

1 Evaluation of combinations of putative anti-biofilm agents and antibiotics to eradicate
2 biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

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18 Running title: Antibiofilm enhancers

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Abstract

OBJECTIVES: To evaluate potential anti-biofilm agents for their ability to enhance the activity of antibiotics for local treatment of localised biofilm infections.

METHODS: *Staphylococcus aureus* and *Pseudomonas aeruginosa* *in vitro* biofilm models were developed. The putative antibiotic enhancers N-acetylcysteine, acetylsalicylic acid, sodium salicylate, rhDNase I, Dispersin B, hydrogen peroxide, and Baby Shampoo were tested for their anti-biofilm activity alone and their ability to enhance the activity of antibiotics for seven or 14 days, against five-day-old biofilms. The antibiotic enhancers were paired with rifampicin and clindamycin against *S. aureus* and gentamicin and ciprofloxacin against *P. aeruginosa*. Isolates from biofilms that were not eradicated were tested for antibiotic resistance.

RESULTS: Antibiotic levels 10xMIC and 100xMIC significantly reduced biofilm but did not consistently eradicate it. Antibiotics at 100xMIC with 10% Baby Shampoo for 14 days was the only treatment to eradicate both staphylococcal and pseudomonal biofilms. RhDNase I significantly reduced staphylococcal biofilm. Emergence of resistance of surviving isolates was minimal and was often associated with Small Colony Variant phenotype.

CONCLUSIONS: Baby Shampoo enhanced the activity of antibiotics and several other promising anti-biofilm agents were identified. Antibiotics with 10% Baby Shampoo eradicated biofilms produced by both organisms. Such a combination might be useful in local treatment of localised biofilm infections.

Introduction

Biofilms are an important cause of persistent and chronic infections such as otitis media with effusion (OME),^{1,2} prosthetic joint infections,³ colonisation of other indwelling devices,⁴ and infections after trauma, either following the injury itself or the surgical treatment.⁵ The biofilm mode of growth has many strategies for persistence and in this state, only essential processes remain active, and therefore many target sites for antibiotics are down-regulated leading to reduced susceptibility to antibiotics.^{6,7} Biofilm eradication requires 10-1,000 times the MIC of antibiotics normally needed to inhibit the planktonic form.^{8,9} Such high levels of antibiotics would be difficult to achieve safely when administered

62 systemically.¹⁰ Many biofilm infections are localised, for instance around spinal and
63 orthopaedic implants, in trauma, or in chronic wounds or facial sinuses. Local administration
64 of antibiotics in such situations results in very high concentrations at the biofilm site while
65 avoiding systemic exposure, but even then, failures occur due to incomplete eradication of
66 the biofilm.

67 Therefore, there is a need for alternative anti-biofilm strategies that might enable
68 locally administered antibiotics to exert anti-biofilm effect more consistently while if possible
69 reducing the concentrations necessary. Agents that disrupt the biofilm matrix may enhance
70 the anti-biofilm activity of antibiotics so that the biofilm cells become once again susceptible
71 to antibiotic treatment. We identified the following agents that could potentially be 'antibiotic
72 enhancers': N-acetylcysteine (NAC),^{11,12} acetylsalicylic acid,^{13,14} sodium salicylate,^{15,16}
73 salicylic acid,^{17,18} recombinant human deoxyribonuclease I (rhDNAse I),^{19,20} Dispersin B,²¹
74 hydrogen peroxide,²² and Johnson's Baby Shampoo (JBS).²³ JBS was included for its
75 reported ability to inhibit biofilm formation *in vitro* and to reduce clinical symptoms after local
76 nasal application for treatment of chronic rhinosinusitis, a biofilm infection.²⁴ Its individual
77 ingredients, specifically the surfactants,²⁵ dyes,^{25,26,27} and preservatives²⁸ were also
78 investigated to determine the antibiofilm activity of its components.

79 To date these agents have been studied individually in different *in vitro* biofilm and
80 species models, and in combination with different antimicrobial agents. However, they have
81 not been compared against each other in a consistent model. In order to do this, we tested
82 them in a systematic manner with the aim of identifying an 'antibiotic enhancer' that could be
83 used as adjunct to antibiotics to eradicate local biofilm infections.

84

85 **Methods**

86 The antibiotic enhancers were tested against two biofilm - forming bacteria,
87 *Staphylococcus aureus* and *Pseudomonas aeruginosa*, grown as biofilms on silicone discs.
88 The agents were paired with rifampicin and clindamycin against *S. aureus*, and gentamicin
89 and ciprofloxacin against *P. aeruginosa*. These antibiotics were chosen based on the
90 isolates' susceptibilities, routine clinical use, and their known anti-biofilm activity.^{29,30}

91

92 **Biofilm Model**

93 The two strains of *S. aureus* and *P. aeruginosa* strains were isolated from
94 clinical biofilm infections (from the middle ear effusion of patients undergoing surgery for
95 treatment of OME). Biofilms were grown on autoclaved silicone disks (6.0mm x 1.0mm,
96 silicone elastomer MQ/VNQ/PMQ/PVMQ, Goodfellow Ltd, Cambridge, UK) in 20mL volumes
97 of tryptone soya broth (TSB, Oxoid, Basingstoke, UK). Disks were added to a 0.5 McFarland

98 bacterial TSB suspension, spectrophotometer - verified to between 0.080 and 0.130
99 absorbance at 490 nm. The 0.5 McFarland suspension containing the disks at the beginning
100 of each “treatment” run (“treatment” in this context refers to exposure *in vitro* to antibiotics
101 and/or potential enhancers) was quantified by viable counting to ensure consistency. The
102 uninoculated control disk was added to an equal volume of TSB alone. Both groups were
103 incubated for one hour with shaking (200 rpm) then one hour statically at 37°C for
104 attachment to take place.

105 After incubation, each disk was added to 1.0mL of TSB in a sterile bijou bottle and
106 incubated for five days at 37°C without replenishing the medium, to achieve biofilm
107 maturation. This process was repeated at the beginning of each treatment run. The model
108 has been validated previously.^{31,32}

109
110 Sensitivity of *S. aureus* to rifampicin (Rifadin infusion, Sanofi – Aventis, New Jersey
111 USA) and clindamycin (clindamycin hydrochloride, Fluka Analytical, Buchs, Switzerland) and
112 of *P. aeruginosa* to gentamicin (gentamicin sulphate, Sigma-Aldrich, St Louis, MO, USA) and
113 ciprofloxacin (ciprofloxacin hydrochloride, PanReac AppliChem, St. Louis MO, USA) was
114 carried out in accordance with EUCAST guidelines³³ and verified before each treatment by
115 Etest (bioMérieux, Craaponne, France).

116

117 **Potential antibiotic enhancers**

118 The concentrations of the antibiotic enhancers were determined by the literature
119 review, using achievable, non-toxic plasma levels as a marker of safe levels (irrespective of
120 the intended local application) of those that can be administered systemically^{10,18-20,22,28-30} as
121 a guide. The following putative antibiotic enhancers were tested in the biofilm model: NAC
122 (neutralised with sodium hydroxide), acetylsalicylic acid, sodium salicylate, salicylic acid (all
123 Sigma-Aldrich), recombinant human deoxyribonuclease I (rhDNAse I) (Dornase alpha,
124 Genentech, California, USA), Dispersin B (Kane Biotech, Winnipeg, Manitoba, Canada),
125 hydrogen peroxide (Scientific Laboratory Supplies, Nottingham UK), and Johnson’s Baby
126 Shampoo (JBS, formulation sold in England, Johnson & Johnson, New Brunswick NJ). NAC
127 was neutralised because it is neutralised for clinical use in solutions given orally and for
128 injection. Two different concentrations of each enhancer were examined in the experiments,
129 with the chosen high and low concentrations added to the biofilm model shown in Table 1.

130

131 Table 1: High and low concentrations of the potential antibiotic enhancers used to treat mature biofilm
132 models

Antibiotic Enhancer Treatments	High concentration	Low concentration
Neutralised N-acetylcysteine	100 mg/mL	25 mg/mL
Acetylsalicylic acid	200 µg/mL	20 µg/mL

Sodium salicylate	175 µg/mL	17.5 µg/mL
Salicylic acid	150 µg/mL	15 µg/mL
rhDNase I	100 µg/mL	10 µg/mL
Hydrogen peroxide	30 mL/L (3%)	3 mL/L (0.3%)
Dispersin B	20 µg/mL	2 µg/mL
Baby Shampoo	10%	1%

133

134 ***Baby Shampoo Ingredients***

135 Surfactants, preservatives and dyes in JBS were investigated for their antibiofilm
136 activity. The surfactants investigated were sodium lauryl ether sulphate (SLES) (Mistral R&D
137 laboratories, Antrim, Northern Ireland), sodium lauroamphoacetate (SLAA) (Colonial
138 Chemical Inc., Pittsburg, Tennessee USA), polysorbate 20 (Sigma-Aldrich), polyethylene
139 glycol 80 (PEG-80) (Tween 28-LQ-(CQ), Croda International Plc, Snaith, England), and
140 polyethylene glycol distearate (PEG-150) (Evonik Industries, Essen, Germany). The
141 preservatives were sodium benzoate and citric acid and the dyes were quinolone yellow (all
142 Sigma-Aldrich) and Sunset Yellow FCF (Aldrich).

143 Preliminary screening for antibacterial activity was determined by broth microdilution
144 based on EUCAST guidance.³³ Briefly, the JBS ingredient and JBS stock solutions were
145 sterilised by autoclaving or membrane filtration. Bacteria were grown overnight on blood
146 agar and a 0.5 McFarland suspension (spectrophotometrically adjusted) in PBS was
147 prepared post-incubation. In triplicate, 100 µL of cation-adjusted Mueller-Hinton broth (MHB,
148 Sigma-Aldrich) was added to each well in the 12 rows on a 96-well plate (Nunclon Delta
149 Surface, Thermo Scientific, Roskilde, Denmark). To the first wells 100 µL of the drug
150 standard was added, mixed, and 100 µL was transferred to the next well. The final well
151 contained only MHB. To all wells including the MHB-only well 10 µL of the bacterial
152 suspension was added. The plates were incubated overnight at 37°C.

153 For preliminary screening for the possible antibiotic - enhancing activity of the
154 ingredients, JBS, the individual ingredients and antibiotics were tested in combination in a
155 modified chequerboard assay.³⁴ A 1:100 dilution of a 0.5 McFarland bacterial suspension in
156 PBS was determined to be equivalent to 5×10^5 cfu/mL for both bacteria. According to the
157 MICs determined in the broth microdilution assay, a 4xMIC stock solution of each JBS
158 ingredient was prepared and sterilised accordingly. A stock solution (8xMIC) of each
159 antibiotic was prepared. To a 96 well plate, 100 µL of MHB was added to each well. The
160 JBS ingredient was serially diluted along the y-axis with a starting concentration of 2xMIC.
161 The two antibiotics were serially diluted along the x-axis, with 50µL of each antibiotic stock
162 solution added to the initial wells also to give a starting concentration of 2xMIC. All wells
163 were inoculated with 10µL of the bacterial suspension. JBS ingredients showing potential
164 antibacterial activity and/or interaction with the antibiotics were tested in the biofilm model at
165 high (10xMIC) and low (1xMIC) concentrations.

166

167 **Treatment of mature biofilm**

168 After five-day incubation of the silicone disks, the TSB was removed and replaced
169 with 1.0mL of fresh TSB plus any treatment. The treatment groups were: no-treatment, 10X
170 MIC alone, 100X MIC alone, low concentration potential antibiotic enhancer alone and
171 paired with 10X and 100X MIC, and high concentration potential antibiotic enhancer (Table
172 1) alone and paired with 10X MIC and 100X MIC. Experiments were performed in triplicate
173 with an additional set of three prepared for resuscitation experiments if necessary. Where
174 biofilms had apparently been eradicated, a further period of antibiotic-free incubation was
175 applied to detect any sublethal suppression. Treatment was for either seven and or 14 days.
176 Dispersin B was tested only against *S. aureus*, as its enzymatic activity is directed against n-
177 acetyl glucosamine and not the *P. aeruginosa* exopolysaccharide matrix.

178

179 At the end of treatment, any surviving bacteria were quantified. Disks were removed
180 and placed into sterile 1.5mL microtubes (Sarstedt, Nümbrecht, Germany) with 400µL of
181 10% trypsin (gamma-irradiated, SAFC Biosciences, Hampshire, UK), which disaggregated
182 bacteria attached to the disk. Microtubes were incubated for 15 minutes at 37°C, trypsin was
183 replaced with 1.0mL of PBS, and all tubes were sonicated (30 kHz) for five minutes to
184 detach bacteria from the disk. Previous work showed that trypsinisation and sonication
185 together yielded more bacteria than each separately without loss of viability.³² The sonicate
186 was serially diluted and 200µL of the dilutions were each spread on three blood agar plates
187 (Oxoid), and incubated for 48 hours at 37°C

188

189 **Resuscitation experiments**

190 Plates were checked for growth after 24 hours. If two or more of the three plates
191 were culture negative after 48hours, resuscitation experiments were undertaken to
192 determine if the treatment had killed the bacteria (resuscitation negative, indicating biofilm
193 eradication) or merely inhibited growth (resuscitation positive, indicating biofilm
194 suppression). Disks were washed in 1.0mL PBS and placed into 1.0mL of fresh TSB to
195 provide optimum growth conditions for six days to revive any bacteria with suppressed
196 growth. The attached bacteria were quantified as described previously.

197

198 **Determining development of resistance after treatment**

199 Colonies that grew after 14 - day treatment, or were culture - negative after 14 - day
200 treatment but then resuscitation - positive, had their MICs determined again by Etest. If small
201 colony variants (SCVs)³⁵ were present alongside typical colonies, each population had their

202 MIC determined separately. Isolates from biofilms that were not eradicated after 14 days
203 were also investigated for resistance using EUCAST breakpoints.

204

205 **Scanning Electron Microscopy (SEM)**

206 Five-day-old biofilms on silicone discs were fixed in 1.0mL of cold acetone, then
207 dried with tetramethylsilane (Sigma-Aldrich), sputter coated with gold for 300 seconds and
208 visualized using Jeol 6060LV variable pressure SEM (Jeol UK Ltd).

209

210 **Statistics**

211 The effect of antibiotics and antibiotic enhancers between groups was compared
212 using two-way ANOVA (Graphpad Prism 7.01, La Jolla California USA). Two-way ANOVA
213 was conducted on the effect of the concentration of antibiotics and the antibiotic enhancer
214 on reduction of biofilm bacteria (cfu/mL). Post-hoc multiple comparisons were only carried
215 out when the ANOVA value was significant ($p < 0.05$) and was corrected using Dunnett's test.

216

217 **Results**

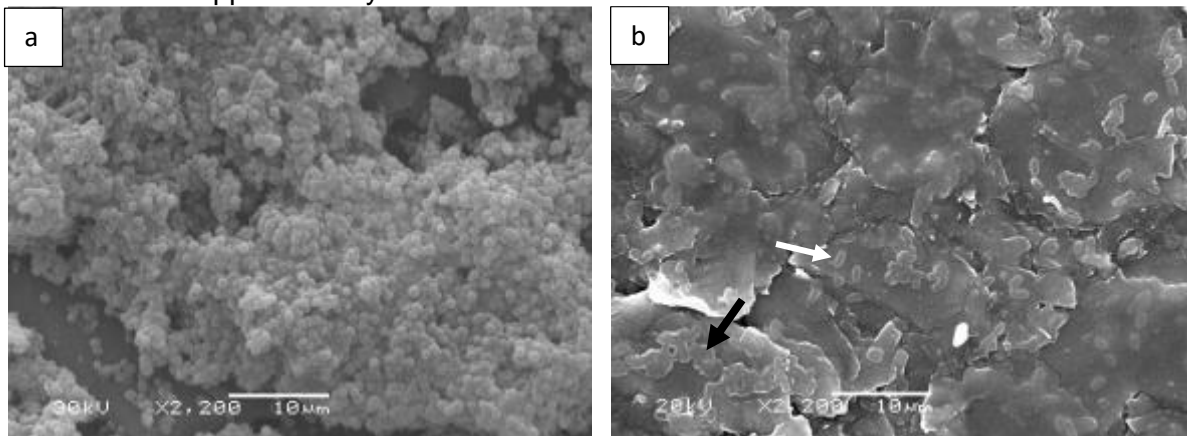
218 **Susceptibility to the chosen antibiotics**

219 *S. aureus* was susceptible to rifampicin (MIC 0.004mg/L) and clindamycin (MIC
220 0.064mg/L), and *P. aeruginosa* was susceptible to gentamicin (MIC 1.0mg/L) and
221 ciprofloxacin (MIC 0.125mg/L). These MIC values were then used to determine the
222 concentrations of 10xMIC and 100xMIC used with or without antibiotic enhancers to treat the
223 biofilm model.

224

225 **Confirmation of biofilm growth in model**

226 *S. aureus* and *P. aeruginosa* appeared structurally as biofilms on SEM. In Figure 1b,
227 the bacteria are more difficult to distinguish as so much polysaccharide extracellular matrix
228 was produced that the bacteria were incompletely exposed. The discs were confirmed to be
229 colonised with approximately 10^8 cfu/mL after sonication.



230

231 **Fig 1.** a. Coccus-shaped bacteria present on a silicone disc 5 days post inoculation with *S. aureus*. b.
232 Rod-shaped bacteria (white arrow and elsewhere) and some encased by a matrix (black arrow) on a
233 silicone disc, five-days post inoculation with *P. aeruginosa*. Both at X2200 magnification SEM.

234

235 *S. aureus* and *P. aeruginosa* both behaved functionally as biofilms in that neither was
236 eradicated with 14 days of treatment with 10x MIC or 100x MIC antibiotics alone, although
237 antibiotics reduced the number of viable bacteria present.

238

239 ***Treatment of mature biofilms with antibiotics alone or in combination with potential***
240 ***antibiotic enhancers***

241 Mature biofilms of *S. aureus* or *P. aeruginosa* were treated with paired antibiotics,
242 with a potential antibiotic enhancer, or with antibiotics and the enhancers together. The
243 results of 14 day treatments of the two biofilms are shown in Fig 2. For *S. aureus*, the main
244 effect of antibiotic concentration (including no antibiotics) was statistically significant at
245 $p < 0.0001$ and the main effect of different antibiotic enhancers was statistically significant at
246 $p < 0.0001$ (Fig 2A). Likewise, the main effect of antibiotic concentration was significant
247 ($p = 0.0005$) for *P. aeruginosa* and main effect of different antibiotic enhancers was significant
248 at $p < 0.0001$ (Fig 2B).

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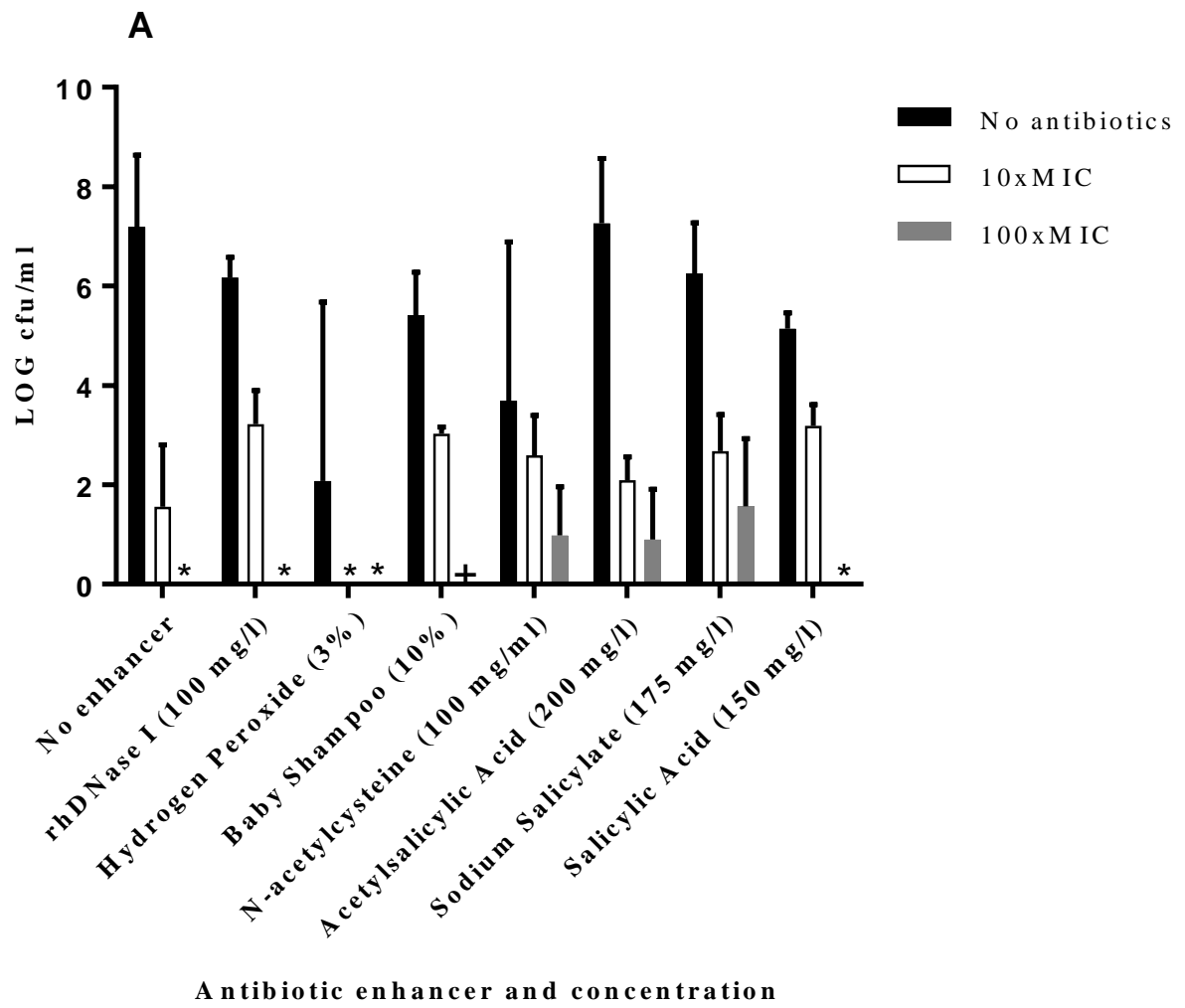
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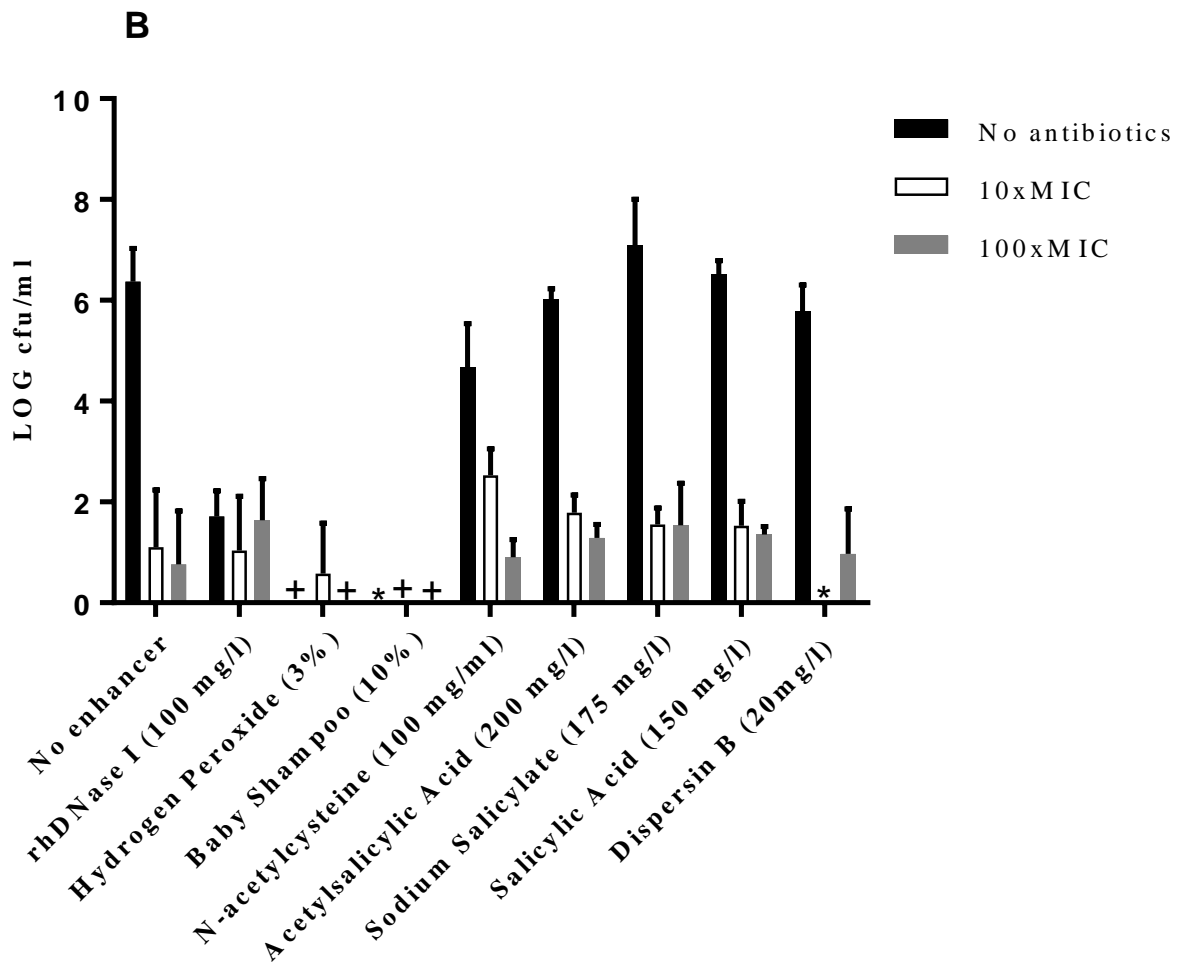
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Antibiotic enhancer and concentration

260

261 **Fig 2:** Log mean colony - forming units per millilitre (Log cfu/mL) and standard deviations for 5 - day
 262 old *P. aeruginosa* (A) and *S. aureus* (B) biofilms treated for 14 days with antibiotic enhancer and/or
 263 antibiotics (10x or 100xMIC antibiotics). The antibiotics against *P. aeruginosa* were ciprofloxacin and
 264 gentamicin and rifampicin and clindamycin for *S. aureus*. * indicates 'inhibited' in that treatment
 265 resulted in bacterial counts of zero but resuscitation experiments were positive for bacterial growth,
 266 whereas + indicates "eradicated" meaning that treatment resulted in bacterial counts of zero and
 267 remained negative for growth after resuscitation experiments.

268

269 Biofilm eradication and inhibition were considered as the most stringent measures of
 270 efficacy rather than bacterial count reduction. JBS was the only potential antibiotic enhancer
 271 capable of eradicating both *S. aureus* and *P. aeruginosa* biofilms. 10% JBS in combination
 272 with 10xMIC antibiotics and 100xMIC antibiotics eradicated *S. aureus* biofilms in 14 days.
 273 JBS appeared to enhance the activity of antibiotics against the mature staphylococcal biofilm
 274 as 10xMIC and 100xMIC antibiotics without JBS were insufficient to eradicate or inhibit

275 biofilm. 10% JBS in combination with 100xMIC antibiotics eradicated the pseudomonas
276 biofilm and was the only treatment to do so.

277 Hydrogen peroxide (3%) alone and paired with antibiotics was capable of eradicating
278 the *S. aureus* biofilm after 14 days of treatment. Hydrogen peroxide with 10xMIC and
279 100xMIC inhibited the *P. aeruginosa* biofilm after 14 days but did not eradicate it.

280 rhDNAse I (100 µg/mL) alone significantly ($p < 0.0001$) reduced staphylococcal biofilm
281 bacteria, but did not enhance the activity of the antibiotics. rhDNAse I in combination with the
282 100xMIC inhibited *P. aeruginosa* biofilm after 14 days of treatment but did not eradicate it.

283

284 **Development of resistance**

285 The majority of biofilms were not eradicated by 100xMIC concentrations of
286 antibiotics. As sub-MBEC levels can result in resistance in the surviving isolates, MICs of
287 those that survived treatment from each of the assays, including SCV populations, were
288 determined.

289 Resistance to rifampicin developed in seven (6.1%) of the 114 *S. aureus* 14 - day
290 surviving or 14 - day resuscitated isolates tested. Of these seven, two were categorized as
291 SCVs and six were isolates that had been resuscitated. None developed resistance to
292 clindamycin. Of the 126 *P. aeruginosa* survivors four (3.2%) developed resistance to
293 gentamicin and one developed resistance to ciprofloxacin. The five resistant *P. aeruginosa*
294 survivors had all been treated with 10xMIC and rhDNAse I, and four were SCVs. Of these,
295 three were resistant to gentamicin and one to ciprofloxacin. None was resistant to both
296 antibiotics.

297

298 **Screening of ingredients in baby shampoo for antibacterial activity**

299

300 Table 2: MIC of ingredients in baby shampoo against *S. aureus* and *P. aeruginosa*

Ingredient	MIC against <i>S. aureus</i>	MIC against <i>P. aeruginosa</i>
Quinolone Yellow	100 mg/mL	> 100 mg/L
Sunset Yellow	100 mg/mL	> 100 mg/L
Citric acid	> 100 mg/L	> 100 mg/L
Sodium benzoate	> 100 mg/L	> 100 mg/L
PEG-80	> 100 mg/L	> 100 mg/L
PEG-150	> 100 mg/L	> 100 mg/L
Polysorbate 20	> 100 mg/L	> 100 mg/L
Sodium lauroamphoacetate	22.5 mg/mL	45 mg/mL
Sodium lauryl ether sulphate	4.2 mg/mL	4.2 mg/mL

301

302

303 These results suggest that JBS may be the most promising antibiofilm agent and potential
304 antibiotic enhancer determined by this systematic comparison. However, JBS is a complex
305 mixture of ingredients (Table 2). To determine the active antibiofilm component of JBS, its

306 ingredients were screened for antibacterial activity and interaction with antibiotics and those
307 of interest were tested in the biofilm model.

308 The MIC of several of the ingredients was greater than any concentrations tested. Therefore,
309 these ingredients were not tested for interaction in the checkerboard assay as they did not
310 demonstrate an antibacterial effect at a concentration within the range of concentrations
311 used in commercially available topical antiseptic solutions.

312

313 **Checkerboard assay to determine interaction of JBS ingredients with antibiotics**

314 Initially the pairs of antibiotics were screened for their drug interaction, defined as the
315 fractional inhibitory concentration index (FICI) where $FICI < 0.5$ indicates synergy, $FICI > 4.0$
316 indicated antagonism and values in between suggest no interaction.³⁶ The FICI between
317 gentamicin and ciprofloxacin against *P. aeruginosa* was 0.0625 indicating synergy (Table 3).

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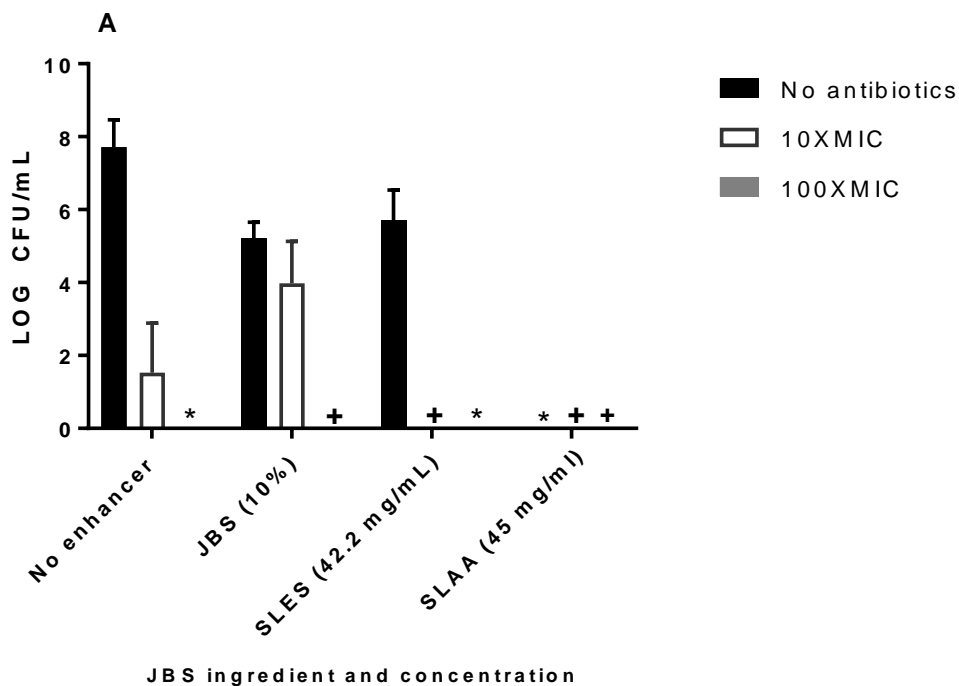
319 Table 3: Drug interactions of baby shampoo and its selected ingredients in a modified checkerboard
320 assay. Σ FICI: fractional inhibitory concentration index

Ingredient in combination with antibiotics	<i>P. aeruginosa</i>		<i>S. aureus</i>	
	FICI	Drug Interaction	FICI	Drug Interaction
Baby Shampoo	0.53	No interaction	0.375	Synergy
Quinolone Yellow			8.0	Antagonism
Sunset Yellow			6.0	Antagonism
Sodium lauroamphoacetate	0.53	No interaction	0.281	Synergy
Sodium lauryl ether sulphate	1.125	No interaction	1.125	No interaction

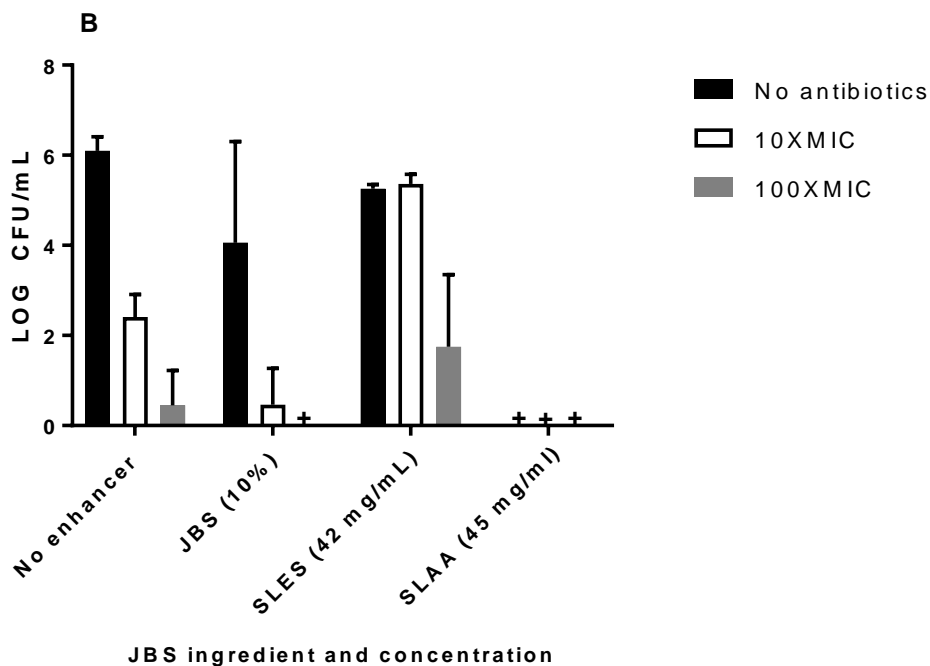
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322 **Biofilm Model**

323 SLAA and SLES were tested in the biofilm model (Fig 3). Quinolone yellow and
324 sunset yellow were excluded as they were antagonistic when paired with antibiotics in the
325 checkerboard assay against *S. aureus*. JBS was run alongside SLAA and SLES for
326 consistency with the original experiment, and with 100xMIC antibiotics was able to eradicate
327 mature *P. aeruginosa* and *S. aureus* biofilms after 14 days. SLAA paired with 10xMIC
328 antibiotics was able to eradicate *P. aeruginosa* and *S. aureus* biofilms demonstrating an
329 enhancing effect since 10xMIC alone was not capable of eradicating either biofilm.



330



331

332 **Fig 3:** Log mean colony - forming units per millilitre (Log cfu/mL) and standard deviations for five - day
 333 old *P. aeruginosa* (A) and *S. aureus* (B) biofilms treated for 14 days with JBS ingredients and/or
 334 antibiotics (10x or 100xMIC antibiotics). The antibiotics against *P. aeruginosa* were ciprofloxacin and
 335 gentamicin and rifampicin and clindamycin for *S. aureus*. * indicates 'inhibited' in that treatment
 336 resulted in bacterial counts of zero but resuscitation experiments were positive for bacterial growth,
 337 whereas + indicates "eradicated" meaning that treatment resulted in bacterial counts of zero and
 338 remained negative for growth after resuscitation experiments. JBS: Baby shampoo, SLES: sodium
 339 lauryl ether sulfate, SLAA: sodium lauroamphoacetate

340

341 **Discussion**

342

343 In this study, several literature-cited potential antibiotic enhancers were
344 systematically evaluated against *S. aureus* and *P. aeruginosa* mature biofilm models. When
345 used alone hydrogen peroxide and rhDNase 1 demonstrated a significant anti-biofilm effect
346 against *S. aureus* ($p < 0.001$ and $p < 0.001$ respectively) when compared to the untreated
347 control. Therefore, it was not possible to demonstrate potentiation of antibiotic activity for
348 these two agents. However, 10% JBS acted as an antibiotic enhancer in combination with
349 10xMIC, eradicating both *S. aureus* and *P. aeruginosa* biofilms with no recovery where both
350 10xMIC and 100xMIC antibiotics alone failed to eradicate these biofilms. When the individual
351 ingredients of the successful JBS were investigated, SLAA showed the greatest antibiofilm
352 activity as it was able to eradicate *S. aureus* and *P. aeruginosa* biofilm in 14 days with
353 10xMIC and 100xMIC antibiotics.

354

355 ***The Biofilm Model***

356 The *in vitro* biofilm model, validated in previous studies,³² and was chosen for its
357 ability to provide individual experimental conditions for each biofilm grown on a silicone disk.
358 A five-day-old biofilm was considered mature and well-established based on our previous
359 studies which demonstrated that one-day-old biofilms were easier to eradicate than five-day-
360 old ones (data not shown), consistent with the findings of Anwar *et al*, who showed that
361 mature *S. aureus* biofilms are more difficult to treat with antibiotics than younger biofilms.³⁷
362 Two antibiotics were used together according to the dual drug principle, which states that
363 using two antibiotics of two different classes at concentrations above their MIC reduces the
364 risk of the development of resistance.³⁸

365

366 ***rhDNase I***

367 rhDNase cleaves bacterial extracellular DNA (eDNA), a component of the biofilm
368 matrix¹⁹. However, it did not reduce pseudomonas biofilm when used alone. The role of
369 eDNA varies between organisms. It is required only for initial biofilm formation of *P.*
370 *aeruginosa*³⁹ but it plays a more important structural role in established *S. aureus* biofilms
371 compared to *S. epidermidis* biofilms.²¹ When Whitchurch *et al* treated a five-day-old
372 pseudomonas biofilm with DNase I it disrupted the biofilm, but not a seven-day old biofilm.³⁹
373 Our results differ from the Whitchurch *et al* study only in that our five-day old biofilm was not
374 affected by rhDNase I alone, possibly due to strain differences as we used a clinically
375 isolated strain and they used *P. aeruginosa* PA01, a common research strain.

376 Izano *et al* demonstrated that the exopolysaccharide matrix and eDNA have different
377 structural roles in *S. aureus* and *S. epidermidis* biofilms, where eDNA had a more major role
378 in the *S. aureus* biofilm structure. This was shown by action of rhDNase I which inhibited
379 biofilm formation and detached pre-formed *S. aureus* biofilms but not *S. epidermidis* biofilms.
380 Our results are consistent with Izano *et al* as 100 µg/mL rhDNase I reduced biofilm in our
381 pre-formed *S. aureus* biofilm model.²¹

382

383 **Hydrogen Peroxide**

384 Hydrogen peroxide eradicated staphylococcal biofilm at seven days (data not
385 shown), alone and in combination with 100xMIC antibiotics. However, it inhibited
386 pseudomonas biofilms only when paired with 100xMIC antibiotics. Sumen *et al* found that
387 hydrogen peroxide had an 'inhibitory effect' on 37 biofilm organisms that they tested, and
388 they demonstrated that its effectiveness against a broad spectrum of microorganisms unlike
389 Dispersin B and other enzymes.²² Toté *et al* found that hydrogen peroxide was active
390 against *P. aeruginosa* and *S. aureus* biofilm bacteria as well as the biofilm matrix.⁴⁰ *S.*
391 *aureus* and *P. aeruginosa* are catalase - positive organisms. Eradication of the biofilm may
392 be a mechanical action in which bubbling physically disrupts the structure, or a chemical
393 action in which the molecule itself reacts with a component of the biofilm.

394

395 **Baby Shampoo**

396 JBS has been proposed as an adjuvant treatment in chronic rhinosinusitis,²³ a
397 biofilm infection, as it contains three surfactants which have also been shown to disrupt
398 biofilms in orthopaedic infections.⁴¹ JBS is well - tolerated by users and is non-irritant. Chiu
399 *et al* found that 10% JBS alone was not capable of eradicating an established pseudomonas
400 biofilm,²³ which concurs with our results. However, JBS with 100xMIC antibiotics did
401 eradicate pseudomonas biofilm in our study and was the only treatment to do so.
402 Furthermore, JBS with 10xMIC antibiotics also eradicated the staphylococcal biofilm, and
403 was in fact the only treatment able to eradicate biofilm with antibiotic levels as low as this.
404 Antibiotics alone at 10xMIC did not eradicate an established biofilm. It therefore appears that
405 JBS enhanced the activity of the antibiotics. An antibiotic level of 10xMIC is readily
406 achievable by local administration without toxicity, making JBS the most promising agent
407 tested in these experiments.

408 From the results, it is clear that SLAA is most likely responsible. Alone, it recreates
409 the same antibiotic - enhancing effect demonstrated by JBS. SLAA is an amphoteric
410 surfactant, with limited data demonstrating some anti-bacterial activity,^{42,43} however, to our
411 knowledge this is the first study to show the anti-biofilm activity of SLAA. Amphoteric
412 compounds, being both anionic and cationic, have an advantage in that they have both the

413 detergent activity seen with anionic compounds and the bactericidal activity seen with
414 cationic compounds.⁴⁴ In the case of biofilm bacteria, it is possible that the detergent activity
415 physically disrupts the biofilm and the bactericidal activity of SLAA and the antibiotics
416 together can then be effective against newly-planktonic cells.

417

418 **Conclusions and Implications for Practice**

419 Based on the results, JBS (in particular, the SLAA component), rhDNase I and
420 hydrogen peroxide might have a role in local therapy for local biofilm conditions such as
421 OME, osteomyelitis, or infection of accessible implantable devices. In any situation where a
422 local infection may be treated with antibiotic beads or in which the infected area may be
423 irrigated with an antibiotic solution, the enhancer could be added alongside. Irrigation of
424 infected wounds is common practice and antibiotics or antiseptics may be added to the
425 irrigation fluid.⁴⁵ Considering the high acceptability of JBS in both medical use and for its
426 original, intended hair shampoo use, that rhDNase I is approved for use in the lungs by
427 inhalation,⁴⁶ and that hydrogen peroxide has many historical antiseptic uses, these three
428 agents are likely to have satisfactory safety profiles. Furthermore, success in this
429 experimental setting was considered to be complete eradication of biofilm, but *in vivo* a
430 significant reduction in bacterial counts might be considered a success in certain
431 circumstances. It is accepted that results of *in vitro* evaluations do not always apply *in vivo*,
432 nevertheless we feel that on safety and *in vitro* grounds the enhancers show clinical
433 promise. Future *in vivo* studies are planned to look at JBS or its active component, SLAA, as
434 an adjunct to local antibiotic treatment for infections such as otitis externa, OME, and
435 infected wounds.

436

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