Investigation of the enhanced antimicrobial activity of combination dry powder inhaler formulations of lactoferrin

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ABSTRACT

The airways of most people with cystic fibrosis are colonized with biofilms of the Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa*. Delivery of antibiotics directly to the lung in the form of aerosols or dry powders offers the potential to achieve high local concentrations directly to the biofilms. Unfortunately, current aerosolised antibiotic regimes are unable to efficiently eradicate these biofilms from the airways. We investigated the ability of the innate antimicrobial, lactoferrin, to enhance the activity of two aminoglycoside antibiotics (tobramycin and gentamicin) against biofilms of *P. aeruginosa* strain PAO1. Biofilms were prepared in 96 well polystyrene plates. Combinations of the antibiotics and various lactoferrin preparations were spray dried. The bacterial cell viability of the various spray dried combinations were determined. Iron-free lactoferrin (apo lactoferrin) induced a 3 log reduction in the killing of planktonic cell by the aminoglycoside antibiotics ($p < 0.01$) and also reduced both the formation and persistence of *P. aeruginosa* biofilms ($p < 0.01$). Combinations of lactoferrin and an aminoglycoside displays potential as an effective new therapeutic strategy in the treatment of *P. aeruginosa* biofilms infections such as those typical of the CF lungs.

KEYWORDS: Antimicrobial protein, spray drying, Pseudomonas aeruginosa, cystic fibrosis, biofilms, dry powder inhalers.

1. INTRODUCTION

P. aeruginosa biofilms have been recognised as one of the most persistent microbe community in the airway of people with cystic fibrosis (CF) (Singh et al., 2002; Banin et al., 2006; Costerton et al., 1999; Musk and Hergenrother, 2008; Van Delden and Iglewski, 1998). We now understand that *P. aeruginosa* are able to form these biofilms by assembling viable cells together to form a cooperative consortium encased within an exopolysaccharide (or glycocalyx) (Gomez and Prince, 2007; Lam et al., 1980; Singh et al., 2000). The exopolysaccharide formed specialises in attaching to a variety of surfaces (Mah and O'Toole, 2001). Consequently, the presence of *P. aeruginosa* biofilms at any stage of infection usually results in recurrent bacterial infections due to the ability of dispersed planktonic daughter cells released from Pseudomonas biofilms to colonise new surfaces (Hoiby et al., 2001).

Currently, in other to manage the high incidences of mortality resulting from chronic colonization of *P. aeruginosa* in the airways, clinicians have employed the use of aerosolised antibiotics (O Riordan, 2000; Ramsey et al., 1993). Many of the established antibiotic therapies such as colistimethate sodium and tobramycin are delivered to the CF airways using nebulisers. Nebulising medication is time consuming and requires the use of expensive nebuliser apparatus that require maintenance. Both these factors increase the treatment burden and may reduce compliance and, therefore, treatment efficacy. New dry powder inhaler (DPI) formulations of colistimethate sodium (Colobreathe; Forest Laboratories, Dartford, UK) and tobramycin (TOBI Podhaler; Novartis Pharmaceuticals, Camberley, UK) have become available,

delivered by convenient portable mechanisms (Turbospin device (Forest Laboratories) and T-326 Inhaler (Novartis Pharmaceuticals)). The benefits of dry powder antibiotics are that they are quicker to use and easier to maintain than traditional nebulised therapy, which may thereby improve adherence to the therapy

Although aerosolised antibiotic therapy has its merits, such as; the ability to achieve high local concentrations in the airways, decrease systemic toxicity and successful suppression of established planktonic *P. aeruginosa* cells it is relatively inefficient in the eradication of persistent biofilms of *P. aeruginosa* (A. L. Smith et al., 1999). In fact, several studies have suggested that when bacterial cells exist in biofilms, they can become 10 – 1000 times more resistant to the effects of antimicrobial agents (Rogan et al., 2004; Smith, 2005) as well as to the effects of immune cells (Leid et al., 2002; Jesaitis et al., 2003). Evidence from several studies suggests that once *P. aeruginosa* biofilms are established in the airways, they become a formidable, highly organized and resistant pathogen with fatal consequences for the host. Hence, loss of any defence mechanism by the host that may facilitate biofilms development could result in catastrophic consequences (Rogan et al., 2004).

The antimicrobial peptides and proteins (AMP) form an important component of innate immune defences- the primary defence system of the majority of living organisms. These AMP's are present in high concentrations on respiratory epithelia and are able to delay or prevent microbial growth following an infection. Usually, they can act for several hours until the adaptive immune system is sufficiently mobilised to the site of infection. An

example of one of these AMP's is lactoferrin, a glycoprotein of about 80 kDa found in high concentration in most exocrine secretions including milk, tears, saliva, genital secretions, intestinal mucus and in the specific granule of neutrophils (Ellison, 1994). Various biological functions of lactoferrin – i.e. anti-microbial, anti-inflammatory and immunomodulatory – have been described (Tomita et al., 2002).

Lactoferrin displays antibacterial effects against planktonic Gram-negative microbes by two mechanisms; (i) impairment of bacterial multiplication by decreasing the availability of iron which is required for bacterial growth (Rogan et al., 2004; Arnold et al., 1982) and (ii) disruption the outer membrane of the microbes by binding to lipopolysaccharide (LPS) this results in an altered permeability eventually resulting in the death of the microbe (Elass-Rochard et al., 1995; Appelmelk et al., 1994). More recently, lactoferrin has been shown to inhibit *P. aeruginosa* biofilm formation by sequestering iron from the microbe (Singh et al., 2002). This biofilm inhibitory property is unique to lactoferrin and is a pivotal role in host defence against invading microbes. Several studies have so far shown that lactoferrin is able to enhance the antimicrobial properties of some antibiotics (Leitch and Willcox, 1999). This is particularly interesting since currently inhaled antibiotics (for example tobramycin) has been frequently associated with emergence of resistant strains (O Riordan, 2000; Costerton et al., 1999; Allison et al., 2011).

The aim of the present study was to investigate the development of combination DPI formulations of lactoferrin with other anit-microbial agents using spray drying. The further focus of our study was to investigate if these

127 treatment strategies permit the prevention of biofilm formation and/or
128 disruption of persistent biofilms of *P. aeruginosa* in patients whose airways
129 have been compromised to *P. aeruginosa* infection.

130

131 ## 2. METHODS AND MATERIALS

132

133 ### 2.1 Bacterial strains.

134

135 *P. aeruginosa* strain PAO1 was routinely grown from CAA medium- nutrient
136 medium containing casamino acids (CAA, 0.5% w/v) supplemented with 1mM
137 magnesium chloride. For the biofilm penetration assays, the BSAC standard
138 *Escherichia coli* NCTC 10418 was used and was grown overnight in Luria-
139 Bertani (LB) broth.

140

141 ### 2.2 Production of iron-free lactoferrin (apolactoferrin; ApoLf).

142

143 Apolactoferrin was prepared by dialyzing bovine lactoferrin (kindly donated by
144 DMV Fonterra, Netherlands) against 20 mM sodium acetate, 20 mM sodium
145 dihydrogen phosphate and 40 mM EDTA (pH 3.5) for 24 hrs at 4°C. The
146 apolactoferrin was dialysed against at least two changes of 900 ml distilled
147 water and either used immediately or stored at -80°C until needed. Ferric ion
148 (Fe^{3+}) removal was confirmed by atomic absorption spectroscopy (data not
149 shown).

150

151 2.3 Production and charcaterisation of mono and combination particles of
152 lactoferrin and apolactoferrin with Tobramycin or Gentamicin.

153

154 A spray drying technique was employed for the preparation of micron-sized
155 particles of lactoferrin (LF), apo-lactoferrin (ApoLf) tobramycin (Tobi) and
156 gentamicin (Genta). Aqueous solutions of each material (2% w/v) was spray-
157 dried using a Büchi 290 Mini Spray Dryer with pneumatic atomizer with a
158 7mm aperture, air (prior relative humidity 20%) aspirator rate 35 m^3/h, feed
159 pump 5 mL/min, spray flow rate 700 L/h, inlet air temperature 180– 190°C,
160 outlet temperature 96–98°C.
161 Inhalable combination particles of each antibiotic and LF and ApoLf were also
162 produced by spray drying. Aqueous solutions of tobramycin and gentamycin
163 with and with micronisation of the different formulations using a (Büchi
164 Laboratoriums-Technik AG, Flawil, Switzerland).
165 For particle sizing, dry powders were dispersed with compressed air (3 bar)
166 and sized by laser diffraction (RODOS dry powder feeder; HELOS laser
167 diffractometer, WINDOX 4.0 software; Sympatec GmbH, Germany). The 10%,
168 50% and 90% undersize particle size values (X10, X50 and X90, respectively)
169 were obtained.
170 The in vitro aerosolisation performance of the dry powders was measured
171 using a Multi-stage liquid impinge (MSLI, Copley Scientific, Nottingham, UK).
172 The four stages of the MSLI were charged with 20 mL of water. A vacuum
173 pump was attached to the MSLI and the flow rate was set at a flow rate of 60

174 L/min using a digital flowmeter (DFM2000, Copley Scientific, Nottingham,
175 UK). Approximately 30 ± 2 mg of each of spray dried powders was manually
176 weighed into a size 3 hard hydroxypropyl methylcellulose (HPMC) capsules
177 (V-caps, Capsugel, Geel, Belgium). A capsule was placed into the capsule
178 compartment of a Cyclohaler™ DPI device (Pharmachemie BV, The
179 Netherlands), pierced and actuated for 4 s. Powders depositing in the four
180 impinger levels were recovered by agitating the apparatus to ensure complete
181 dissolution. The powder which deposited in the throat and the back filter were
182 also collected and the amount of protein depositing in each of these stages
183 determined by carrying out bicinchoninic acid (BCA) assay or using a high
184 performance liquid chromatography (HPLC) assay for the detection and
185 quantification of either Tobi or Genta.
186 Quantitation of total protein was carried out according to the manufacturing
187 instructions accompanying a BCA assay kit (Sigma Aldrich, Poole, UK).
188 Briefly, bovine serum albumin standards were prepared by diluting the
189 contents of a 1 mL ampoule containing 2 mg of albumin (to prepare standard
190 concentrations of 20 – 2000 µg/mL). The working reagent was prepared
191 mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. Then a 200
192 µL aliquot of the working reagent was added into each of the wells already
193 containing 25 µL of either standard or test protein. The plates were covered
194 and incubated at 37 °C for 30 minutes, then cooled to room temperature and
195 then the absorbance measured at 562 nm using a Multiskan Spectrum -
196 UV/Vis Microplate Spectrophotometer.
197 Tobi was separated and quantified by HPLC (Agilent 1260, London, UK) using

198 an isocratic mobile phase consists of buffer 0.05 M diammonium hydrogen
199 phosphate, pH adjusted to 10.0 using tetramethyl ammonium hydroxide.
200 Chromatography was carried out at 25°C on a Purosphere RP-8e, 250 mm ×
201 4.6 mm, 5mm (Merck KGaA, Darmstadt, Germany). The detection was carried
202 out using variable wavelength UV-Vis detector set at 210 nm. The compounds
203 were eluted isocratically at a steady flow rate of 1.0 mL/min. Gentamicin was
204 separated and quantified by HPLC (Agilent 1260, London, UK) using an
205 isocratic mobile phase was 48.5mM TFA–MeOH (97:3, v/v) and ran at flow
206 rate of 0.7 ml/min. Chromatography was carried out at 25°C on a Hypurity RP
207 18, 3µm, 125 mm x 4 mm i.d. (Thermo Hypersil, Runcorn, UK. The detection
208 was carried out using an evaporative light scattering detector (Agilent,
209 London, UK).
210 The aerosolisation performance of the different spray-dried powders were
211 characterised by comparing the percentage emitted fraction (%EF,
212 percentage collected from all stages of MSLI as a function of recovered dose)
213 and fine particle fraction of the recovered dose (FPF_{RD}, percentage of FPD to
214 RD) of the respective spray- dried powders. The %EF, FPF_{RD} were expressed
215 as the means of quintuplicate runs (n = 5).
216
217 2.4 Viability assay for planktonic bacterial killing.
218
219 The antimicrobial activity of Lf and apoLf, alone and in combination with the
220 aminoglycosides, towards planktonic bacteria was assessed using a viability
221 assay. Briefly, 10µL of ~ 6.6 x 10^7 CFU/ml PAO1 was incubated with 90 µL of

222 2 µg/ml of Lf, ApoLf or combinations of protein with aminoglycoside. Samples
223 were taken every 10 mins, diluted in deionised water and plated on Mueller-
224 Hinton agar. Following 24 h incubation at 37°C, colonies were enumerated
225 and the percentage of viable cells was determined relative to the cell count in
226 the absence of proteins/antibiotics.
227
228 2.5 Qualitative analysis of biofilms.
229
230 Crystal violet staining was used to assess the effects of spray-dried
231 formulations of Lf, ApoLf and combinations of proteins with aminoglycosides
232 on biofilm formation by PAO1. Overnight cultures were diluted 1:100 (v/v) into
233 fresh medium supplemented with magnesium chloride, glucose and casamino
234 acids. In order to examine the effects of compounds on biofilm *formation*,
235 aliquots were dispensed into a 96 well plate in the presence of the
236 compounds at 2 g/ml. Plates were incubated at 37°C for 24 h, after which
237 time excess broth was removed and the biofilm was stained with crystal violet.
238 In order to examine the effects of the compounds on a pre-formed biofilm,
239 overnight cultures, diluted 1:100 as above, were incubated at 37°C for 24 h
240 before compounds were added and plates incubated for a further 24h prior to
241 biofilm staining. In both cases, following the appropriate incubation times, the
242 adherent biofilm was washed twice with distilled water before staining with
243 0.1% (w/v) crystal violet in distilled water for 15 mins at room temperature.
244 Following vigorous washing with water, the crystal violet-stained biofilms were
245 solubilised in 30% (v/v) acetic acid and the absorbance at 550nm was

246 determined (Multiskan spectrum plate reader) using 30% acetic acid in water
247 as the blank.
248
249 2.6 Preparation of biofilms for penetration assays.
250
251 Biofilms of *P. aeruginosa* were prepared according to a method adapted from
252 Anderl *et al.* (2000). Briefly, cultures of *P. aeruginosa* were grown overnight at
253 37°C in casamino acid media supplemented with magnesium chloride. The
254 bacterial culture was diluted to give ca. 10^6 cfu/ml and 5 µL of this diluted
255 inoculum was used to seed a UV-sterilised 13 mm diameter, 0.2 m pore size
256 polycarbonate nucleopore membrane (Whatman). Membranes were placed
257 on Mueller-Hinton agar (MHA) plates and incubated for 24h at 37°C after
258 which time they were transferred onto fresh MHA for a further 24h. This
259 established biofilm was then used in the assembly depicted in Fig. 1.
260
261 2.7 Biofilm penetration assay.
262
263 *E coli* NCTC 10148 was grown overnight in Luria-Bertani (LB) broth before the
264 density was adjusted to a 0.5 McFarland standard and applied to MHA plates
265 to enable the growth of a confluent lawn. The biofilm penetration of
266 tobramycin and gentamicin, either alone or in combination with the lactoferrin
267 preparations, was determined using a method modified from Singh *et al.*
268 (2010). Briefly, the membranes supporting biofilm growth were transferred to
269 MHA plates inoculated with *E. coli*. A UV-sterilised 6 mm diameter, 0.2 m

pore size nucleopore polycarbonate membrane (Whatman) was placed on top of each biofilm together with the antibiotic disc, containing the compounds of interest. All plates were incubated at 37°C for 24 h before the diameters of the clear zones of inhibition of growth were measured.

Controls comprising of membranes and antibiotic discs without biofilms and biofilms with “empty” discs were also set up. Antibiotic discs containing tobramycin, gentamicin and combinations of both antibiotics with the different preparations of lactoferrin were all prepared locally. Antibiotic discs were applied to the agar following inoculation with *E coli* and plates were incubated for 24 hours at 37°C. Zones of inhibition were measured after this incubation period and used to construct a calibration curve for antibiotic effects. A calibration plot of the diameter of the zone of inhibition against log concentration (for antibiotic discs containing 1 – 10,000 µg) was used to determine the concentration of the antibiotic in the combination discs that passed through the biofilms. The diameter of the zones of inhibition in the controls (no biofilm) were taken to signify 100% penetration and used to determine the percent penetration of the antibiotics through the biofilms. All experiments were performed in triplicates and a two-tailed, paired t-test was used for statistical analysis. In order to avoid false negative results arising from side diffusion from the antibiotic disc around the biofilm, rather than through the biofilm, only biofilms <13 mm in diameter were used in this assay.

2.8 Confocal imaging of non-viable bacteria in biofilms.

294

295 Reusable silicone incubation chambers (Flexwell™, Grace Bio-Labs) were
296 used to create 8 chambers on glass coverslips into which *P. aeruginosa* was
297 seeded and incubated for 72 hours in order to produce biofilms. A 1ml aliquot
298 of the cell suspension (~ 10^7 cfu/ml) was added into each of the chambers
299 and incubated at 37°C, with CAA medium refreshed every 16 hours. Following
300 72 hours growth of the biofilms on the coverslip, the CAA medium was
301 removed and wells were carefully washed twice with phosphate-buffered
302 saline (PBS) to remove planktonic bacteria. Biofilms were treated for 24 hours
303 at 37°C with 200 µl of 1 g/ml tobramycin, ApoLf or Tobi+ApoLf. Positive
304 controls (viable bacteria) contained 200 µL of CAA media without any
305 antibiotic, whilst the negative control (dead bacteria) was treated with 200 µL
306 of isopropanol. The biofilms were washed with PBS and stained with
307 LIVE/DEAD® *Bac*Light™ bacterial viability kit (Invitrogen, Life Technologies,
308 Ltd, Paisley, UK) using a mixture of 3 µl of SYTO® 9 with 3 µl of propidium
309 iodide per ml of saline, as per the manufacturer's instructions.
310 Visualisation of antibiotic/combination treated and untreated biofilms was
311 carried out using a Leica tandem confocal scanner SP5 confocal laser
312 scanning microscope (CLSM; Leica Microsystems, Milton Keynes, UK).
313 Images were obtained using 20x HC PL APO with 20x/0.70 CS lens. For
314 detection of SYTO® 9 (green channel), the 488 nm line of the argon laser with
315 a detection bandwidth of 495 – 515 nm was employed. For detection of
316 propidium iodide (red channel), the 561 nm HeNe laser was used with a
317 detection bandwidth of 615 – 600 nm. Image analysis was carried out using

318 Leica LAS AF 2.2.1 software.

319

320 2.9 Statistical analysis.

321

322 All experiments were performed in independent triplicate analysis of multiple
323 repeats. Two-tailed, unpaired t-tests or two-way Anova with Tukey's post hoc
324 were used for statistical analysis. Statistical analyses were performed using
325 GraphPad Prism 5.

326 3. RESULTS

327

328 The effect of spray drying on the antimicrobial activity of Lf and ApoLf was
329 evaluated for planktonic *P. aeruginosa* PAO1 over a 60-minute incubation
330 period (Fig. 2). Lf produced over 2 log reductions in bacterial number after 60
331 minutes, whereas SD Lf was slightly less effective (<2 log reductions in 60
332 minutes). The kinetics of the action of Lf and SDLf were very similar, with
333 most activity apparent in the first 10 minutes of incubation. ApoLf was more
334 effective than Lf - 60 minutes incubation with ApoLf reduced bacterial
335 numbers by over 5 $\log_{10}$ cfu/ml. Although spray drying reduced the activity of
336 ApoLf, this was still greater than Lf - indicating that ApoLf, even when spray
337 dried, is the more active antimicrobial form of the protein.

338

339 Aminoglycosides were spray dried with the antimicrobial proteins and tested
340 for their ability to kill planktonic organisms. The data in Fig. 4A indicated that
341 spray dried tobramycin at 2μg/ml produced a 2 log reduction in cfu/ml over 60
342 minutes, but that this activity was significantly ($p<0.05$) increased when the
343 antibiotic was co-spray dried with either Lf or ApoLf. The combination of TOBI
344 with ApoLf was the most effective and resulted in over 5 log reductions in cfu
345 over duration of the assay. The combination of Genta with Lf or ApoLf was
346 also more effective in terms of planktonic bacterial killing in comparison to the
347 mono-treatment with the aminoglycoside alone (Fig. 4B). Here, spray dried
348 Genta was shown to be effective and reduce the cfu by 2 logs over 60
349 minutes, but when Gm was co-spray dried with Lf, this killing was enhanced to

almost 4 log reductions and again, ApoLf with Gm was most effective and resulted in 100% bacterial eradication within 40 minutes.

The particle size distribution (PSD) and aerodynamic performance of the spray dried and co-spray dried formulations is shown in Table 1A and B. The d_{50} of the spray dried and co-spray dried particles was between 1.5 – 1.9 μm with 90% of particles below 5μm.

Following aerosolisation of the different spray dried formulations, the %EF of ranged between 88 – 97%. Whilst %EF is a measure of how well the powder is removed from the device, the FPF_{RD} is the measure of the aerosolisation efficiency. The %FPF of the spray dried Lf and ApoLf was lower than that of Tobi and Genta. The co-spray dried formulation also had high %EF (range: 91 – 94% for both agents) and the %FPF ranged from 35 – 42% for both the antibiotic and protein fraction of the co-spray dried particles. Moreover, the %FPF of each agent was very similar suggesting that were likely to be co-delivered.

The effects of the spray dried antibiotics and combinations were examined for their effects on biofilms of PAO1 using the crystal violet assay. Figure 5A shows that SD TOBI reduced the biofilm intensity in the first 24 hours to 57.4±0.1 % control. However, co-spray drying TOBI with either Lf or ApoLf significantly ($P<0.01$) enhanced this reduction to 24.8±0.8 or 22.9±0.8 % control, respectively. A similar pattern of biofilm inhibition was observed after

24-hour treatment with SD Genta, which suggested that biofilm intensity was reduced to 56.9±7.8%. Co-spray drying Genta with Lf or ApoLf showed significant ($P<0.01$) reduction in biofilm intensity to 30.5±1.5 or 23±0.6 % control following co-spray drying Genta with Lf or ApoLf, respectively. Interestingly, the combination treatments appeared more effective on pre-formed biofilms (Fig. 5B). Here, biofilms were allowed to form for 24 hours prior to the addition of SD aminoglycosides or co-spray-dried protein-antibiotic formulations. Under these conditions, the effects of Lf-aminoglycoside combinations were less impressive, with SD TOBI+Lf reducing the biofilm intensity to 63.7±11.6% whilst co-spray dried Genta+Lf only reduced intensity to 86.7±10.3 %. However, spray-dried combinations of the aminoglycosides with ApoLf were still effective at significantly ($P<0.01$) reducing biofilm intensity to 27.4±7 or 31.5±7.8% for co-spray dried TOBI+ApoLF or Genta+ApoLf respectively.

Since the SD combinations were effective at reducing biofilm intensity, the mechanism of action of this effect was investigated by examining the effects of the antimicrobial proteins on penetration of the SD antibiotics through PAO1 biofilms. The zones of growth inhibition of the indicator organism in the absence of a biofilm were taken to represent 100% penetration and used to determine the percentage retardation of the penetration of antibiotics and combinations through PAO1 biofilms. The limit of detection was taken to be 13mm, since this was size of the polycarbonate membrane filter on which the biofilms were grown.

Figure 6 indicated that PAO1 biofilms reduced the penetration of SD TOBI to 15.3±6.3% and spray dried combinations of Lf with TOBI did not significantly alter this (drug penetration 23±4% control). In contrast, the spray-dried combination of ApoLf with TOBI significantly ($P<0.05$) increased penetration of the antibiotic through PAO1 biofilms to 42.4±11.7 %. Visualisation of viable bacteria within the biofilm (Fig. 7) indicated that SD combinations of ApoLf with TOBI are more effective at reducing bacterial viability than SD TOBI alone. Biofilms were grown on glass slides for 72 hours in order to allow confocal fluorescence microscopy using the nucleic acid stains, SYTO 9 and propidium iodide. While SYTO 9 stains all nuclei acid in a population (and therefore bacteria with intact and damaged membranes appear green), propidium iodide stains only bacteria with damaged membranes. When the two dyes are used together, propidium iodide staining of damage bacteria will cause a reduction in their green fluorescence, visible as an increase in the yellow intensity seen on overlay of the green and red channel images. These experiments evaluated the effects of co-spray dried TOBI+ApoLf, since this combination consistently performed most effectively. Fig. 7B shows that SD ApoLf reduces bacterial viability (apparent from the yellow staining in the right panel), whereas SD TOBI is not altering bacterial viability and the staining is predominantly in the green channel (Fig. 7C). The images in Fig. 7D indicated that co-spray dried TOBI+ApoLf was most effective in terms of reducing bacterial viability in the biofilm- thus red fluorescence was greater here than with the other compounds tested and the intensity of the yellow

overlay indicating dead bacteria is greatest after incubation with co-spray dried TOBI+ApoLf.

4. DISCUSSION

The idea of combining various antibiotics to increase their efficacy has been around for decades. In the present study, we investigated the in vitro antibacterial activities of a novel inhaled antibiotic combination consisting of a 1:1 spray dried combination of aminoglycosides and an antimicrobial peptide (Lactoferrin). Upon spray drying our various preparations, we obtained an average residual moisture content of between 3.2 – 5.9 % indicating that the drying process was efficient in evaporating most of the moisture.

To better understand the activities of our paired agents against both planktonic and biofilms of *P.aeruginosa*, we carried out various time-kill experiments to ascertain the rate and degree of bacterial killing by the individual agents as well as in combination (Fig. 2 and 4). Our results, indicate that following spray drying all the antibiotics were able to reduce the colony forming unit per mililitre (cfu/ml) by 3 Log units within 60 mins when administered at high concentrations, but on combining both agents, we discovered that there was a much rapid bactericidal effect at very low concentrations compared to the individual administrations.

Similarly, in other to ascertain the effectiveness of our spray dried

446 combinations either in preventing biofilm formation or in depleting already
447 formed biofilms. We employed a method which effectively measured biofilm
448 mass by quantifying the amount of crystal violet retained by a microtitre plate
449 in which biofilms had been previously grown, washed and stained. Our
450 findings from these experiments, showed that there was a significant
451 decrease in both the initiation and persistence of biofilms when incubated with
452 the various lactoferrin preparations. Interestingly, this observed decrease in
453 both initiation and persistence were both absent on exposing the biofilms to
454 the aminoglycoside alone but reappears on combining the aminoglycosides
455 with lactoferrin.
456
457 Furthermore, to better understand how our paired agents are able to disrupt
458 the biofilms matrix, we employed the method whose schematic is briefly
459 described in figure 1. Results obtained from our penetration experiments
460 indicated that our tobramycin:lactoferrin combination permitted a significantly
461 higher amount of solute through the model biofilms. However, it is understood
462 that in vitro antibiotic activity may not always correlate with in vivo activity,
463 particularly in CF because CF sputum has been shown to inhibit the activity of
464 aminoglycosides (Mendelman et al., 1985; Hunt et al., 1995).
465
466 Previous studies have demonstrated that lactoferrin, tobramycin and
467 gentamicin are able to impart negatively on the viability of *P. aeruginosa*
468 biofilms (P. K. Singh et al., 2002; Tré-Hardy et al., 2008; Khan et al., 2011).
469 However, the effect of micron sized combinations of lactoferrin-tobramycin

and lactoferrin-gentamicin has not been investigated. Thus, these studies show the potential benefit such a formulation would have.

Images obtained by confocal laser scanning microscopy CLSM show that both apo lactoferrin and tobramycin caused minimal cell membrane damage to majority of the biofilm cells. However, treatment of the biofilms with a 1 mg/ml spray dried combination of lactoferrin and tobramycin caused significant membrane damage to all cells indicating cell lysis in the *P. aeruginosa* PAO1 biofilm. These observations were consistent with our biofilm and penetration assays. Taken together, our confocal microscopy results indicate that on treating the biofilms with a spray dried combination therapy of lactoferrin and tobramycin there is a complete suppression of metabolic activity of biofilms due to the enhanced antibiotic penetration through disrupted cell membranes.

Overall, results from our study show that the spray dried combinations of aminoglycosides and lactoferrin appear to have a superior antimicrobial activity in *P. aeruginosa* PAO1 biofilms compared to monotherapy. Tobramycin:lactoferrin combination could be a potentially new therapeutic agent for biofilm associated infections. Also the fact that as a combination they can be homogeneously micronized means that they could potentially be employed in the management of chronic airway infections as seen amongst people with cystic fibrosis. The clinical applicability of a tobramycin:lactoferrin combination warrants future in vivo studies

Drug resistant bacterial infections are becoming more widespread with new drug approvals for inhaled antibiotics constantly declining year on year. The rise in drug resistance especially due to the ability of bacteria to exist in biofilms has limited the repertoire of effective antimicrobials with considerable potency against infections as commonly seen amongst people with cystic fibrosis. The potential for employing a combination therapy of lactoferrin and aminoglycoside as described here provides a new and large playing field for proper utilisation of our currently available antibiotic resources in the provision of therapeutic solutions to some of the current bacterial menace. It will be interesting to translate most of our understanding into clinical treatment so as to effectively fight resistant *P. aeruginosa* infections.

5. CONCLUSIONS

In conclusion, we have developed combination therapies comprising an antimicrobial protein (lactoferrin) and an aminoglycoside antibiotic (tobramycin or gentamicin), which are more effective than monotherapy at reducing planktonic cell viability and preventing biofilm formation. We have shown that these combinations have parameters compatible with delivery to the airways and sug- gest, therefore, that they represent an important and exciting new therapeutic strategy for treating biofilm-forming infections such as those typical of the CF lungs.

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613
614

615 TABLES

616

617 Table 1A. Particle size distribution of the spray-dried particles and their
618 aerodynamic performance as determined by the percentage emitted fraction
619 (%EF) and percentage fine particle fraction of recovered dose ($\%FPF_{RD}$).

620

Formulation	*d*[*v*,10] (μm)	*d*[*v*,50] (μm)	*d*[*v*,90] (μm)	%EF	$\%FPF_{RD}$
SD Lf	0.8 ± 0.2	1.9 ± 0.3	4.08 ± 0.4	96.2 ± 0.4	28.6 ± 0.3
SD ApoLf	0.7 ± 0.3	1.7 ± 0.2	4.01 ± 0.5	93.9 ± 0.5	31.9 ± 0.6
SD Tobi	0.8 ± 0.6	1.7 ± 0.6	4.78 ± 0.8	88.9 ± 3.2	38.7 ± 1.4
SD Genta	0.8 ± 0.1	1.8 ± 0.2	4.38 ± 0.2	90.5 ± 0.1	40.7 ± 1.7

621

622

623 Table 1B. Particle size distribution of the co-spray dried particles and their
624 aerodynamic performance as determined by the percentage emitted fraction
625 (%EF) and percentage fine particle fraction of recovered dose ($\%FPF_{RD}$).

626

Formulation	*d*[*v*,10] (μm)	*d*[*v*,50] (μm)	*d*[*v*,90] (μm)	%EF (Aminoglycoside)	%EF (Protein)	$\%FPF_{RD}$ (Aminoglycoside)	$\%FPF_{RD}$ (Protein)
SDLf + Tobi	0.9 ± 0.4	1.8 ± 0.6	4.56 ± 0.8	92.6 ± 0.3	91.9 ± 0.4	35.3 ± 2.2	36.8 ± 1.8
SD	0.9 ±	1.8 ±	4.01 ±	91.7 ±	90.9 ±	38.9 ±	37.2 ±

ApoLf + Tobi	0.3	0.3	0.3	0.8	0.9	3.9	1.5
SD Lf + Genta	0.7 ± 0.4	1.6 ± 0.5	4.05 ± 0.1	91.6 ± 0.5	92.3 ± 0.7	42.3 ± 2.3	40.9 ± 1.9
SD ApoLf + Genta	0.7 ± 0.4	1.5 ± 0.2	4.28 ± 0.3	93.5 ± 0.8	94.2 ± 0.6	35.1 ± 2.8	36.8 ± 1.2

627

628

629 FIGURES

630

631 Figure 1. Schematic depiction of the experimental setup used to analyse the
632 penetration of antibiotics and combinations of antibiotics with lactoferrin/apo-
633 lactoferrin, through *P. aeruginosa* biofilms. Biofilms were grown for 48 hours
634 before use in this penetration assay.

635

636 Figure 2. Spray drying antimicrobial proteins antimicrobial activity.
637 Apolactoferrin and SD apolactoferrin were significantly more effective than the
638 iron-rich form ($P<0.05$) and resulted in 4-5 log reductions in bacterial numbers
639 over 60 minutes. Control (no antimicrobial protein), ■; Lactoferrin, ●; SD
640 lactoferrin, ○; Apolactoferrin, ▲; SD apolactoferrin, △. Data are presented as
641 mean±SD, n=3 independent experiments.

642

643 Figure 3. Effects of the compounds on initiation of the biofilm, where
644 compounds were present throughout 24 hours of culture (A) and the effects of

645 the compounds on an established biofilm, here compounds were added 24
646 hours after biofilm formation and incubated for a further 24 hours (B). Data are
647 presented as a percentage of control- where the intensity of the biofilm in the
648 absence of any agents (control) was given the value 100 % and all other data
649 are expressed relative to this. Data are shown as mean ± standard deviation
650 of 8 repeats from triplicate analysis.
651
652 Figure 4. Effect of spray dried combinations of aminoglycosides with
653 antimicrobial proteins on planktonic bacterial killing. A: Combinations of
654 tobramycin with Lf or ApoLf and B: Combinations of gentamicin with Lf and
655 ApoLf.
656
657 Figure 5. Effects of the combinations of tobramycin and on initiation of the
658 biofilm, where the antibiotic or combination was present throughout 24 hours
659 of culture (A) and effects of the combinations on an established biofilm (B).
660
661 Figure 6. Investigation of the penetration of combination therapies of antibiotic
662 with lactoferrin or apolactoferrin PAO1 biofilms.
663
664 Figure 7. Confocal imaging of bioflms following exposure to spray-dried
665 combinations of Tobi and ApoLf.
666

Figure 1

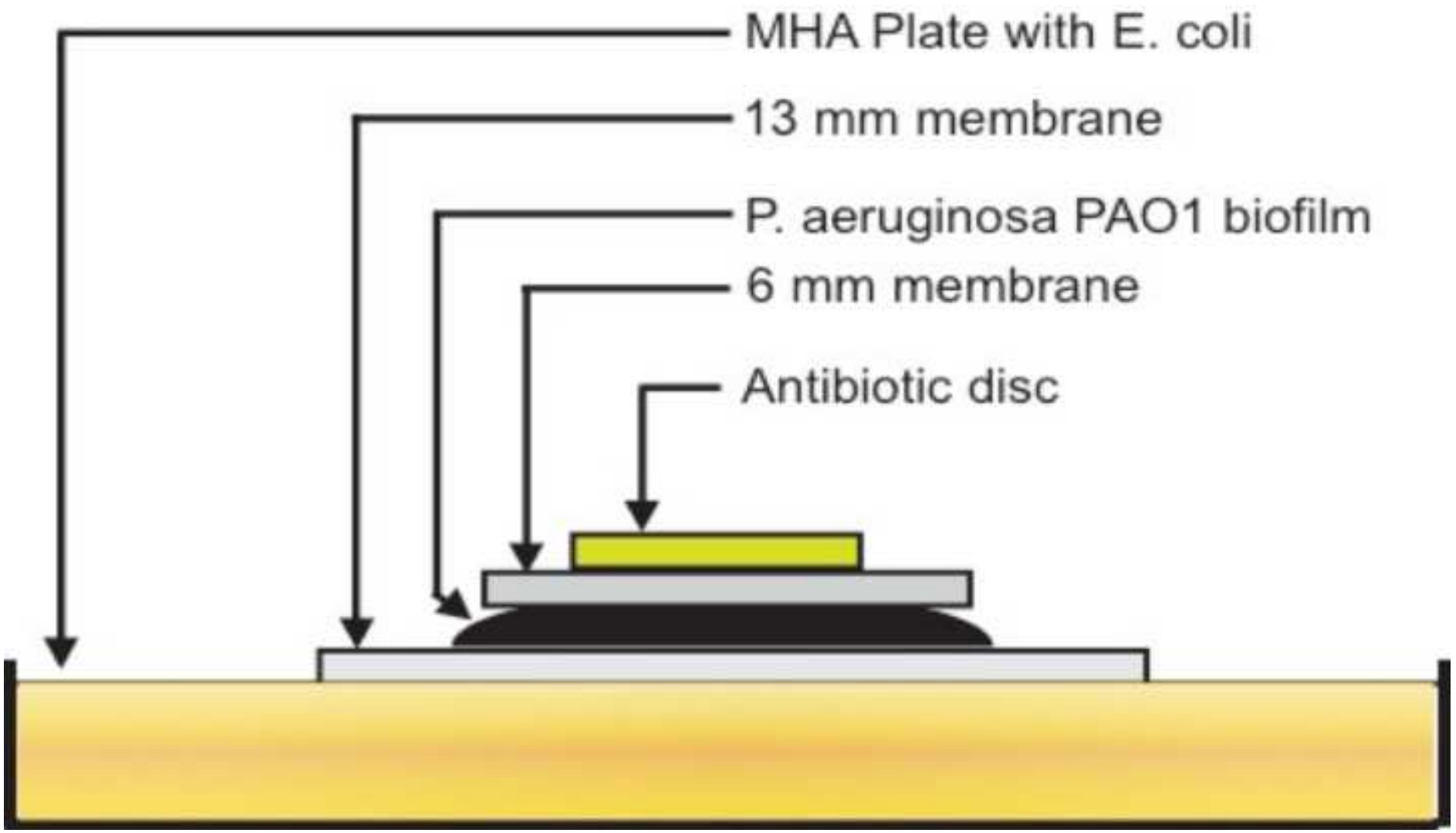
MHA Plate with E. coli
13 mm membrane
P. aeruginosa PAO1 biofilm
6 mm membrane
Antibiotic disc

Figure 2

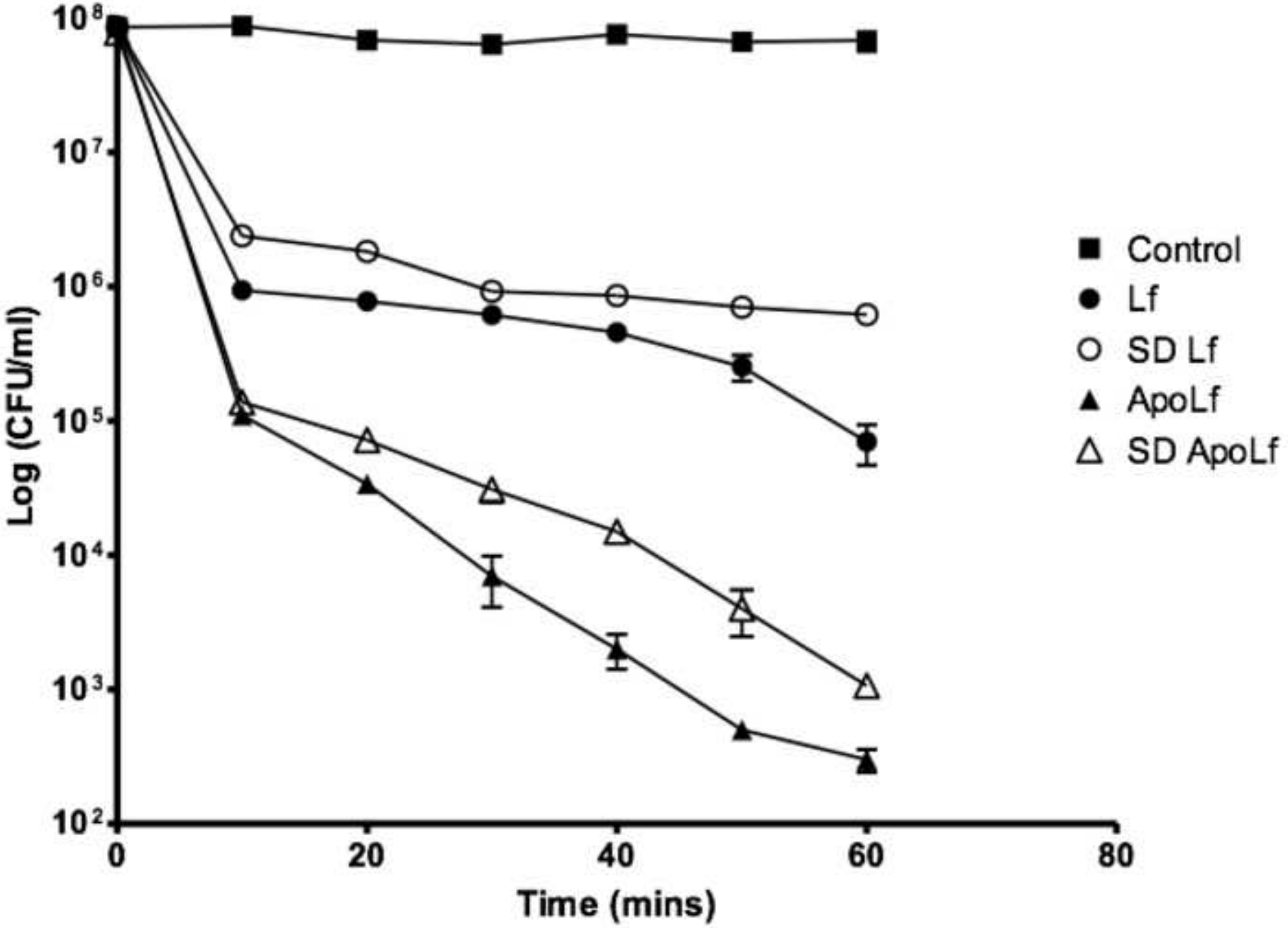
Log (CFU/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
20
40
60
80
Time (mins)
Control
Lf
SD Lf
ApoLf
SD ApoLf

Figure 3

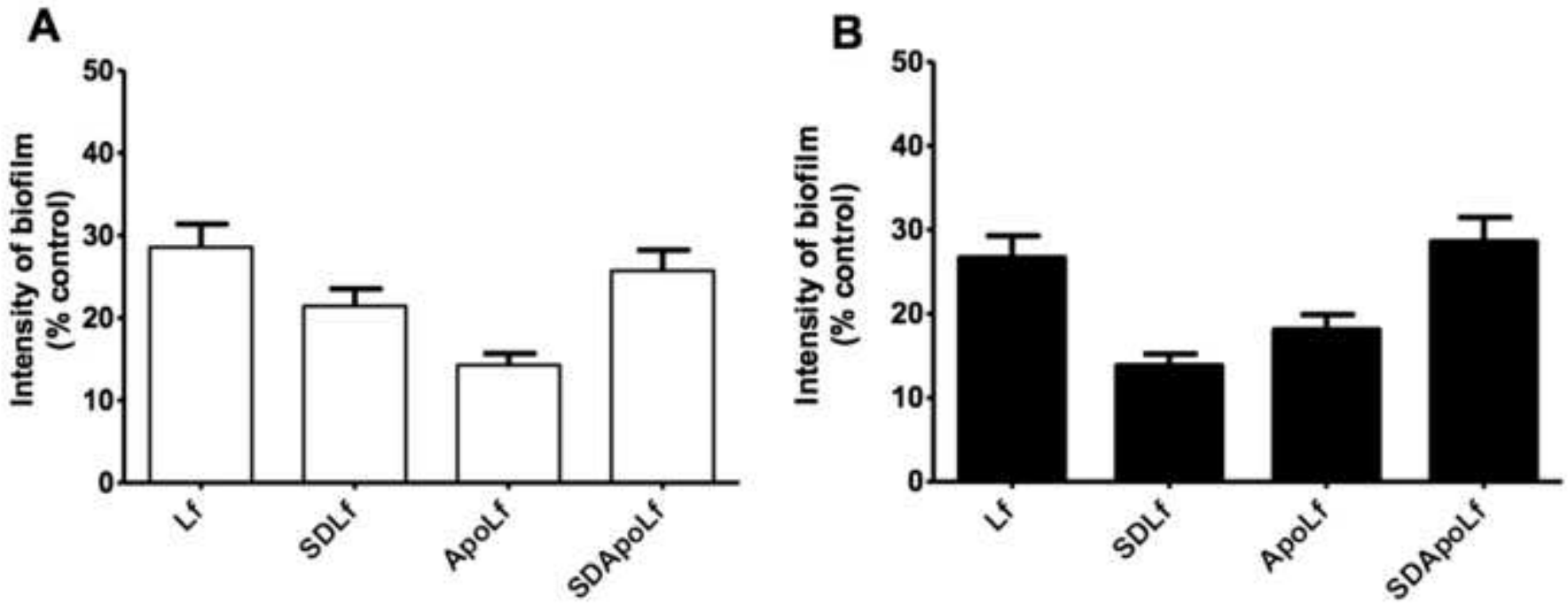
A
Intensity of biofilm (% control)
50
40
30
20
10
0
Lf
SDLf
ApoLf
SDApoLf
B
Intensity of biofilm (% control)
50
40
30
20
10
0
Lf
SDLf
ApoLf
SDApoLf

Figure 4

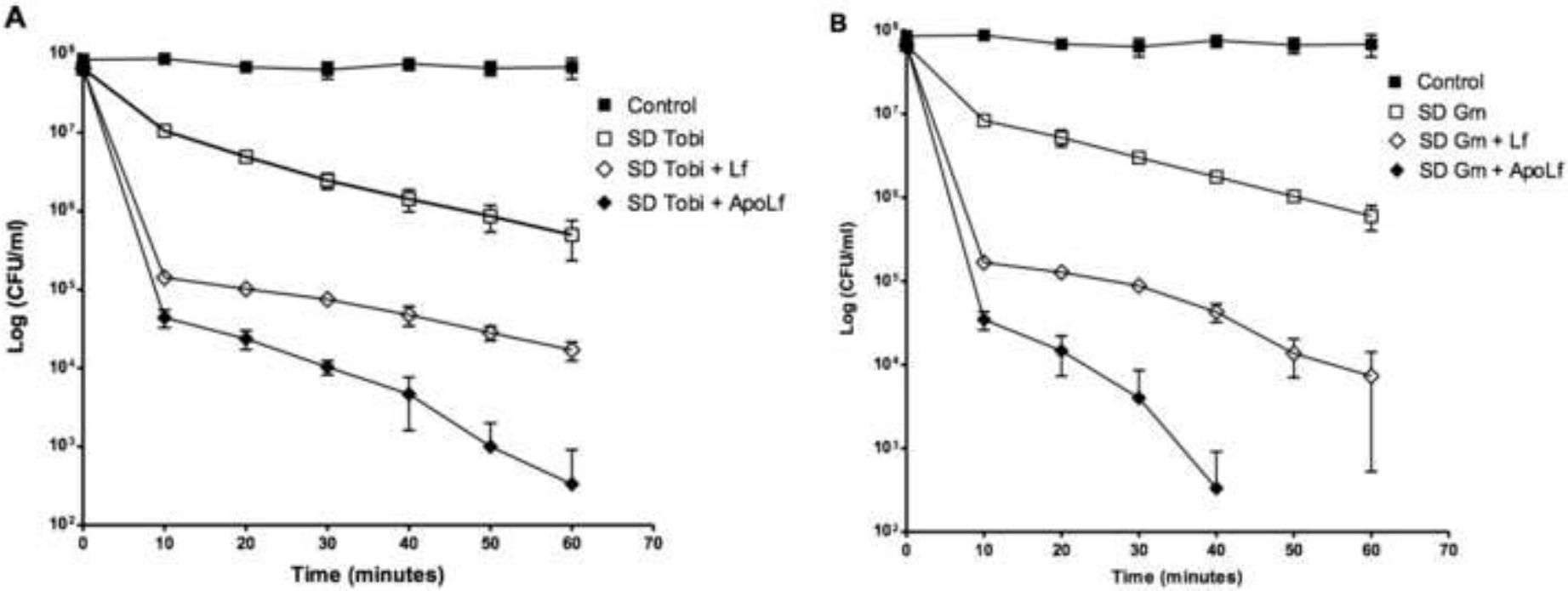
A
Log (CFU/ml)
Time (minutes)
Control
SD Tobi
SD Tobi + Lf
SD Tobi + ApoLf
B
Log (CFU/ml)
Time (minutes)
Control
SD Gm
SD Gm + Lf
SD Gm + ApoLf

Figure 5

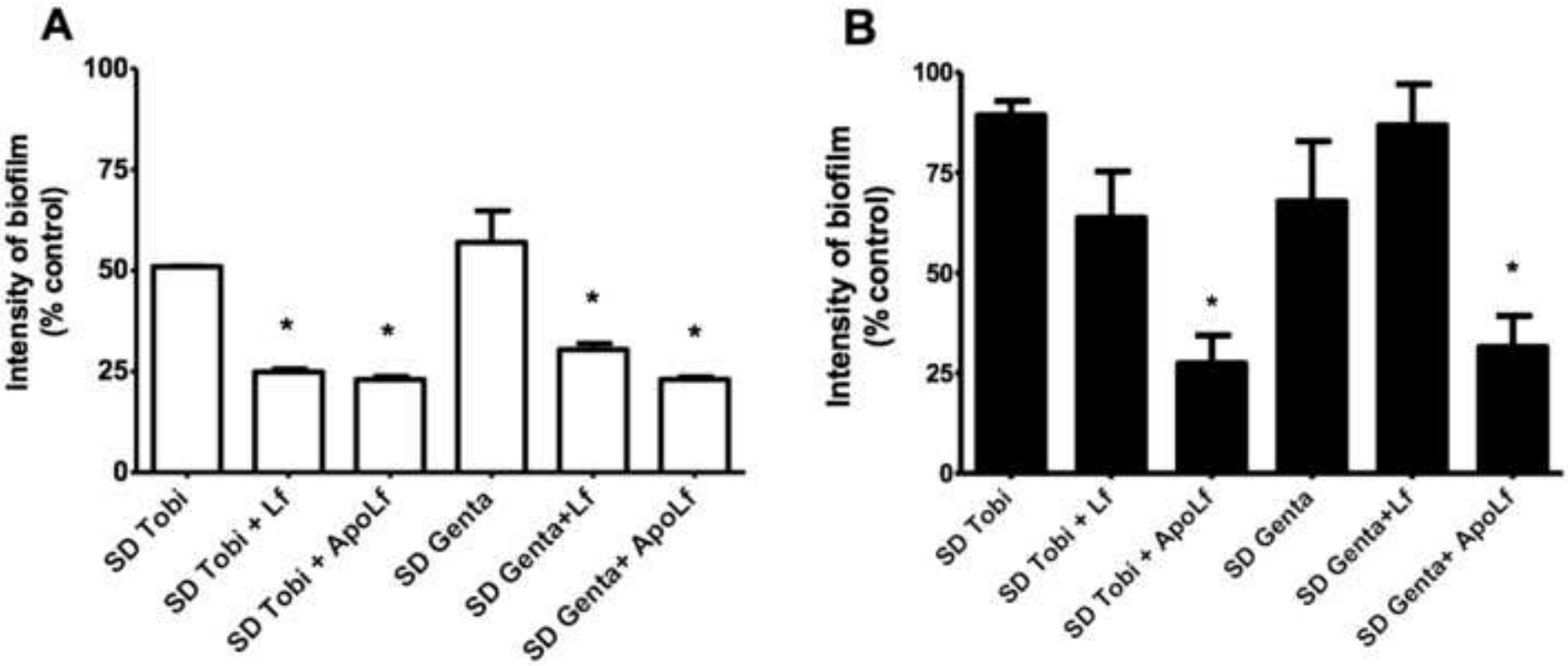
A
Intensity of biofilm (% control)
100
75
50
25
0
*
*
*
SD Tobi
SD Tobi + Lf
SD Tobi + ApoLf
SD Genta
SD Genta+Lf
SD Genta+ ApoLf
B
Intensity of biofilm (% control)
100
75
50
25
0
*
*
SD Tobi
SD Tobi + Lf
SD Tobi + ApoLf
SD Genta
SD Genta+Lf
SD Genta+ ApoLf

Figure 6

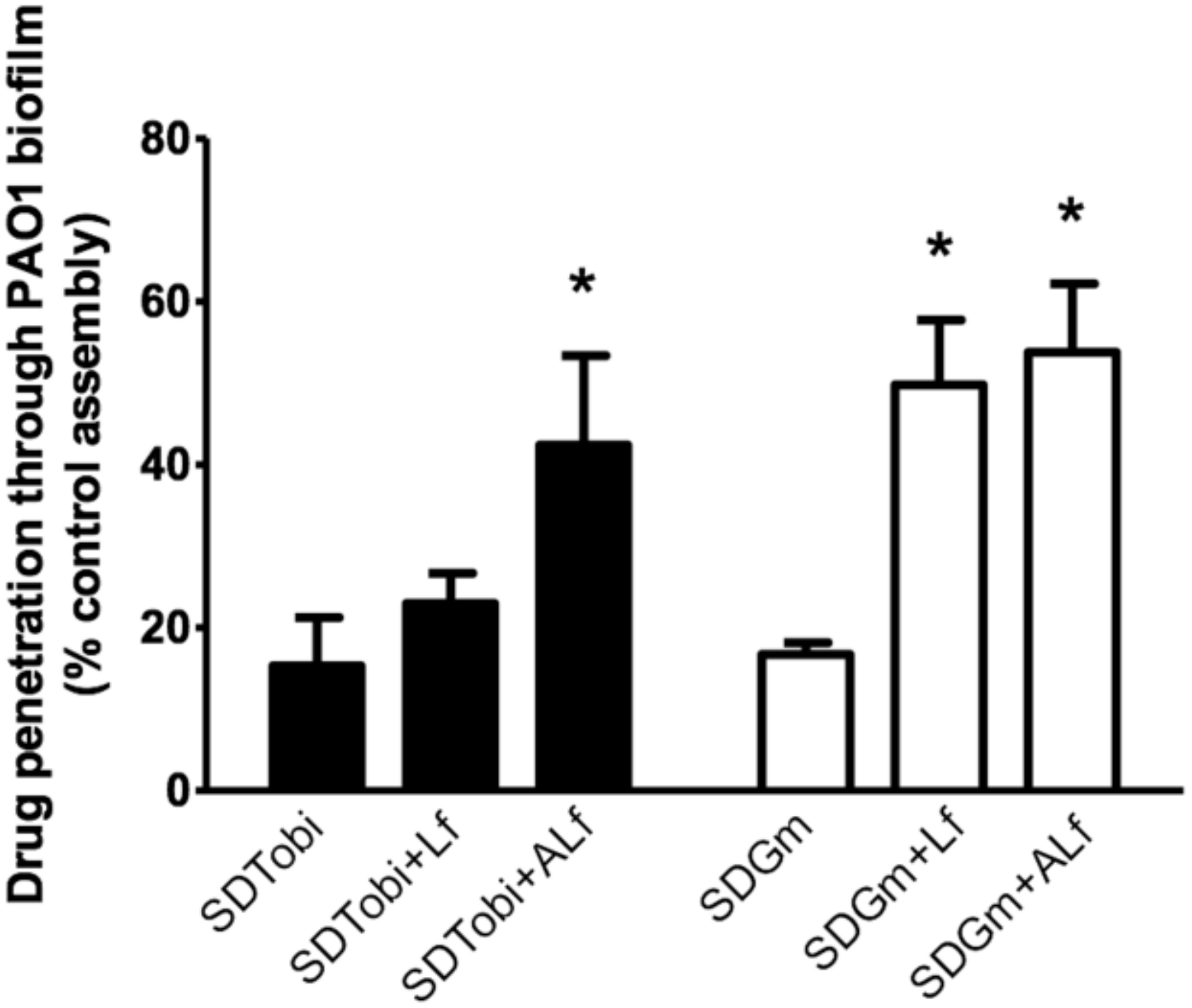
Drug penetration through PAO1 biofilm
(% control assembly)
80
60
40
20
0
*
*
*
SDTobi
SDTobi+Lf
SDTobi+ALf
SDGm
SDGm+Lf
SDGm+ALf

Figure 7