensity of the inoculum control, from those corresponding to the antimicrobials as well as from those of their combinations, were analyzed using a Mann–Whitney nonparametric test. To maintain the overall boundary for statistical significance at 0.05, the threshold p-value was divided by our 5 independent hypotheses (comparison of the intensity obtained in the inoculum control with the intensity obtained in FOF-MIC, FOF-MBIC, TOB-MIC, TOB-MBIC, and FT-MBIC) so a value of $p<0.01$ was considered statistically significant. Stata statistical software was used (Data Analysis and Statistical Software version 11.0).

Acknowledgments

María Díez-Aguilar was supported by the Innovative Medicines Initiative (IMI)- European Commission-funded project (iABC grant 115721-2) and Fundación Francisco Soria Melguizo (Madrid. Spain). The content and scientific background of this work were also supported by Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015/0004 and RD12/0015/0006 and REIPI RD16/0016/0004 and RD16/0016/0011) and grants PI15/00088 and PI12/00734) - cofinanced by European Development Regional Fund "A way to achieve Europe" (EDRF), Operative program Intelligent Growth 2014-2020.

We thank Alfonso Muriel (Unidad de Bioestadística Clínica, Hospital Universitario Ramón y Cajal) for the statistical advice during manuscript preparation.

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450

451 **Figure 1.** Biofilm formed by each isolate in the *Calgary* device, represented by the
452 OD_{450} difference between 0 and 6 hours (ΔOD).

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454 **Figure 2**. Percentage of the remaining biofilm in the microchannel after 24-hour
455 incubation (48-hour in the case of *Pab5*). Standard deviation was calculated considering
456 the maximum intensity areas. Fosfomycin-Tobramycin combination was not tested in the
457 ATCC 27853 strain as it did not exhibit synergy with the *Calgary* device.

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460

461 **Table 1.** Characteristics of the *P. aeruginosa* used in the biofilm assays

Strain	Morphotype	FOF[a] MIC (µg/ml)	TOB[b] MIC (µg/ml)	Infection	Patients' age in years
Pab1	Mucoid	64	1	Initial infection	21
Pab2	Smooth	64	1	Initial infection	15
Pab3	Smooth	64	2	Chronic infection	45
Pab4	Mucoid	64	1	Chronic infection	22
Pab5	Smooth	64	4	Chronic infection	22
Pab6	Small colony	64	4	Chronic infection	26
ATCC 27853	-	4	0.5	-	-

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463 [a]FOF: fosfomycin; [b]TOB: tobramycin

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Table 2. Fosfomycin, tobramycin and fosfomycin-tobramycin MBICs (µg/ml) results obtained with the *Calgary* device. Fosfomycin-tobramycin concentrations that were synergistic for each strain are gray marked.

	FOF[a] MBIC (µg/ml)	TOB[b] MBIC (µg/ml)	FT[c] MBIC (µg/ml)					
Pab1	>1,024	8	-	256/1	256/2	256/4	<1/8	<1/16
Pab2	>1,024	32	-	-	>512/2	>512/4	128/8	<1/16
Pab3	1,024	32	-	-	64/2	32/4	16/8	<1/16
Pab4	>1,024	8	-	256/1	64/2	<1/4	<1/8	<1/16
Pab5	>1,024	16		>512/1	>512/2	256/4	128/8	<1/16
ATCC 27853	>1,024	2	512/0.5	64/1	<1/2	<1/4	<1/8	-

[a]FOF: fosfomycin; [b]TOB: tobramycin; [c]FT: fosfomycin-tobramycin combination

479 **Table 3.** Fractional inhibitory concentration results for the fosfomycin-tobramycin
480 combination concentrations tested in the *Calgary* device. The lowest tobramycin
481 concentration of the combination that resulted synergistic is grey marked.

[a]TOB (µg/ml)	∑FIC					
	Pab1	***Pab2***	***Pab3***	***Pab4***	***Pab5***	**ATCC 27853**
16	1	0.5	0.5	-	-	-
8	1	0.375 [b](128/8)	0.265	-	0.625	-
4	0.75	1.125	0.156	-	0.5 [b](256/4)	-
2	0.5	1.06	0.125 [b](64/2)	0.562	-	-
1	0.375 [b](256/1)	-	-	0.5 [b](256/1)	-	0.562
0.5	-	-	-		-	0.75

482 [a]TOB: tobramycin

483 [b]Fosfomycin/tobramycin concentrations (µg/ml)

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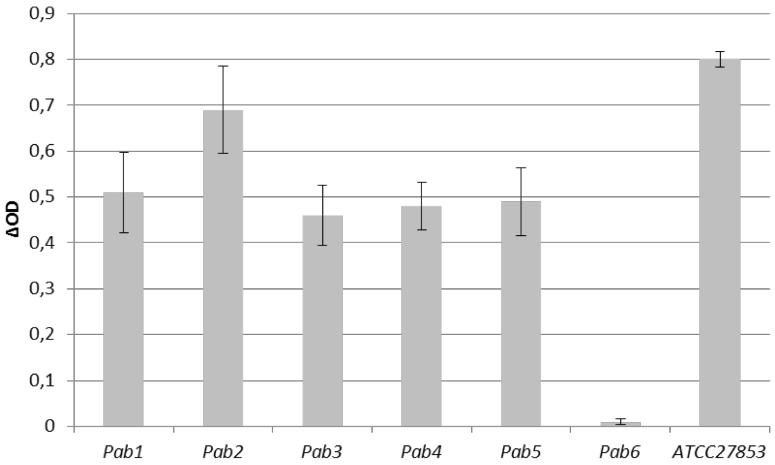
485 **Table 4.** Fosfomycin and tobramycin biofilm prevention concentration (BPC) and
486 BPC/MIC results obtained for each isolate by the *Calgary* device

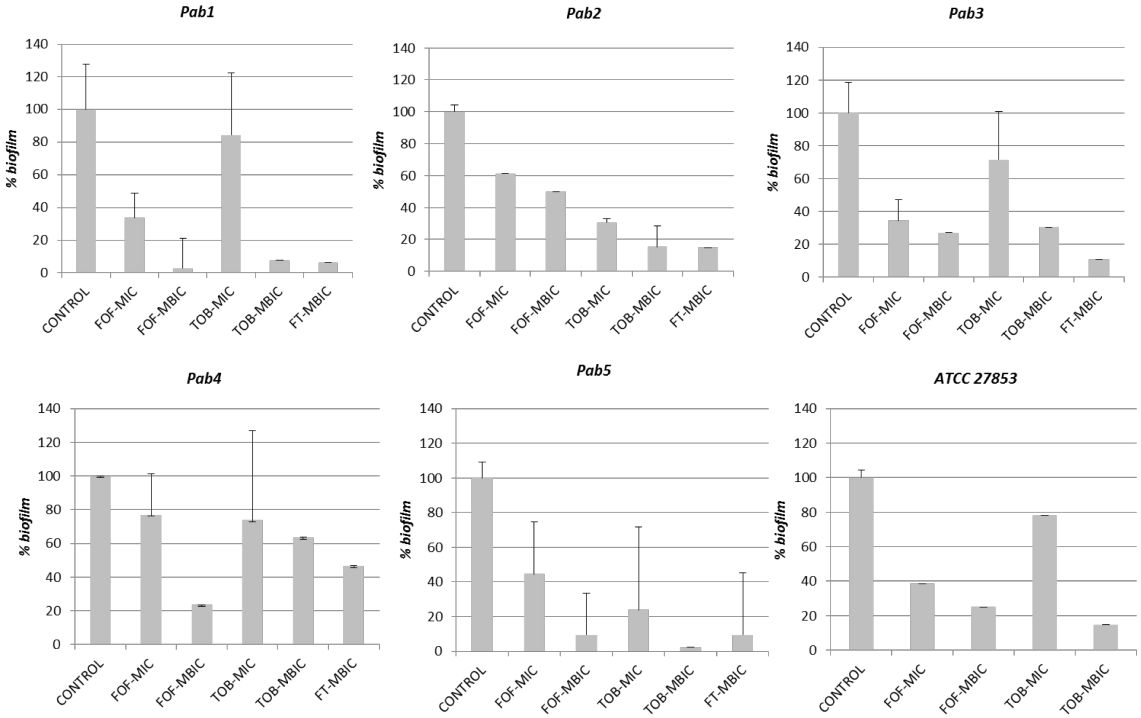
STRAIN	**[a]TOB BPC** (µg/ml)	**TOB BPC/MIC**	**[b]FOF BPC** (µg/ml)	**FOF BPC/MIC**
Pab1	≤1	1	128	2
Pab2	≤1	1	>1024	32
Pab3	≤1	0.5	512	8
Pab4	≤1	1	64	1
Pab5	≤1	0.25	64	1
ATCC 27853	≤0.5	1	8	2

487 [a]TOB: tobramycin; [b]FOF: fosfomycin.



Strain	ΔOD±SD
Pab1	0.51 ± 0.09
Pab2	0.69 ± 0.09
Pab3	0.46 ± 0.07
Pab4	0.48 ± 0.05
Pa5b	0.49 ± 0.07
Pab6	0.01 ± 0.006
ATCC27853	0.8 ± 0.02

Figure 1. Biofilm formed by each isolate in the *Calgary* device, represented by the OD_{450} difference between 0 and 6 hours (ΔOD).



***p values:** **FOF-MIC: 0.0374, FOF-MBIC: 0.0039, TOB-MIC: 0.0547, TOB-MBIC: 0.0065, FT-MBIC: 0.0062**

Figure 2. Percentage of the remaining biofilm in the microchannel after incubation with antibiotics. Standard deviation was calculated considering the maximum intensity areas. Fosfomycin-Tobramycin combination was not tested in the ATCC 27853 strain as it did not exhibit synergy with the *Calgary* device.