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

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Running title: Antibiofilm enhancers

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

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

Therefore, there is a need for alternative anti-biofilm strategies that might enable locally administered antibiotics to exert anti-biofilm effect more consistently while if possible reducing the concentrations necessary. Agents that disrupt the biofilm matrix may enhance the anti-biofilm activity of antibiotics so that the biofilm cells become once again susceptible to antibiotic treatment. We identified the following agents that could potentially be 'antibiotic enhancers': N-acetylcysteine (NAC),[11,12] acetylsalicylic acid,[13,14] sodium salicylate,[15,16] salicylic acid,[17,18] recombinant human deoxyribonuclease I (rhDNAse I),[19,20] Dispersin B,[21] hydrogen peroxide,[22] and Johnson's Baby Shampoo (JBS).[23] JBS was included for its reported ability to inhibit biofilm formation *in vitro* and to reduce clinical symptoms after local nasal application for treatment of chronic rhinosinusitis, a biofilm infection.[24] Its individual ingredients, specifically the surfactants,[25] dyes,[25,26,27] and preservatives[28] were also investigated to determine the antibiofilm activity of its components.

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

Methods

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

Biofilm Model

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

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

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

Sensitivity of *S. aureus* to rifampicin (Rifadin infusion, Sanofi – Aventis, New Jersey USA) and clindamycin (clindamycin hydrochloride, Fluka Analytical, Buchs, Switzerland) and of *P. aeruginosa* to gentamicin (gentamicin sulphate, Sigma-Aldrich, St Louis, MO, USA) and ciprofloxacin (ciprofloxacin hydrochloride, PanReac AppliChem, St. Louis MO, USA) was carried out in accordance with EUCAST guidelines[33] and verified before each treatment by Etest (bioMérieux, Craponne, France).

Potential antibiotic enhancers

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

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

Baby Shampoo Ingredients

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

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

For preliminarily screening for the possible antibiotic - enhancing activity of the ingredients, JBS, the individual ingredients and antibiotics were tested in combination in a modified chequerboard assay.[34] A 1:100 dilution of a 0.5 McFarland bacterial suspension in PBS was determined to be equivalent to 5 x10^5 cfu/mL for both bacteria. According to the MICs determined in the broth microdilution assay, a 4xMIC stock solution of each JBS ingredient was prepared and sterilised accordingly. A stock solution (8xMIC) of each antibiotic was prepared. To a 96 well plate, 100 µL of MHB was added to each well. The JBS ingredient was serially diluted along the y-axis with a starting concentration of 2xMIC. The two antibiotics were serially diluted along the x-axis, with 50µL of each antibiotic stock solution added to the initial wells also to give a starting concentration of 2xMIC. All wells were inoculated with 10µL of the bacterial suspension. JBS ingredients showing potential antibacterial activity and/or interaction with the antibiotics were tested in the biofilm model at high (10xMIC) and low (1xMIC) concentrations.

Treatment of mature biofilm

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

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

Resuscitation experiments

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

Determining development of resistance after treatment

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

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

Scanning Electron Microscopy (SEM)

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

Statistics

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

Results

Susceptibility to the chosen antibiotics

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

Confirmation of biofilm growth in model

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

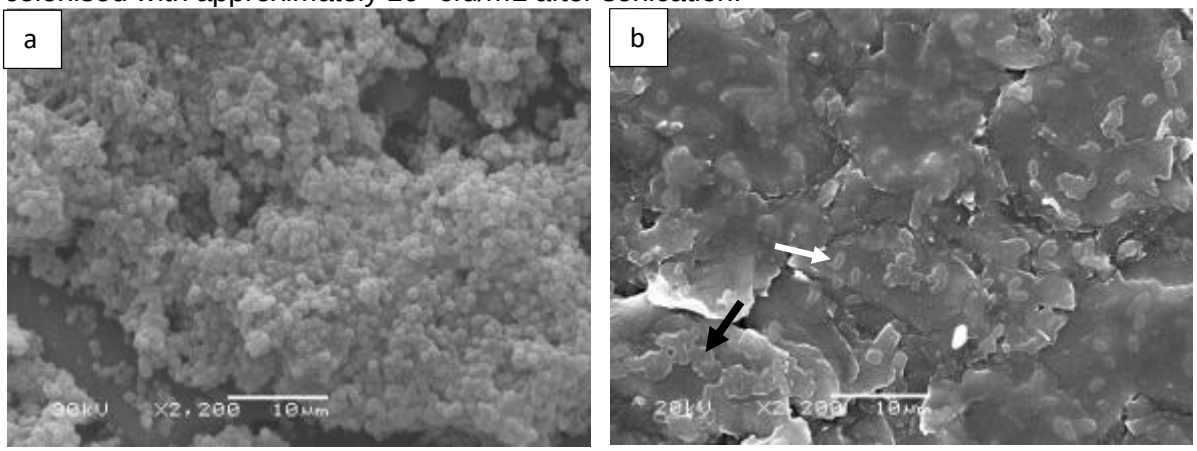
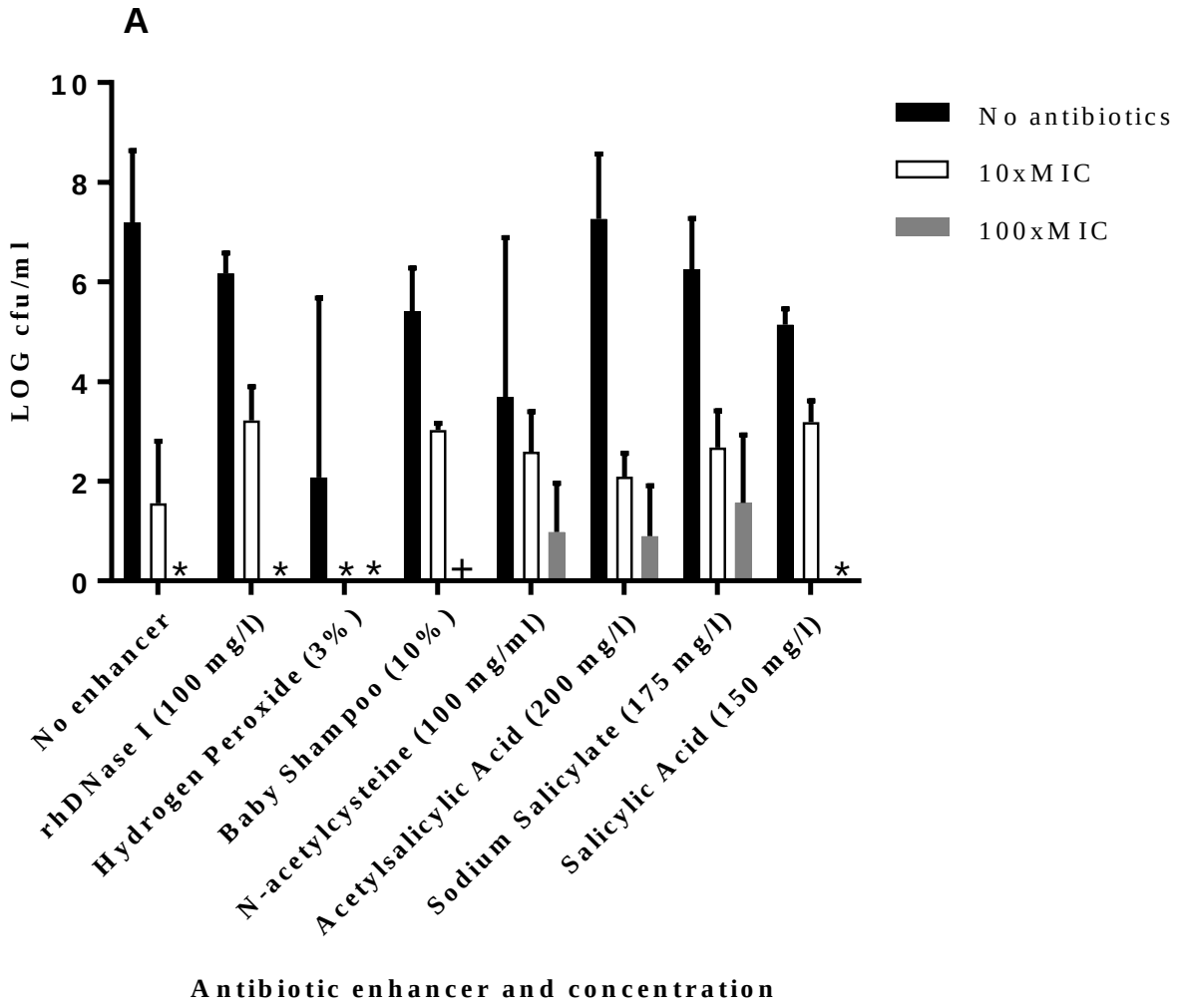


Fig 1. a. Coccus-shaped bacteria present on a silicone disc 5 days post inoculation with *S. aureus*. b. Rod-shaped bacteria (white arrow and elsewhere) and some encased by a matrix (black arrow) on a silicone disc, five-days post inoculation with *P. aeruginosa*. Both at X2200 magnification SEM.

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

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

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


A
10
8
6
4
2
0
LOG cfu/ml
No antibiotics
10xMIC
100xMIC
No enhancer
rhDNase I (100 mg/l)
Hydrogen Peroxide (3%)
Baby Shampoo (10%)
N-acetylcysteine (100 mg/ml)
Acetylsalicylic Acid (200 mg/l)
Sodium Salicylate (175 mg/l)
Salicylic Acid (150 mg/l)
Antibiotic enhancer and concentration

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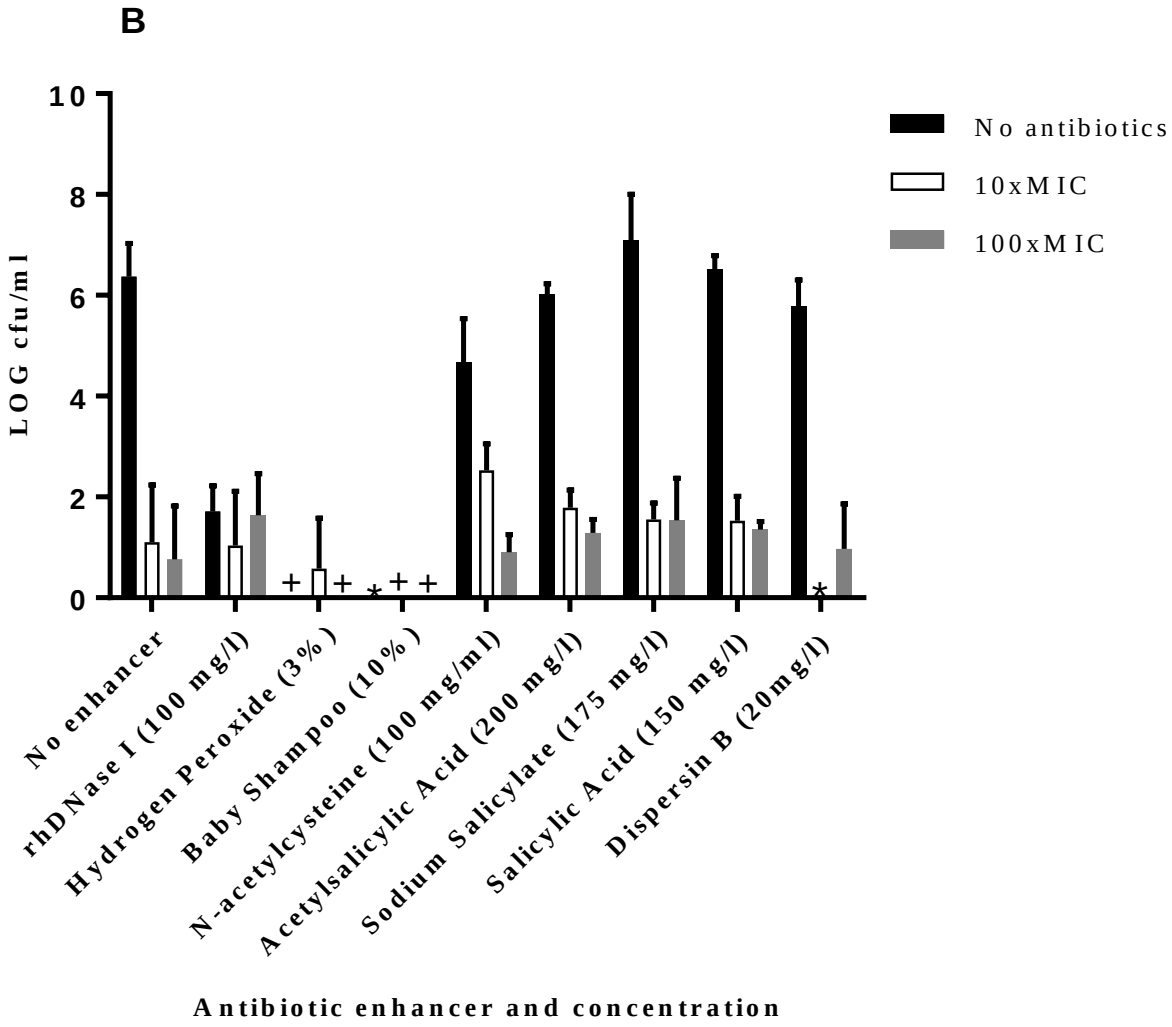


Fig 2: Log mean colony - forming units per millilitre (Log cfu/mL) and standard deviations for 5 - day old *P. aeruginosa* **(A)** and *S. aureus* **(B)** biofilms treated for 14 days with antibiotic enhancer and/or antibiotics (10x or 100xMIC antibiotics). The antibiotics against *P. aeruginosa* were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

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

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

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

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

Development of resistance

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

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

Screening of ingredients in baby shampoo for antibacterial activity

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

Ingredient	**MIC against *S. aureus***	**MIC against *P. aeruginosa***
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

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

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

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

Checkerboard assay to determine interaction of JBS ingredients with antibiotics

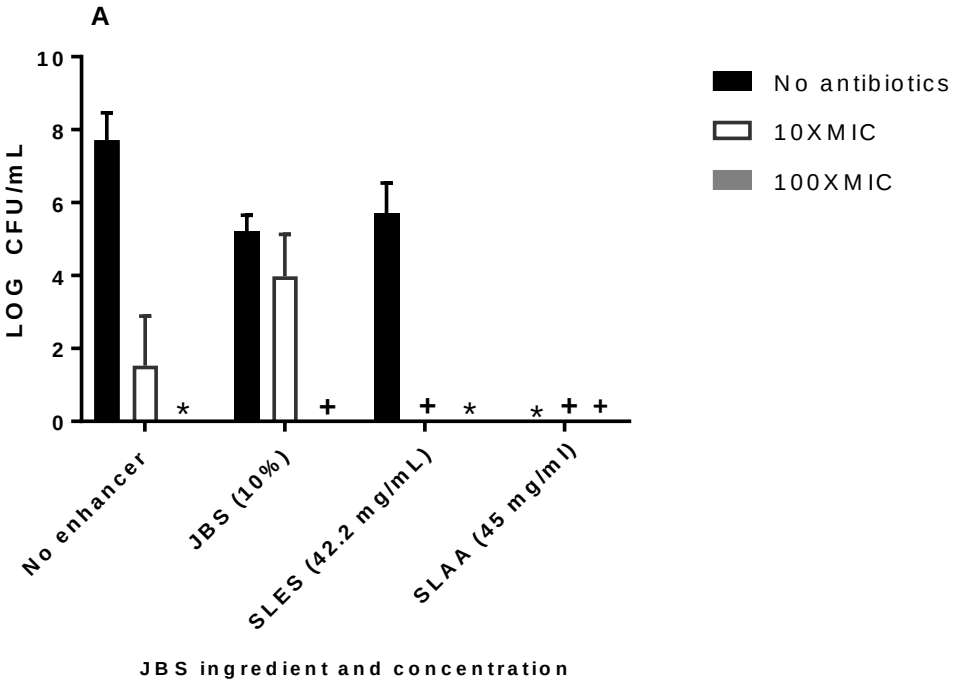
Initially the pairs of antibiotics were screened for their drug interaction, defined as the fractional inhibitory concentration index (FICI) where FICI<0.5 indicates synergy, FICI>4.0 indicated antagonism and values in between suggest no interaction.[36] The FICI between gentamicin and ciprofloxacin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

Table 3: Drug interactions of baby shampoo and its selected ingredients in a modified chequerboard assay. ∑FICI: fractional inhibitory concentration index

Ingredient in combination with antibiotics	*P. aeruginosa*		*S. aureus*	
	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

Biofilm Model

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



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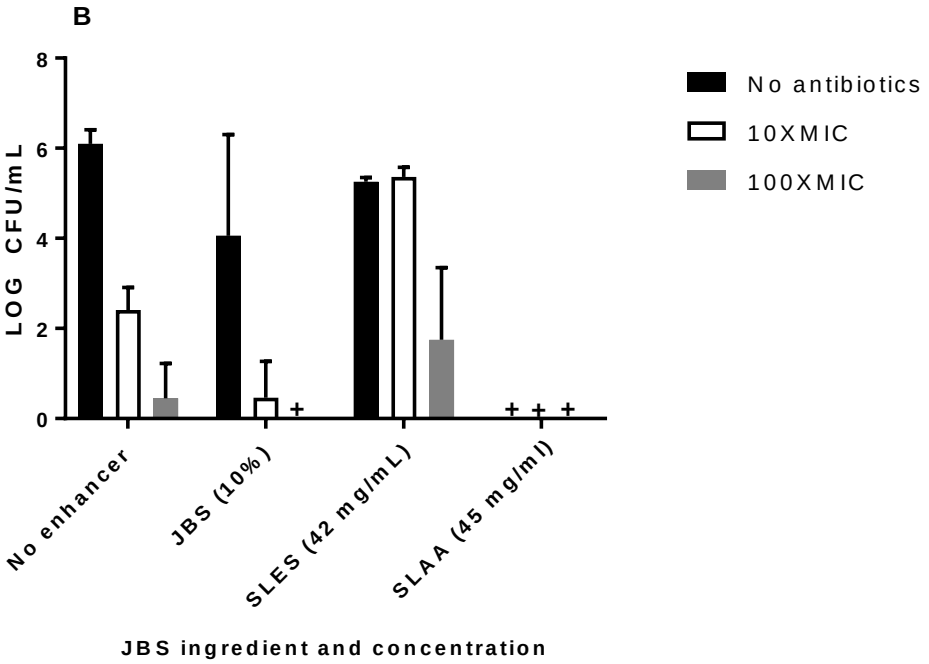

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332 **Fig 3:** Log mean colony - forming units per millilitre (Log cfu/mL) and standard deviations for five - day
333 old *P. aeruginosa* **(A)** and *S. aureus* **(B)** biofilms treated for 14 days with JBS ingredients and/or
334 antibiotics (10x 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

Discussion

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

The Biofilm Model

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

rhDNase I

rhDNAse cleaves bacterial extracellular DNA (eDNA), a component of the biofilm matrix [19]. However, it did not reduce pseudomonas biofilm when used alone. The role of eDNA varies between organisms. It is required only for initial biofilm formation of *P. aeruginosa* [39] but it plays a more important structural role in established *S. aureus* biofilms compared to *S. epidermidis* biofilms.[21] When Whitchurch *et al* treated a five-day-old pseudomonas biofilm with DNAse I it disrupted the biofilm, but not a seven-day old biofilm.[39] Our results differ from the Whitchurch *et al* study only in that our five-day old biofilm was not affected by rhDNase I alone, possibly due to strain differences as we used a clinically isolated strain and they used *P. aeruginosa* PA01, a common research strain.

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

Hydrogen Peroxide

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

Baby Shampoo

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

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

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

Conclusions and Implications for Practice

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

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