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1 Investigation of the enhanced antimicrobial activity of combination dry powder

2 inhaler formulations of lactoferrin

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31   ABSTRACT

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33   The airways of most people with cystic fibrosis are colonized with biofilms of

34   the Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa*.

35   Delivery of antibiotics directly to the lung in the form of aerosols or dry

36   powders offers the potential to achieve high local concentrations directly to

37   the biofilms. Unfortunately, current aerosolised antibiotic regimes are unable

38   to efficiently eradicate these biofilms from the airways. We investigated the

39   ability of the innate antimicrobial, lactoferrin, to enhance the activity of two

40   aminoglycoside antibiotics (tobramycin and gentamicin) against biofilms of *P.*

41   *aeruginosa* strain PAO1. Biofilms were prepared in 96 well polystyrene plates.

42   Combinations of the antibiotics and various lactoferrin preparations were

43   spray dried. The bacterial cell viability of the various spray dried combinations

44   were determined. Iron-free lactoferrin (apo lactoferrin) induced a 3 log

45   reduction in the killing of planktonic cell by the aminoglycoside antibiotics ($p <$

46   0.01) and also reduced both the formation and persistence of *P. aeruginosa*

47   biofilms ($p < 0.01$). Combinations of lactoferrin and an aminoglycoside

48   displays potential as an effective new therapeutic strategy in the treatment of

49   *P. aeruginosa* biofilms infections such as those typical of the CF lungs.

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52   KEYWORDS: Antimicrobial protein, spray drying, Pseudomonas aeruginosa,

53   cystic fibrosis, biofilms, dry powder inhalers.

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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,

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79 delivered by convenient portable mechanisms (Turbospin device (Forest

80 Laboratories) and T-326 Inhaler (Novartis Pharmaceuticals)). The benefits of

81 dry powder antibiotics are that they are quicker to use and easier to maintain

82 than traditional nebulised therapy, which may thereby improve adherence to

83 the therapy

84 Although aerosolised antibiotic therapy has its merits, such as; the ability to

85 achieve high local concentrations in the airways, decrease systemic toxicity

86 and successful suppression of established planktonic *P. aeruginosa* cells it is

87 relatively inefficient in the eradication of persistent biofilms of *P. aeruginosa*

88 (A. L. Smith et al., 1999). In fact, several studies have suggested that when

89 bacterial cells exist in biofilms, they can become 10 – 1000 times more

90 resistant to the effects of antimicrobial agents (Rogan et al., 2004; Smith,

91 2005) as well as to the effects of immune cells (Leid et al., 2002; Jesaitis et

92 al., 2003). Evidence from several studies suggests that once *P. aeruginosa*

93 biofilms are established in the airways, they become a formidable, highly

94 organized and resistant pathogen with fatal consequences for the host.

95 Hence, loss of any defence mechanism by the host that may facilitate biofilms

96 development could result in catastrophic consequences (Rogan et al., 2004).

97 The antimicrobial peptides and proteins (AMP) form an important component

98 of innate immune defences- the primary defence system of the majority of

99 living organisms. These AMP's are present in high concentrations on

100 respiratory epithelia and are able to delay or prevent microbial growth

101 following an infection. Usually, they can act for several hours until the

102 adaptive immune system is sufficiently mobilised to the site of infection. An

4

103 example of one of these AMP's is lactoferrin, a glycoprotein of about 80 kDa

104 found in high concentration in most exocrine secretions including milk, tears,

105 saliva, genital secretions, intestinal mucus and in the specific granule of

106 neutrophils (Ellison, 1994). Various biological functions of lactoferrin – i.e.

107 anti-microbial, anti-inflammatory and immunomodulatory – have been

108 described (Tomita et al., 2002).

109 Lactoferrin displays antibacterial effects against planktonic Gram-negative

110 microbes by two mechanisms; (i) impairment of bacterial multiplication by

111 decreasing the availability of iron which is required for bacterial growth

112 (Rogan et al., 2004; Arnold et al., 1982) and (ii) disruption the outer

113 membrane of the microbes by binding to lipopolysaccharide (LPS) this results

114 in an altered permeability eventually resulting in the death of the microbe

115 (Elass-Rochard et al., 1995; Appelmelk et al., 1994). More recently, lactoferrin

116 has been shown to inhibit *P. aeruginosa* biofilm formation by sequestering

117 iron from the microbe (Singh et al., 2002). This biofilm inhibitory property is

118 unique to lactoferrin and is a pivotal role in host defence against invading

119 microbes. Several studies have so far shown that lactoferrin is able to

120 enhance the antimicrobial properties of some antibiotics (Leitch and Willcox,

121 1999). This is particularly interesting since currently inhaled antibiotics (for

122 example tobramycin) has been frequently associated with emergence of

123 resistant strains (O Riordan, 2000; Costerton et al., 1999; Allison et al., 2011).

124 The aim of the present study was to investigate the development of

125 combination DPI formulations of lactoferrin with other anit-microbial agents

126 using spray drying. The further focus of our study was to investigate if these

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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).

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

A spray drying technique was employed for the preparation of micron-sized particles of lactoferrin (LF), apo-lactoferrin (ApoLf) tobramycin (Tobi) and gentamicin (Genta). Aqueous solutions of each material (2% w/v) was spray-dried using a Büchi 290 Mini Spray Dryer with pneumatic atomizer with a 7mm aperture, air (prior relative humidity 20%) aspirator rate 35 m^3/h, feed pump 5 mL/min, spray flow rate 700 L/h, inlet air temperature 180– 190°C, outlet temperature 96–98°C.

Inhalable combination particles of each antibiotic and LF and ApoLf were also produced by spray drying. Aqueous solutions of tobramycin and gentamycin with and with micronisation of the different formulations using a (Büchi Laboratoriums-Technik AG, Flawil, Switzerland).

For particle sizing, dry powders were dispersed with compressed air (3 bar) and sized by laser diffraction (RODOS dry powder feeder; HELOS laser diffractometer, WINDOX 4.0 software; Sympatec GmbH, Germany). The 10%, 50% and 90% undersize particle size values (X10, X50 and X90, respectively) were obtained.

The in vitro aerosolisation performance of the dry powders was measured using a Multi-stage liquid impinge (MSLI, Copley Scientific, Nottingham, UK). The four stages of the MSLI were charged with 20 mL of water. A vacuum pump was attached to the MSLI and the flow rate was set at a flow rate of 60

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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^{TM} 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

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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.

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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

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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

10

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

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270 pore size nucleopore polycarbonate membrane (Whatman) was placed on top

271 of each biofilm together with the antibiotic disc, containing the compounds of

272 interest. All plates were incubated at 37°C for 24 h before the diameters of the

273 clear zones of inhibition of growth were measured.

274

275 Controls comprising of membranes and antibiotic discs without biofilms and

276 biofilms with "empty" discs were also set up. Antibiotic discs containing

277 tobramycin, gentamicin and combinations of both antibiotics with the different

278 preparations of lactoferrin were all prepared locally. Antibiotic discs were

279 applied to the agar following inoculation with *E coli* and plates were incubated

280 for 24 hours at 37°C. Zones of inhibition were measured after this incubation

281 period and used to construct a calibration curve for antibiotic effects. A

282 calibration plot of the diameter of the zone of inhibition against log

283 concentration (for antibiotic discs containing 1 – 10,000 μg) was used to

284 determine the concentration of the antibiotic in the combination discs that

285 passed through the biofilms. The diameter of the zones of inhibition in the

286 controls (no biofilm) were taken to signify 100% penetration and used to

287 determine the percent penetration of the antibiotics through the biofilms. All

288 experiments were performed in triplicates and a two-tailed, paired t-test was

289 used for statistical analysis. In order to avoid false negative results arising

290 from side diffusion from the antibiotic disc around the biofilm, rather than

291 through the biofilm, only biofilms <13 mm in diameter were used in this assay.

292

293 2.8 Confocal imaging of non-viable bacteria in biofilms.

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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

13

318   Leica LAS AF 2.2.1 software.

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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

15

350   almost 4 log reductions and again, ApoLf with Gm was most effective and

351   resulted in 100% bacterial eradication within 40 minutes.

352

353   The particle size distribution (PSD) and aerodynamic performance of the

354   spray dried and co-spray dried formulations is shown in Table 1A and B. The

355   d_{50} of the spray dried and co-spray dried particles was between 1.5 – 1.9 μm

356   with 90% of particles below 5μm.

357

358   Following aerosolisation of the different spray dried formulations, the %EF of

359   ranged between 88 – 97%. Whilst %EF is a measure of how well the powder

360   is removed from the device, the FPF_{RD} is the measure of the aerosolisation

361   efficiency. The %FPF of the spray dried Lf and ApoLf was lower than that of

362   Tobi and Genta. The co-spray dried formulation also had high %EF (range: 91

363   – 94% for both agents) and the %FPF ranged from 35 – 42% for both the

364   antibiotic and protein fraction of the co-spray dried particles. Moreover, the

365   %FPF of each agent was very similar suggesting that were likely to be co-

366   delivered.

367

368   The effects of the spray dried antibiotics and combinations were examined for

369   their effects on biofilms of PAO1 using the crystal violet assay. Figure 5A

370   shows that SD TOBI reduced the biofilm intensity in the first 24 hours to

371   57.4±0.1 % control. However, co-spray drying TOBI with either Lf or ApoLf

372   significantly (P<0.01) enhanced this reduction to 24.8±0.8 or 22.9±0.8 %

373   control, respectively. A similar pattern of biofilm inhibition was observed after

16

374   24-hour treatment with SD Genta, which suggested that biofilm intensity was

375   reduced to 56.9±7.8%. Co-spray drying Genta with Lf or ApoLf showed

376   significant (P<0.01) reduction in biofilm intensity to 30.5±1.5 or 23±0.6 %

377   control following co-spray drying Genta with Lf or ApoLf, respectively.

378   Interestingly, the combination treatments appeared more effective on pre-

379   formed biofilms (Fig. 5B). Here, biofilms were allowed to form for 24 hours

380   prior to the addition of SD aminoglycosides or co-spray-dried protein-antibiotic

381   formulations. Under these conditions, the effects of Lf-aminoglycoside

382   combinations were less impressive, with SD TOBI+Lf reducing the biofilm

383   intensity to 63.7±11.6% whilst co-spray dried Genta+Lf only reduced intensity

384   to 86.7±10.3 %. However, spray-dried combinations of the aminoglycosides

385   with ApoLf were still effective at significantly (P<0.01) reducing biofilm

386   intensity to 27.4±7 or 31.5±7.8% for co-spray dried TOBI+ApoLF or

387   Genta+ApoLf respectively.

388

389   Since the SD combinations were effective at reducing biofilm intensity, the

390   mechanism of action of this effect was investigated by examining the effects

391   of the antimicrobial proteins on penetration of the SD antibiotics through

392   PAO1 biofilms. The zones of growth inhibition of the indicator organism in the

393   absence of a biofilm were taken to represent 100% penetration and used to

394   determine the percentage retardation of the penetration of antibiotics and

395   combinations through PAO1 biofilms. The limit of detection was taken to be

396   13mm, since this was size of the polycarbonate membrane filter on which the

397   biofilms were grown.

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398

399   Figure 6 indicated that PAO1 biofilms reduced the penetration of SD TOBI to

400   15.3±6.3% and spray dried combinations of Lf with TOBI did not significantly

401   alter this (drug penetration 23±4% control). In contrast, the spray-dried

402   combination of ApoLf with TOBI significantly (P<0.05) increased penetration

403   of the antibiotic through PAO1 biofilms to 42.4±11.7 %. Visualisation of viable

404   bacteria within the biofilm (Fig. 7) indicated that SD combinations of ApoLf

405   with TOBI are more effective at reducing bacterial viability than SD TOBI

406   alone. Biofilms were grown on glass slides for 72 hours in order to allow

407   confocal fluorescence microscopy using the nucleic acid stains, SYTO 9 and

408   propidium iodide. While SYTO 9 stains all nuclei acid in a population (and

409   therefore bacteria with intact and damaged membranes appear green),

410   propidium iodide stains only bacteria with damaged membranes. When the

411   two dyes are used together, propidium iodide staining of damage bacteria will

412   cause a reduction in their green fluorescence, visible as an increase in the

413   yellow intensity seen on overlay of the green and red channel images. These

414   experiments evaluated the effects of co-spray dried TOBI+ApoLf, since this

415   combination consistently performed most effectively. Fig. 7B shows that SD

416   ApoLf reduces bacterial viability (apparent from the yellow staining in the right

417   panel), whereas SD TOBI is not altering bacterial viability and the staining is

418   predominantly in the green channel (Fig. 7C). The images in Fig. 7D

419   indicated that co-spray dried TOBI+ApoLf was most effective in terms of

420   reducing bacterial viability in the biofilm- thus red fluorescence was greater

421   here than with the other compounds tested and the intensity of the yellow

18

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

424

425   4. DISCUSSION

426

427   The idea of combining various antibiotics to increase their efficacy has been
428   around for decades. In the present study, we investigated the in vitro
429   antibacterial activities of a novel inhaled antibiotic combination consisting of a
430   1:1 spray dried combination of aminoglycosides and an antimicrobial peptide
431   (Lactoferrin). Upon spray drying our various preparations, we obtained an
432   average residual moisture content of between 3.2 – 5.9 % indicating that the
433   drying process was efficient in evaporating most of the moisture.

434

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

444

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

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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

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470 and lactoferrin-gentamicin has not been investigated. Thus, these studies

471 show the potential benefit such a formulation would have.

472

473 Images obtained by confocal laser scanning microscopy CLSM show that

474 both apo lactoferrin and tobramycin caused minimal cell membrane damage

475 to majority of the biofilm cells. However, treatment of the biofilms with a 1

476 mg/ml spray dried combination of lactoferrin and tobramycin caused

477 significant membrane damage to all cells indicating cell lysis in the *P.*

478 *aeruginosa* PAO1 biofilm. These observations were consistent with our biofilm

479 and penetration assays. Taken together, our confocal microscopy results

480 indicate that on treating the biofilms with a spray dried combination therapy of

481 lactoferrin and tobramycin there is a complete suppression of metabolic

482 activity of biofilms due to the enhanced antibiotic penetration through

483 disrupted cell membranes.

484

485 Overall, results from our study show that the spray dried combinations of

486 aminoglycosides and lactoferrin appear to have a superior antimicrobial

487 activity in *P. aeruginosa* PAO1 biofilms compared to monotherapy.

488 Tobramycin:lactoferrin combination could be a potentially new therapeutic

489 agent for biofilm associated infections. Also the fact that as a combination

490 they can be homogeneously micronized means that they could potentially be

491 employed in the management of chronic airway infections as seen amongst

492 people with cystic fibrosis. The clinical applicability of a tobramycin:lactoferrin

493 combination warrants future in vivo studies

21

494   Drug resistant bacterial infections are becoming more widespread with new

495   drug approvals for inhaled antibiotics constantly declining year on year. The

496   rise in drug resistance especially due to the ability of bacteria to exist in

497   biofilms has limited the repertoire of effective antimicrobials with considerable

498   potency against infections as commonly seen amongst people with cystic

499   fibrosis. The potential for employing a combination therapy of lactoferrin and

500   aminoglycoside as described here provides a new and large playing field for

501   proper utilisation of our currently available antibiotic resources in the provision

502   of therapeutic solutions to some of the current bacterial menace. It will be

503   interesting to translate most of our understanding into clinical treatment so as

504   to effectively fight resistant *P. aeruginosa* infections.

505

506   5. CONCLUSIONS

507   In conclusion, we have developed combination therapies comprising an

508   antimicrobial protein (lactoferrin) and an aminoglycoside antibiotic (tobramycin

509   or gentamicin), which are more effective than monotherapy at reducing

510   planktonic cell viability and preventing biofilm formation. We have shown that

511   these combinations have parameters compatible with delivery to the airways

512   and sug- gest, therefore, that they represent an important and exciting new

513   therapeutic strategy for treating biofilm-forming infections such as those

514   typical of the CF lungs.

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516   6. ACKNOWLEDGEMENTS.

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529 7. REFERENCES

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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 ±

28

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

29

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

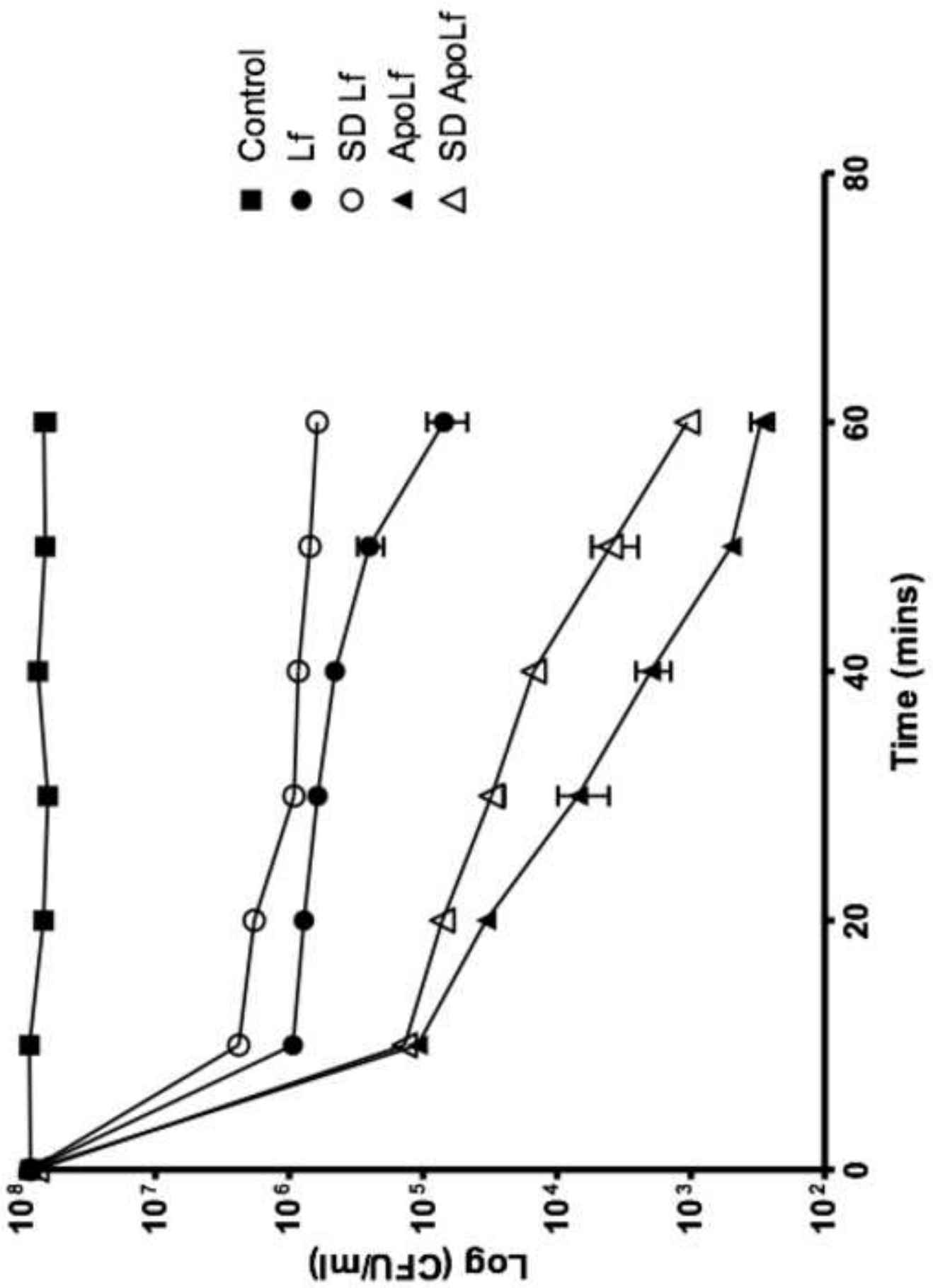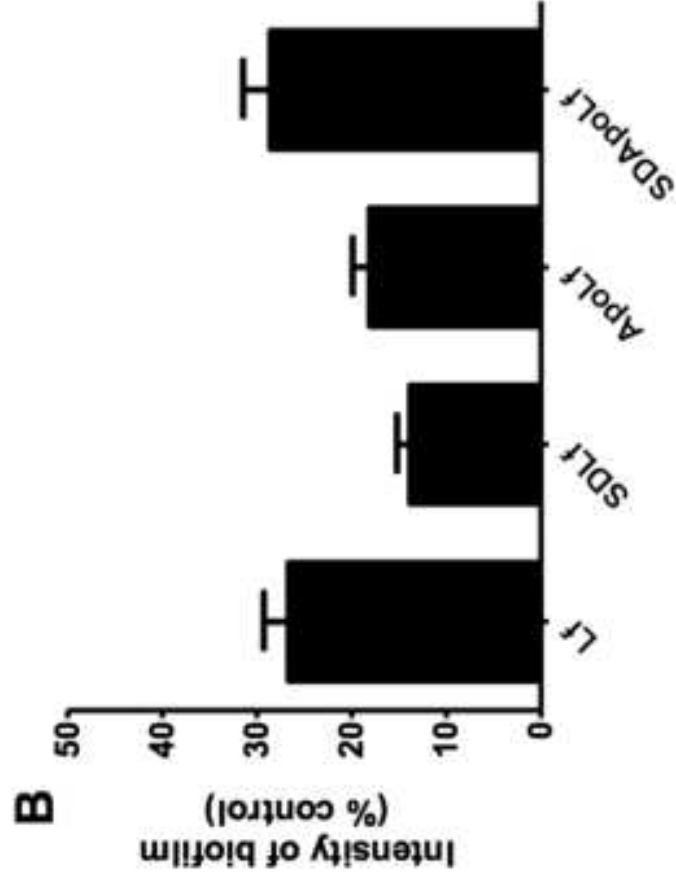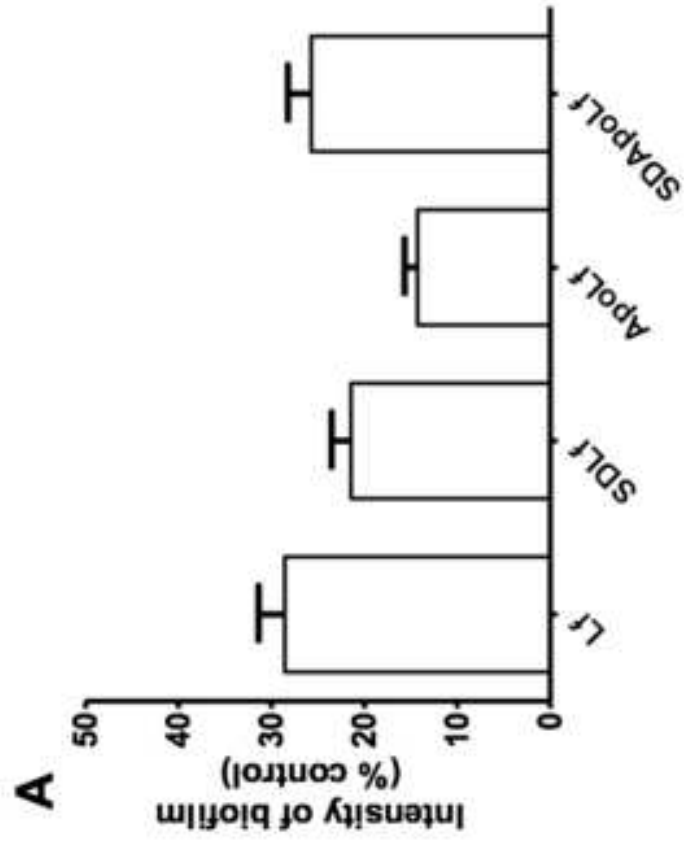665 combinations of Tobi and ApoLf.

666

30

Figure 1

MHA Plate with E. coli

13 mm membrane

P. aeruginosa PAO1 biofilm

6 mm membrane

Antibiotic disc
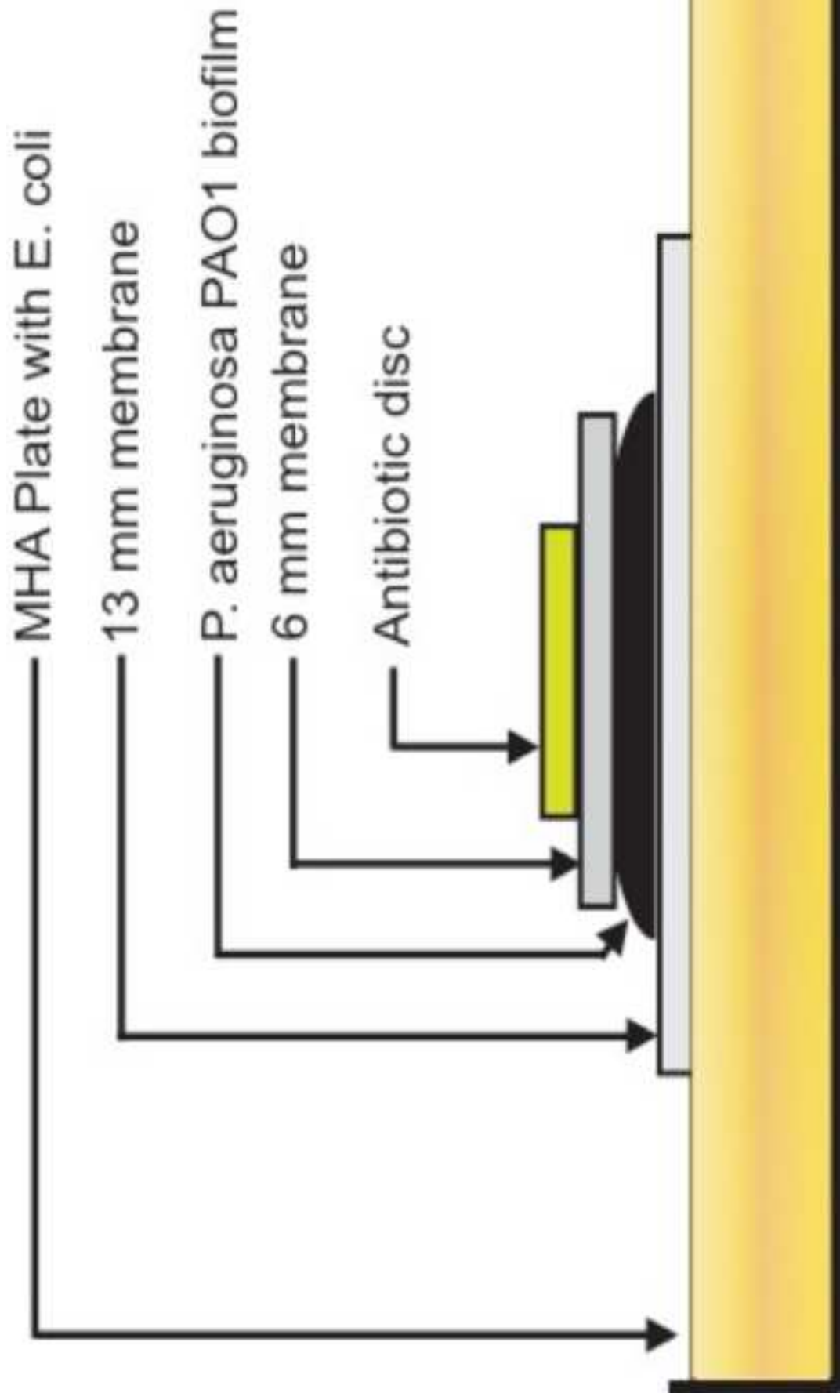
Figure 2

Figure 3

Figure 4
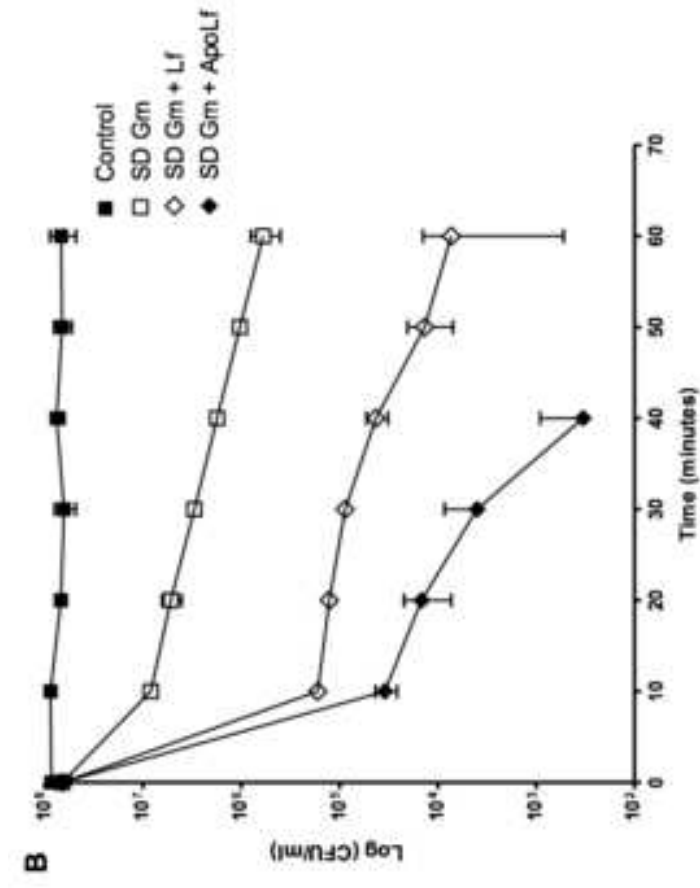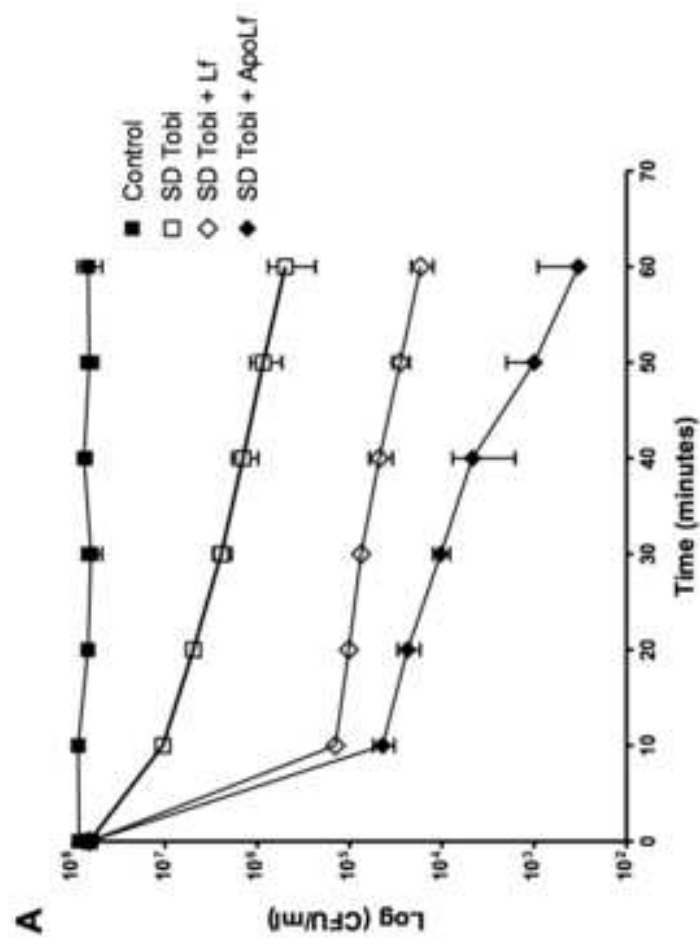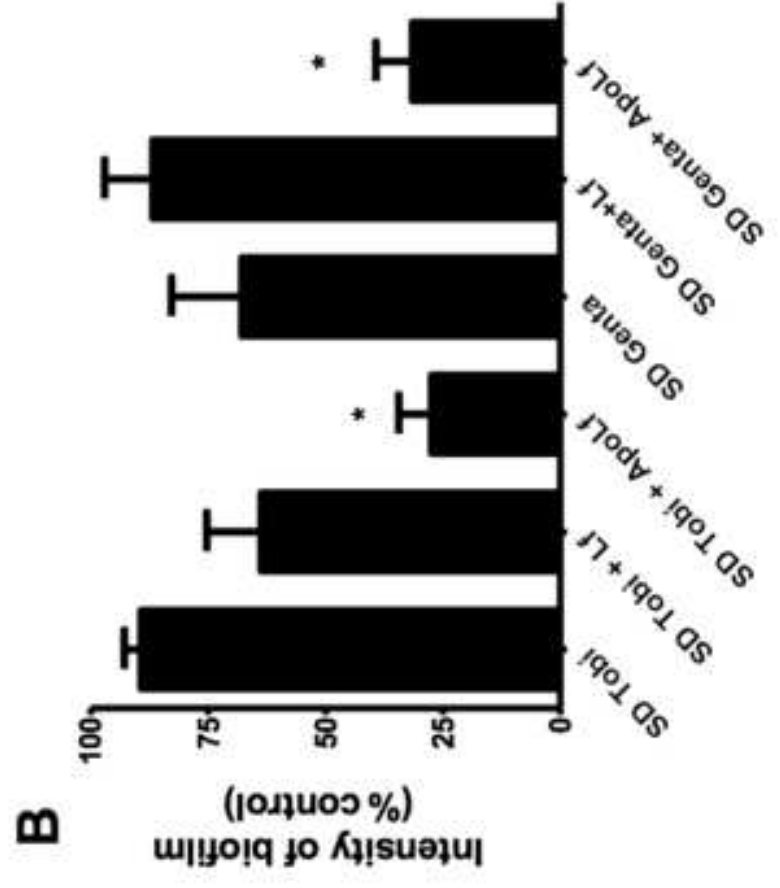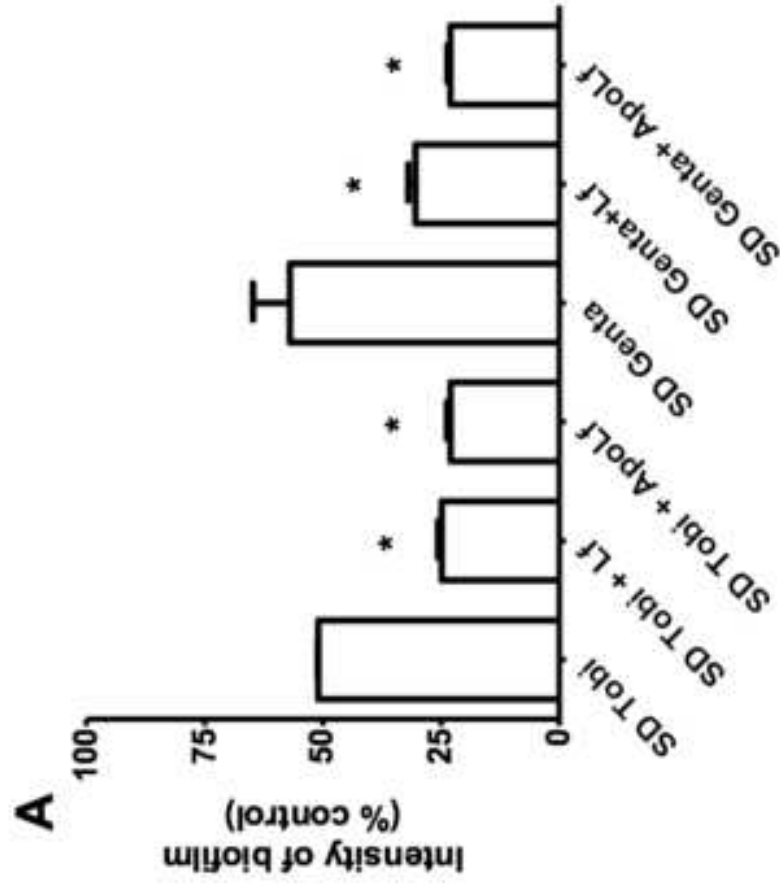
Figure 5

Figure 6

Figure 7

(a)

(b)

(c)

(d)