Anti-biofilm efficacy of a medieval treatment for bacterial infection requires the combination of multiple ingredients.

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Abstract

Novel antimicrobials are urgently needed to combat the increasing occurrence of drug-resistant bacteria and to overcome the inherent difficulties in treating biofilm-associated infections. Research into natural antimicrobials could provide candidates to fill the antibiotic discovery gap, and the study of plants and other natural materials used in historical infection remedies may enable further discoveries of natural products with useful antimicrobial activity. We previously reconstructed a 1,000-year-old remedy containing onion, garlic, wine, and bile salts, which is known as 'Bald's eyesalve', and showed it to have promising antibacterial activity. In this paper, we have found this remedy has bactericidal activity against a range of Gram-negative and Gram-positive wound pathogens in planktonic culture and, crucially, that this activity is maintained against *Acinetobacter baumannii, Stenotrophomonas maltophilia, Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* in a model of soft-tissue wound biofilm. While the presence of garlic in the mixture is sufficient to explain activity against planktonic cultures, garlic alone has no activity against biofilms. We have found the potent anti-biofilm activity of Bald's eyesalve cannot be attributed to a single ingredient and requires the combination of all ingredients to achieve full activity. Our work highlights the need to explore not only single compounds but also mixtures of natural products for treating biofilm infections. These results also underline the importance of working with biofilm models when exploring natural products for the anti-biofilm pipeline.

Importance

Bacteria can live in two ways, as individual planktonic cells or as a multicellular biofilm. Biofilm helps protect bacteria from antibiotics and makes them much harder to treat. Both the biofilm lifestyle and the evolution of antibiotic resistance mean we urgently need new drugs to treat infections. Here, we show that a medieval remedy made from onion, garlic, wine, and bile can kill a range of problematic

bacteria grown both planktonically and as biofilms. A single component of the remedy – allicin, derived from garlic – is sufficient to kill planktonic bacteria. However, garlic or allicin alone do nothing against bacteria when they form a biofilm. All four ingredients are needed to fully kill bacterial biofilm communities, hinting that these ingredients work together to kill the bacteria. This suggests that future discovery of antibiotics from natural products could be enhanced by studying combinations of ingredients, rather than single plants or compounds.

Introduction

Widespread multidrug resistance of once-susceptible pathogens, combined with a lack of success in developing novel antimicrobials, has resulted in a looming crisis. Antimicrobial resistance leads to problematic infections, threatens the success of routine surgery and cancer treatments (1), and is estimated to kill 10 million people per year by 2050 (2,3). One particularly troublesome area is biofilm-associated infection. Biofilm infections are estimated to cost the UK's National Health Service over a billion pounds every year, with this cost only set to increase (4,5). Biofilms are communities of bacteria that produce a protective extracellular matrix and are especially persistent (6). Biofilm eradication often requires 100-1,000 times higher antibiotic concentrations to achieve clearance than the same bacteria growing planktonically (as individual free-floating cells)(7). *In vivo*, biofilms may essentially be completely impervious to antibiotic treatment (8).

The path to overcoming biofilm infections requires a multifaceted response, including the discovery and clinical deployment of novel antimicrobials. This search for novel candidates must be directed towards those pathogenic bacteria with the highest health and economic

79 impact (e.g. ESKAPE group; (9–11)) and will be expedited if early discovery work on leads takes
80 into account the inherent difficulties of treating biofilm-associated infections (6,12).

81 The heightened urgency for novel antibiotics has resulted in natural products being revisited.
82 Just 200 years ago, our pharmacopoeia was dominated by herbal medicines. These medicines
83 have had varying levels of success for the treatment of infections in modern research, with a
84 number of potent compounds against planktonic cultures being identified (13,14); however,
85 these have been far less successful against biofilm cultures. We do not fully understand the
86 reasons for this lack of translational success of plant extracts.

87 It could be that the conventional process for developing drugs may miss key aspects of those
88 herbal remedies which could be effective against biofilms. Conventional drug development
89 calls for the isolation of single active compounds, whereas historical medicine usually calls for
90 combinations of whole plants (and other natural materials). There is some evidence that
91 whole plant extracts can have stronger biological effects than individual isolated compounds
92 (15). Examples of such synergy include observations that the anti-malarial activity of
93 artemisinin is enhanced by the presence of other compounds from the same plant (16,17) or
94 the combination of flavonoids used in Citrox® (15). Synergy may result from the presence of
95 molecules which potentiate the activity of antimicrobial compounds, or from multiple active
96 compounds with different mechanisms of action. It is also possible that in purifying individual
97 compounds to achieve readily quantifiable and characterised treatments, we may lose vital
98 interactions of natural products within the original mixture which prevent irritation or
99 toxicity.

100 Additionally, research into natural antimicrobial products is often limited by a methodological
101 focus on planktonic activity testing. The current gold standard for antibacterial testing is broth

102 microdilution, usually conducted in cation-adjusted Mueller-Hinton Broth (18), and this is
103 used extensively for the discovery of novel antibacterial compounds. However, whilst
104 providing a great high-throughput screening method, this does not provide information on
105 how the test substance interacts with bacteria in biofilms, or in settings which better mimic
106 the chemical environment experienced by pathogens *in vivo* (18,19). This focus may in part
107 explain the lack of translational success of isolated plant compounds, as their activity may be
108 sufficient to kill planktonic cells, but not to penetrate or kill biofilms – i.e. by not using biofilm
109 assays, we create a high false positive rate in the early stages of activity testing.

110 In this paper, we investigate the importance of the combination of ingredients for the
111 anti-biofilm activity of a 10th century remedy used for eye infection from the manuscript
112 known as Bald's Leechbook (London, British Library, Royal 12, D xvii). This remedy has
113 previously been shown to kill *S. aureus* biofilms by Harrison *et al.* (2015) (20), *Pseudomonas*
114 *aeruginosa* planktonic cultures (21) and recently *Neisseria gonorrhoeae* in a disk diffusion
115 assay (22). The recipe, known as 'Bald's eyesalve', requires equal volumes of garlic (*Allium*
116 *sativum*) and another *Allium* species (referred to as *cropleac* in the original Old English) to be
117 crushed together and mixed with equal volumes of wine and ox gall (bovine bile). We now
118 report two key new findings. First, we show the bactericidal activity of Bald's eyesalve against
119 a panel of clinically relevant strains of ESKAPE pathogens in planktonic culture, and against
120 biofilms of a subset of these strains. Second, we conducted a detailed assessment of whether
121 (a) any individual ingredient or (b) the sulphur-containing compound allicin (from garlic) can
122 explain this activity. No single ingredient recaptures the anti-biofilm activity of Bald's
123 eyesalve, and the presence of allicin cannot fully explain the ability of the whole mixture to
124 kill either planktonic cultures grown in host-mimicking medium (synthetic wound fluid), or

125 biofilms grown in an *in vivo*-like model of a soft tissue wound. The combination of ingredients
126 in the full mixture is the key to the remedy's apparent efficacy, and this is only apparent when
127 it is tested in host-mimicking models as opposed to planktonic cultures in standard laboratory
128 medium.

129

130 **Materials & Methods**

131 **Bacterial strains and culture conditions**

132 Strains used are listed in Table 1. All cultures were grown aerobically at 37°C and Miller's LB
133 agar (Melford) was used for plating.

134 **Table 1.** Bacterial strains used in the study

Species	Strain	Notes
Gram-positive		
Staphylococcus aureus	Newman	Standard laboratory strain, used in initial activity testing of all fresh eyesalve batches
Staphylococcus aureus	USA300 Los Angeles County clone	Well-studied example of community-acquired MRSA
Staphylococcus aureus	6 human carriage isolates	1 MRSA + 5 MSSA, gifted by Ruth Massey
Staphylococcus aureus	6 clones isolated from a chronic post-surgical wound	MSSA, with thanks to Tim Sloan
Staphylococcus epidermidis	1457	Standard laboratory strain, with thanks to Kim Hardie
Streptococcus pyogenes	Clinical isolate	Nottingham University Hospitals biobank, strain ref. 15W905066
Gram-negative		
Acinetobacter baumannii	Clinical isolate	Nottingham University Hospitals biobank, strain ref. 12W125768

Enterobacter cloacae	Clinical isolate	Nottingham University Hospitals biobank, training isolate
Escherichia coli	K12	Standard laboratory strain
Pseudomonas aeruginosa	PA14	Standard laboratory strain
Stenotrophomonas maltophilia	Clinical isolate	Nottingham University Hospitals biobank, training isolate

135

136 **Media**

137 Synthetic wound fluid (SWF) comprised equal volumes of foetal bovine serum (Gibco) and
138 peptone water (Sigma-Aldrich), (23). Mueller-Hinton Broth (MHB) was obtained from VWR.
139 HyClone water (GE Healthcare) was used throughout.

140 **Bald's Eyesalve Reconstruction**

141 Bald's eyesalve was prepared as previously reported (20). The outer skin of the garlic and
142 onion (sourced from local greengrocers) was removed. The garlic and onion were finely
143 chopped, and equal volumes of garlic and onion were crushed together using a mortar and
144 pestle for 2 minutes. Various sized batches of Bald's eyesalve were used throughout this
145 paper, ranging from final volumes of 30 ml – 400 ml, the average weight used was 14.1 ± 1.5
146 g of onion and 15.0 ± 1.3 g of garlic per 100 ml of Bald's eyesalve.

147 The crushed onion and garlic were then combined with equal volumes of wine (Pennard's
148 organic dry white, 11% ABV, sourced from Avalon Vineyard, Shepton Mallet) and bovine bile
149 salts (Sigma Aldrich) made up to 89 $mg \cdot ml^{-1}$ in water and sterilised by exposing to UV radiation
150 for 10 minutes (Carlton Germicidal Cabinet fitted with a 2537Å, 8-Watt UV tube). The mixture
151 was stored in sterilised glass bottles in the dark at 4°C for 9 days, after which it was strained
152 and centrifuged for 5 minutes at 1,811 g. The supernatant was then filtered using Whatman™

153 1001-110 Grade 1 Qualitative Filter Paper, Diameter: 11 cm, Pore Size: 11 µm. Filtered Bald's
154 eyesalve was stored in sterilised glass vials in the dark at 4°C.

155 For ease of reading, batches are numbered as they appear within the paper. The key for their
156 batch name is provided in the Data Supplement.

157 **Preparation of Individual Ingredients and Dropouts**

158 Individual ingredients were prepared such that their concentrations were equal to
159 concentrations present in the full remedy, a schematic is provided in Figure S1. To achieve
160 comparable concentrations, each ingredient was prepared as it would be for the full remedy
161 (onion and garlic crushed separately from each other), with all other ingredients substituted
162 with the same volume of water. A similar process was followed for "dropout" batches, where
163 one ingredient at a time was systematically excluded from the remedy and replaced with an
164 equal volume of water to maintain comparable concentrations to the original remedy.

165 **Planktonic killing assay**

166 Bacteria were cultured aerobically at 37°C on LB agar for 18-24 hours. Several colonies were
167 then inoculated into 5 ml SWF and incubated for 6 hours at 37°C on an orbital shaker. Aliquots
168 of each bacterial culture (100 µl) were added to wells of Corning® Costar® TC-Treated 96-Well
169 Plate and 50 µL of the eyesalve batch to be tested was added to 5 wells per strain. 50 µL of
170 water was used as a negative control. Plates were incubated in Tecan SPARK 10M at 37°C for
171 18 hours with periodic orbital shaking (10 seconds at 20 min intervals). Serial dilutions were
172 then performed and plated on LB agar plates.

173 **Minimum Inhibitory Concentration Assay by Broth Microdilution**

174 The minimum inhibitory concentrations (MIC) of eyesalve, single ingredients, dropout batches
175 and allicin were determined as described by Wiegand, Hilpert and Hancock (2008) in MHB
176 and in SWF. Treatments, or water as a negative control, were serially diluted with media in
177 Corning® Costar® TC-Treated 96-Well Plates, following the scheme in Table 2. Bacterial
178 isolates were streaked onto LB agar to obtain single colonies. After 18 - 24 hours incubation
179 at 37°C, three to five morphologically similar colonies were transferred to phosphate-
180 buffered saline (PBS) and diluted to 0.5 McFarland standard (OD_{600} 0.08-0.1). This suspension
181 was diluted 1 in 100 in media, resulting in 5 x 10^5 CFU/ml. The bacterial suspension was then
182 added to the treatment dilutions, resulting in another twofold dilution. The highest final
183 concentration of eyesalve, single ingredient or dropout batch tested was 50%. Plates were
184 incubated for 18 hours at 37°C. Results were visually checked for turbidity, and MIC values
185 are the lowest concentration where growth was not visible.

186 **Table 2**. Final volumes and calculated percentages of Bald's eyesalve in wells of 96-well plates
187 used for MIC.

Well	Bald's eyesalve (µl)	Media (µl)	Bacterial suspension (µl)	Final volume (µl)	% ESO in well
A	50	0	50	100	50
B	33	17	50	100	33
C	25	25	50	100	25
D	12.5	37.5	50	100	12.5
E	6.25	43.75	50	100	6.25
F	3.13	46.87	50	100	3.13
G	1.56	48.44	50	100	1.56
H	0.78	49.22	50	100	0.78

188

189 **Biofilm Killing Assay in Synthetic Wounds**

190 Biofilms were created in a synthetic soft-tissue wound model as described by Werthén *et al.*
191 (2010; (23)) and as used in our previous work (20). Briefly, synthetic wounds were created on
192 ice and comprised 2 mg·ml^{-1} collagen, 0.01% acetic acid, 60% [vol/vol] SWF, 10 mM sodium
193 hydroxide. Synthetic wounds were incubated at 37°C for 1 hour to allow collagen to
194 polymerise and placed under UV light for 10 minutes to ensure no contamination. Wounds
195 were either 400 µl in 24-well culture plates or 200 µl in 48-well plates.

196 Bacteria were incubated aerobically in SWF on an orbital shaker for 6 hours at 37°C, after
197 which cultures were diluted to OD_{600} of 0.1 - 0.2 with fresh SWF. Bacteria suspension was
198 added to each synthetic wound (100 µl in 400 µl wounds or 50 µl in 200 µl wounds) and
199 incubated at 37°C for 24 hours to allow biofilm formation.

200 Wounds containing mature biofilms were then exposed to Bald's eyesalve or water (200 µl in
201 400 µl wounds or 100 µl in 200 µl wounds). Bacteria were recovered by treating with 300 µl
202 (400 µl wounds) or 150 µl (200 µl wounds) of 0.5 mg·ml^{-1} collagenase type 1 (EMD Millipore
203 Corp, USA) for 1 hour at 37°C to break down the matrix; the resulting liquid was serially diluted
204 and plated on LB plates. Plates were incubated at 37°C overnight, colonies were counted, and
205 CFU/wound calculated.

206 **High-Performance Liquid Chromatography Quantification of Allicin**

207 Allicin is an unstable and reactive compound under gas chromatography settings (24),
208 therefore, high-performance liquid chromatography (HPLC) was used to determine allicin
209 concentrations (24).

210 Various preparations of Bald's eyesalve were analysed by reversed-phase HPLC on an Agilent
211 1200 series system fitted with an Agilent ZORBAX Eclipse XDB-C18, 150 x 4.6 mm, 5 µm
212 particle size column and diode array detection at 210 nm. A gradient of methanol (5- 95%) in
213 water was used at a flow rate of 1 $ml \cdot min^{-1}$ over 30 minutes. Injection volume was 10 µl, and
214 the column temperature was 25°C.

215 To identify the concentration of allicin in eyesalve preparations, a calibration curve was
216 prepared by serially diluting an external allicin standard (Abcam, Cambridge) in water
217 (twofold dilutions, from 750 $\mu g \cdot ml^{-1}$ to 0.5 $\mu g \cdot ml^{-1}$). External standards were run through HPLC
218 as described above (n = 2) and allicin peak area, in the 210 nm reading, was plotted against
219 concentration ($\mu g \cdot ml^{-1}$) (Figure S2A and Data Supplement). Allicin external standards had a
220 retention time of approximately 15 minutes (Figure S2B). This peak was confirmed in Bald's
221 eyesalve by comparing fresh eyesalve to the same batch spiked with additional allicin
222 standard. The suspected peak increased as expected (Figure S2C-D).

223 **Statistics**

224 Data were analysed in R v3.5.1 (R Core Team, 2018) using the *car* (25), *lsmeans* (26), *multcomp*
225 (27) and *FSA* (28) packages. Raw data and R code for all experiments are provided in the Data
226 Supplement.

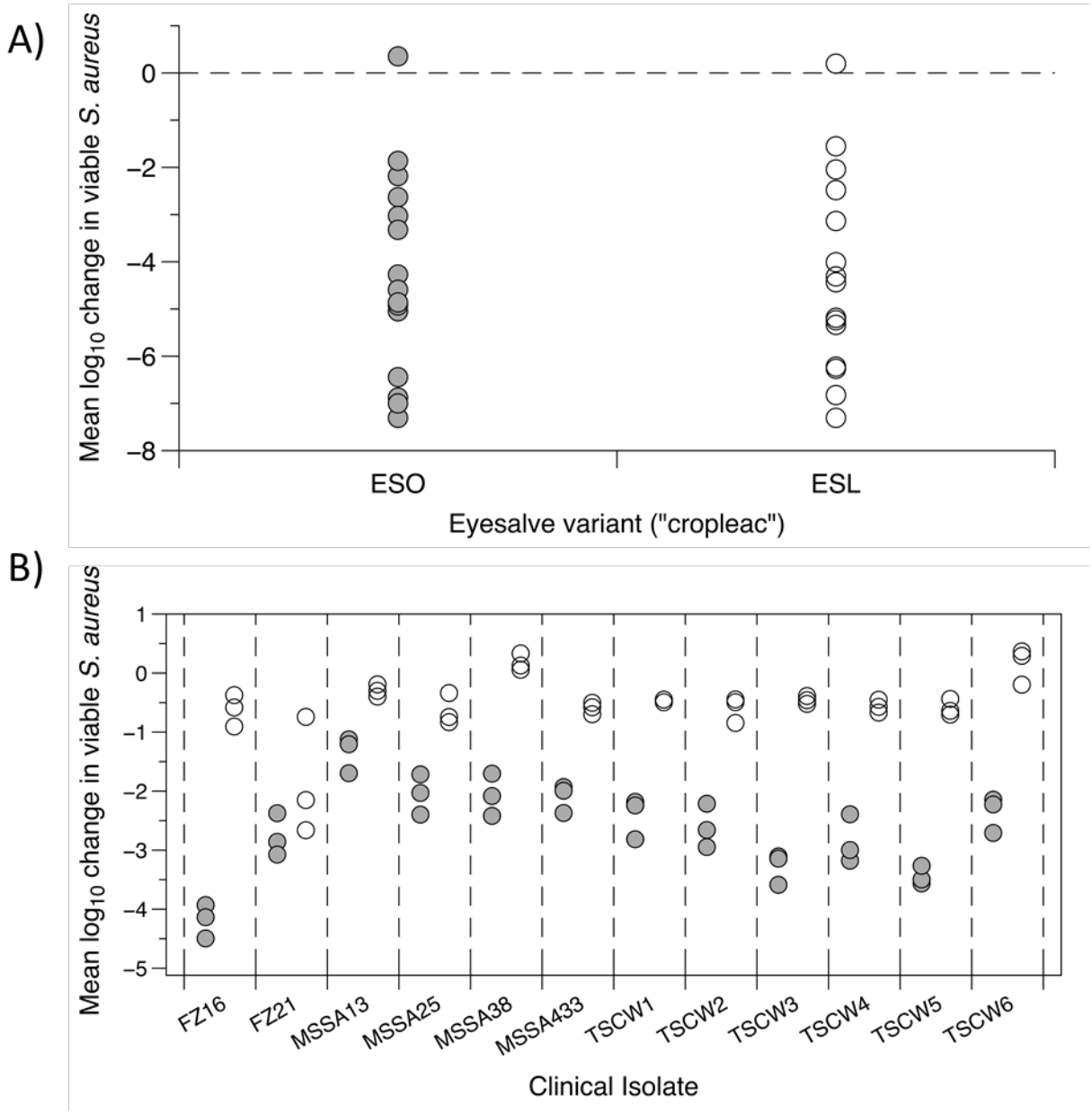
227 **Results**

228 ***Consistent anti-biofilm activity of Bald's eyesalve and decision to use onion (*Allium cepa*) as***
229 ***"cropleac"***

230 The meaning of Old English *cropleac,* as used in the original remedy, is ambiguous and may
231 refer to a variety of *Allium* species (29,30). Two likely translations are onion (*Allium cepa*) or
232 leek (*Allium porrum*). For this reason, multiple batches of the remedy using either *Allium*
233 species were made in our laboratory between 2014 and 2019. After the nine-day brewing
234 period, the activity of the remedies was tested against mature biofilms of *S. aureus* Newman
235 in an *in vivo*-like model of a soft tissue wound. In this assay, 24-hour-old biofilms, that have
236 been grown at 37°C in collagen-based synthetic wounds, are exposed to Bald's eyesalve or
237 water for 24 hours, and surviving bacteria are counted (20,23).

238 In total, 75 batches of Bald's eyesalve were made, including 15 pairs of batches where onion
239 (ESO) and leek (ESL) variants were made at the same time, using the same garlic, wine and
240 bile. As shown in Figure 1A, biofilm killing was achieved by 14/15 paired batches, with 22/30
241 preparations causing a >3-log drop in viable bacteria, when compared with water-treated
242 control biofilms. The mean log drop was not significantly different between paired ESO and
243 ESL batches (paired *t*-test t_{14}= 0.025, p = 0.981). We tested one example pair of ESO and ESL
244 variants (batch 6) against a panel of twelve clinical isolates of *S. aureus* grown in the synthetic
245 wound biofilm model. As shown in Figure 1B, ESO consistently showed more bactericidal
246 activity than ESL. This result may be due to the presence of antimicrobial compounds unique
247 to onion, or it may simply be due to onions being easier to crush in a mortar and pestle,
248 potentially making the extraction of natural compounds more efficient. To simplify further
249 analysis, follow on work was focussed on ESO.

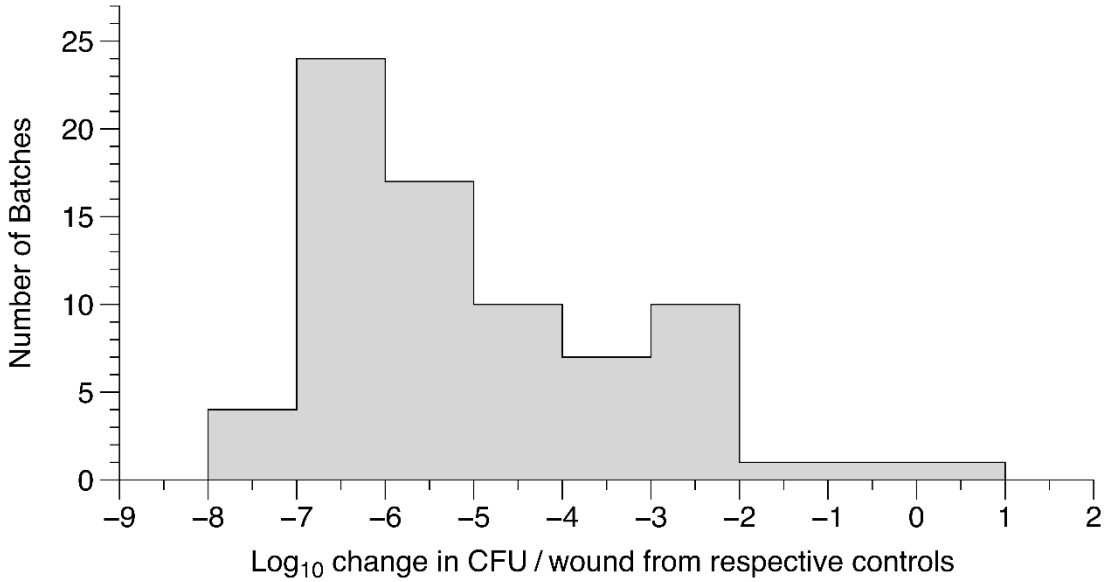
250 As shown in Figure 2, biofilm killing was consistently achieved by multiple batches of ESO. Of
251 these batches, 62 of the 75 caused a >3-log drop in viable CFU, with 45/75 causing >5 log
252 drop, and 28 of these causing > 6-log drop, all relative to control biofilms treated with sterile
253 water.



254

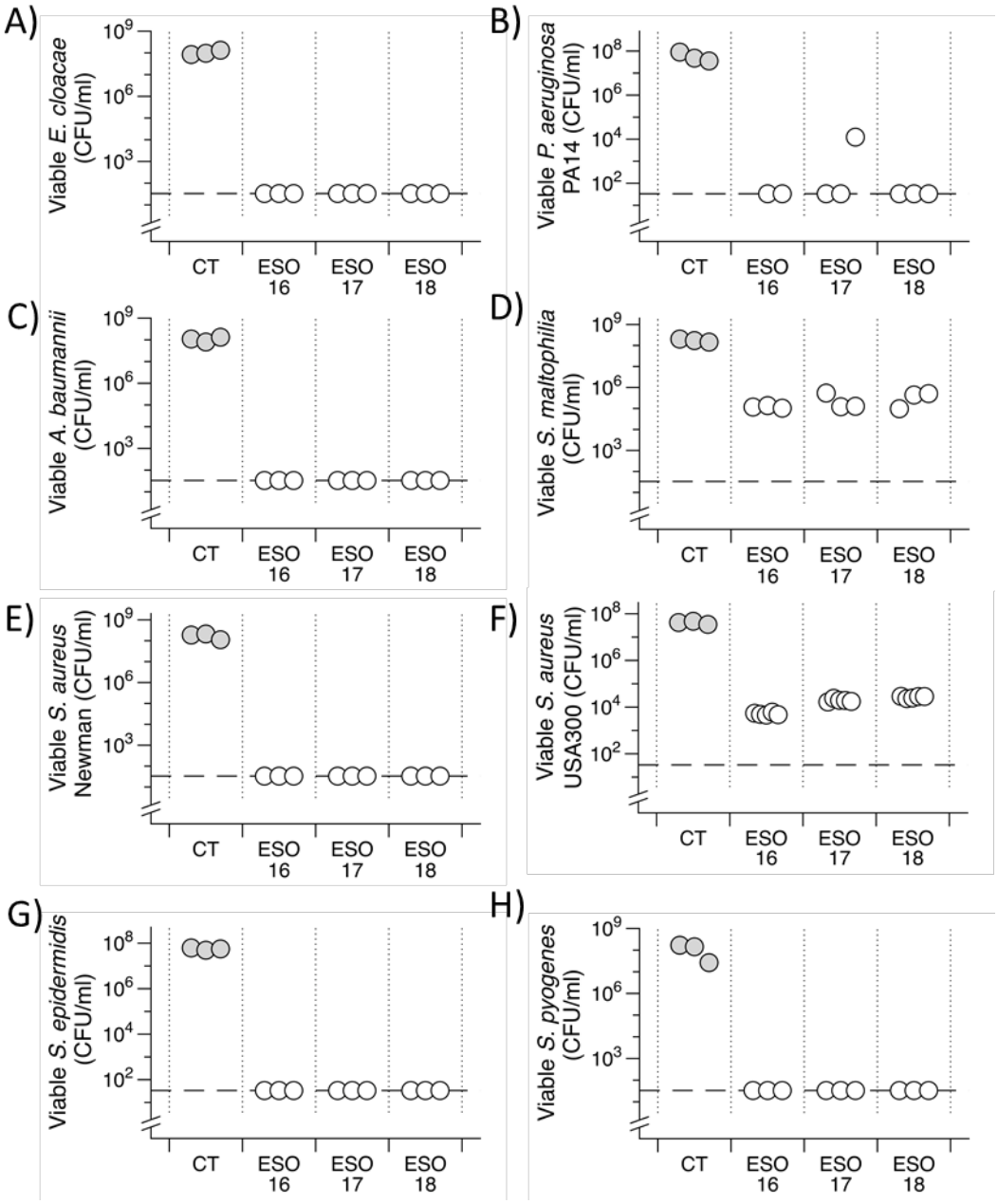
255 **Figure 1**. **The activity of Bald's eyesalve, ESO and ESL variants, against *S. aureus*.** (A) 15 pairs of Bald's
256 eyesalve translating *cropleac* as either onion (ESO, grey) and leek (ESL, white), were prepared at the
257 same time (batches 1-15). Their activity was assessed against *S. aureus* Newman biofilms. Mature

biofilms of *S. aureus* Newman were grown in a model of a soft tissue wound, then treated with either sterile water or Bald's eyesalve (n = 3-5 replicates per treatment) for 24 hours before recovering bacteria for CFU counts. The mean log change in viable bacteria in treated vs control wounds was calculated for each preparation and no significant difference was seen (paired *t*-test t_{14}= 0.025, p = 0.981). Raw data are supplied in the Data Supplement. (B) Six carriage isolates of *S. aureus*, and six isolates from a chronic post-surgical wound (detailed in Table 1), were grown in the synthetic wound biofilm model and treated with ESO, ESL or water (n=3 per treatment). Each strain grew to different densities in untreated wounds (range approx. 10^6–10^8). The $\log_{10}$ drop in bacteria associated with treatment was calculated for each treated wound relative to the mean CFU in the three untreated wounds. Log drop data was analysed by ANOVA which revealed significant differences in log drop between eyesalve variants ($F_{1,48}$ = 732, $p < 0.001$) and strains ($F_{11,48}$ = 14.4, $p < 0.001$) and a significant strain-dependent difference in the magnitude of the effect of eyesalve variant (strain*variant interaction $F_{11,48}$ = 8.66, $p < 0.001$). The family-wise error rate was calculated from the ANOVA table and used to conduct planned contrasts of ESO vs ESL treatment effects for each strain using *t*-tests. ESO caused a larger reduction in viable cells than ESL for all strains (all $p < 0.001$). Raw data and R scripts are supplied in the Data Supplement.



276 **Figure 2. The activity of 75 Bald's eyesalve (ESO) batches made by our group against *S. aureus* Newman**
277 **biofilms.** Mature *S. aureus* Newman biofilms were grown in a model of a soft tissue wound, then treated with
278 either sterile water (control) or eyesalve (n = 3-5 replicates per treatment) for 24h before recovering bacteria
279 for CFU counts. All batches showed killing, and the log drop in treated vs control wounds was calculated. Raw
280 data are provided in the Data Supplement.
281
282 ***Broad-spectrum antibacterial activity of Bald's eyesalve against common wound pathogens***

283 The antibacterial activity of three batches of ESO was tested in both planktonic and biofilm
284 cultures of eight strains of bacteria that commonly cause chronic wound infections. As shown
285 in Figure 3, ESO had potent activity against planktonic cultures of Gram-negative
286 (*P. aeruginosa* PA14, *A. baumannii* clinical isolate, *E. cloacae, S. maltophilia*) and Gram-
287 positive (*S. aureus* Newman, *S. aureus* USA300, *S. epidermidis* and *S. pyogenes*) wound
288 pathogens. ESO eradicated all planktonic cultures in all strains tested with the exception of
289 *S. aureus* USA300 and *S. maltophilia*, where a 3-4 log drop in viable bacteria was seen.



290

291

292 **Figure 3**. **Bald's eyesalve (ESO) activity against eight example strains of bacteria that commonly cause chronic**

293 **wound infections.** Planktonic cultures were grown for 6 hours in synthetic wound fluid. Cultures were treated

294 with ESO (batches 16-18) or sterile water (CT) to a final concentration of 33% v/v (n = 3-5 replicates per

295 treatment). After 18 hours bacteria were recovered for CFU counts. The dashed line represents the limit of

296 detection by plating. For *S. maltophila* and *S. aureus* USA300, where we did not observe complete killing, we

297 used ANOVA to determine that the CFU recovered from ESO-treated wounds was significantly different from

298 control wounds (*S. aureus* USA300: $F_{2,14} = 3458$, $p < 0.001$; *S. maltophilia*: $F_{3,8} = 87.41$, $p < 0.001$). Data were log

transformed to meet the assumptions of linear modelling. A Dunnett's test was conducted to compare the CFU of the three batches with the CFU of the controls. All 3 batches of ESO were significantly different from their respective controls for both isolates (all $p < 0.001$). Raw data and R scripts are supplied in the Data Supplement.

Mature biofilms of the above isolates were grown in the synthetic wound model and treated with the same batches of eyesalve used for planktonic killing experiments. A 2-6-log drop in viable cells was observed for the Gram-positives *S. aureus* Newman, *S. aureus* USA300*, S. epidermidis, S. pyogenes* and the Gram-negative *A. baumannii* (Figure 4). No or inconsistent killing was observed for *P. aeruginosa*, *E. cloacae* and *S. maltophilia* biofilms (Figure S3).

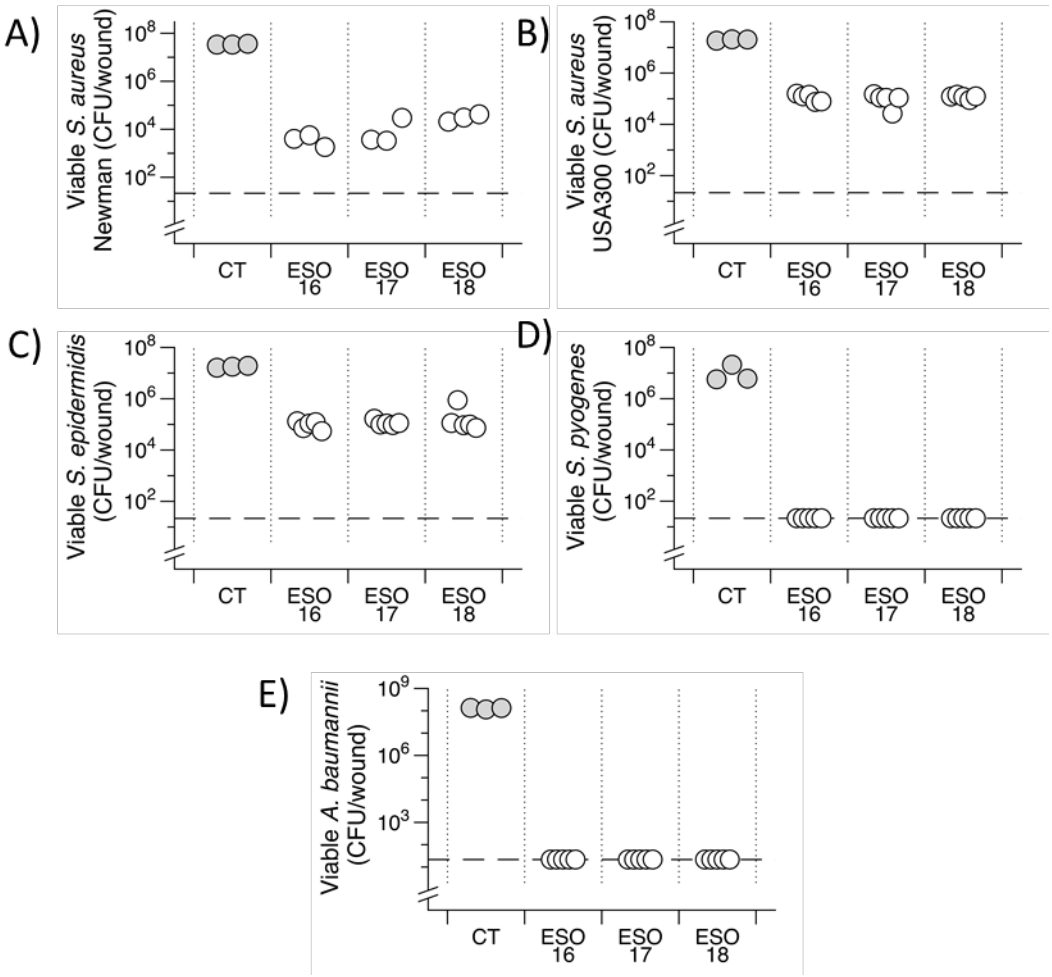


Figure 4. Bald's eyesalve (ESO) anti-biofilm activity. Mature biofilms of various isolates were grown in a model of a soft tissue wound, then treated with either sterile water (control, CT) or eyesalve (batches 16-18), to a final

312 concentration of 33% (v/v), for 24h before recovering bacteria for CFU counts (n = 3-5 replicates per treatment).
313 The dashed line represents the limit of detection by plating. For *S. aureus* Newman, *S. aureus* USA300, and *S.*
314 *epidermidis*, where we did not observe complete killing, we used ANOVA to determine that the CFU recovered
315 from ESO-treated wounds was significantly different from control wounds (*S. aureus* Newman: $F_{3,8} = 107$, $p <$
316 0.001; *S. aureus* USA300: $F_{3,14} = 103.5$, $p < 0.001$; *S. epidermidis*: $F_{3,14} = 63.86$, $p < 0.001$). Data were log
317 transformed to meet the assumptions of linear modelling. A Dunnett's test was conducted to compare the CFU
318 of the three batches with the CFU of the controls. All 3 batches of ESO were significantly different from their
319 respective controls for all three isolates (all $p < 0.001$). Raw data and R scripts are supplied in the Data
320 Supplement.
321
322 ***Garlic is responsible for the majority of planktonic killing by ESO***
323 Our initial work indicated that all four ingredients in ESO were required to kill biofilms of *S.*
324 *aureus* Newman in synthetic wound biofilms (20). A recent publication by Fuchs *et al.* (2018;
325 (21)) concluded that the bactericidal activity of ESO was due to the presence of allicin from
326 garlic, however, the Fuchs *et al.* study only investigated planktonic killing in standard Mueller-
327 Hinton Broth (MHB). It is well known that planktonic cultures of bacteria can be up to 1,000
328 times more sensitive to antibiotics than the same isolates grown as biofilms (7), and that
329 antibiotic sensitivity is highly dependent on growth medium (31). This means conducting
330 assays only on planktonic MHB cultures risks underestimating the number or concentration
331 of bioactive agents in ESO required for killing in *in vivo*-like conditions (18,19).
332
333 To more robustly determine if the activity of ESO stems from one ingredient or several, we
334 prepared individual ingredients and preparations omitting one ingredient, such that the
335 concentrations of each ingredient in the whole remedy and the single ingredient or dropout
336 variants were equal. The MICs of these preparations, along with MICs of the full recipe, were
337 assessed in MHB and in SWF, in standard broth microdilution assays using four of the isolates

338 previously tested (two Gram-negatives, *A. baumannii* clinical isolate strain and *P. aeruginosa*
339 PA14, and two Gram-positives, *S. aureus* Newman and *S. aureus* USA300).
340
341 As shown in Table 3, the bacterial isolates varied in their sensitivity to the full ESO. Crucially,
342 there was an effect of the growth medium, such that MICs at least doubled in SWF compared
343 with MHB for *S. aureus* Newman and *A. baumannii*. Wine, bile or onion alone were much less
344 effective than the full remedy. In MHB, the MIC of garlic alone was the same as the ESO MIC
345 for all strains except *P. aeruginosa*, and in SWF the garlic and ESO MICs were either equal or
346 exhibited a max. 2-fold difference. This suggests that the planktonic activity of the remedy is
347 due to the presence of garlic. The results of MIC testing with ingredients omitted gave similar
348 results (Table 4). Preparations omitting garlic lost all or most of their antibacterial activity.
349 Removal of any other ingredient left activity largely unaffected, although the omission of wine
350 or bile doubled the MIC against *P. aeruginosa* PA14 in SWF. The discordance between MICs
351 in different media is more prominent here, with the removal of an ingredient having a minimal
352 effect in MHB but a large loss in activity when tested in SWF. This stresses the common
353 discordance between testing in standard rich lab medium and host-mimicking medium.

354 **Table 3**. **Minimum inhibitory concentration (MIC) of ESO or individual ingredients.** MICs were tested in both
355 Mueller-Hinton Broth (MHB) or synthetic wound fluid (SWF). MICs are presented as modal values of 3 different
356 batches (ESO 19-21; with 3 replicate MIC tests per batch) and are the percentage of treatment present at MIC
357 (v/v).

Treatment	***S. aureus* Newman**		***S. aureus* USA300**		***A. baumannii* clinical isolate**		***P. aeruginosa* PA14**	
	MHB	SWF	MHB	SWF	MHB	SWF	MHB	SWF
ESO	1.56	6.25	3.13	3.13	1.56	3.13	25	25
Onion	>50	>50	>50	>50	>50	>50	>50	>50

Garlic	1.56	12.5	3.13	3.13	1.56	6.25	33	33
Wine	>50	>50	>50	>50	>50	>50	>50	>50
Bile	>50	>50	>50	>50	>50	>50	>50	>50

358

359

360 **Table 4**. **Minimum inhibitory concentration (MIC) of ESO or preparations omitting a single ingredient.** MICs
361 were tested in both Mueller-Hinton Broth (MHB) and synthetic wound fluid (SWF). MICs are presented as modal
362 values of 3 different batches (ESO 22-24; with 3 replicate MIC tests per batch) and are the percentage of
363 treatment present at MIC (v/v).

	Treatment	***S. aureus* Newman**		***S. aureus* USA300**		***A. baumannii* clinical isolate**		***P. aeruginosa* PA14**	
		MHB	SWF	MHB	SWF	MHB	SWF	MHB	SWF
	ESO	<0.78	12.5	3.13	12.5	1.56	6.25	25	25
Omitting	Onion	<0.78	12.5	3.13	12.5	3.13	6.25	25	25
Omitting	Garlic	50	50	50	50	50	>50	>50	>50
Omitting	Wine	<0.78	6.25	3.13	12.5	1.56	6.25	25	50
Omitting	Bile	<0.78	6.25	3.13	12.5	1.56	6.25	25	50

364

365 ***All four ingredients are necessary for activity against mature* S. aureus *biofilms in synthetic wounds***

366 Consistent with previous results from our group, no single ingredient alone had any
367 bactericidal activity against mature *S. aureus* Newman biofilms in the synthetic wound model,
368 and removing any single ingredient resulted in a reduction in the anti-biofilm activity (Figure
369 5). Surprisingly, removal of wine caused a decrease in activity on par with that seen with the
370 removal of garlic, despite wine possessing very limited antimicrobial activity on its own in
371 either biofilm or planktonic assays (Table 3). For comparison with planktonic MIC data, the
372 final concentration of Bald's eyesalve in synthetic wound biofilm killing assays (Figures 1-6),
373 is 33% vol/vol. Therefore, in more *in vivo*-like biofilm conditions, combining all the ingredients
374 is necessary for full activity against *S. aureus* Newman.

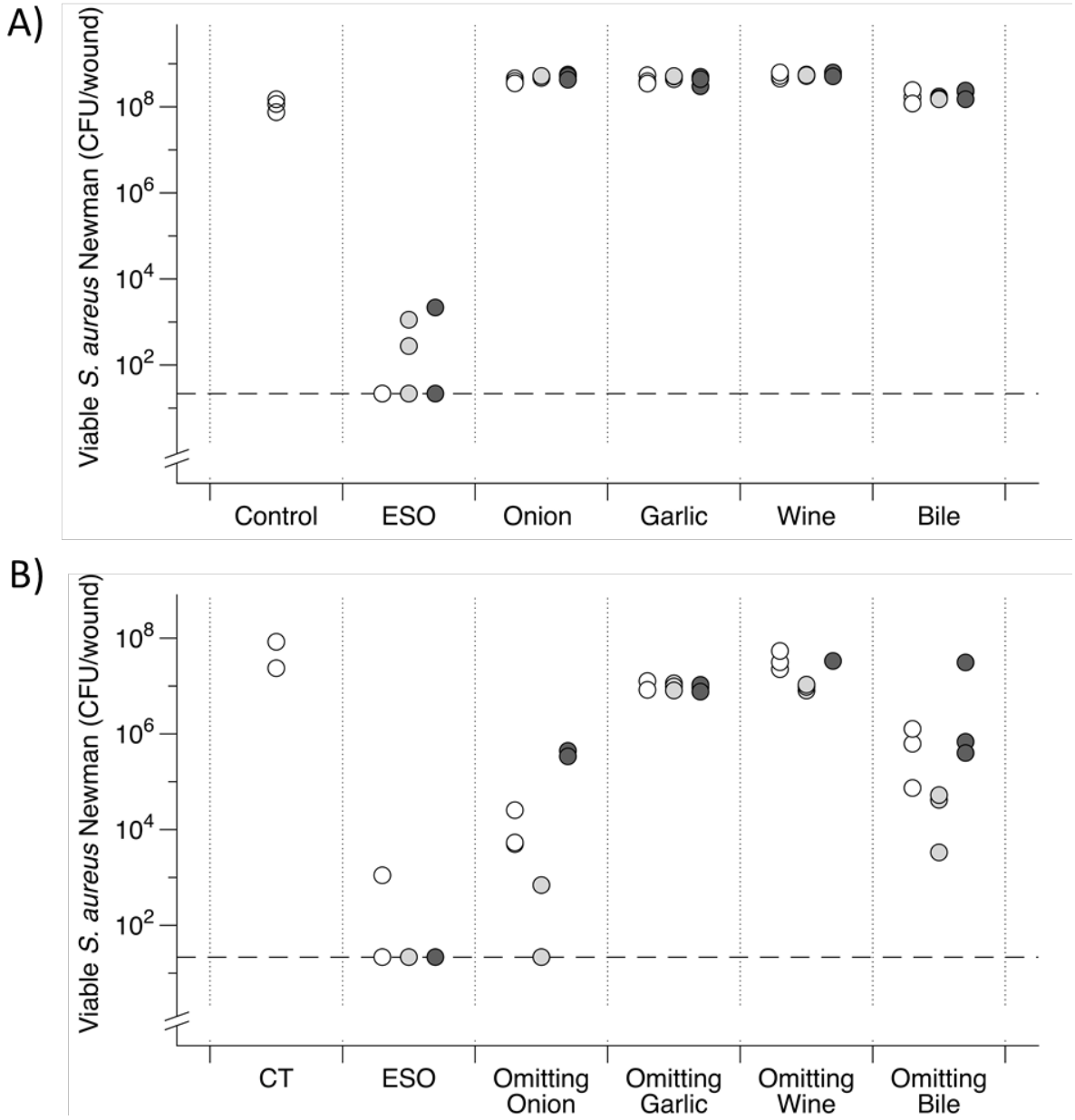


Figure 5. **Contribution of each ingredient to Bald's eyesalve (ESO) anti-biofilm activity.** Mature biofilms of *S. aureus* Newman were grown in a model of a soft tissue wound, then treated with either water (CT) or treatment for 24 hours, before recovering bacteria for CFU counts. The dashed lines represent the limit of detection by plating. A) ESO and individual ingredients prepared such that the concentrations of each ingredient in the whole remedy and individual preparations are equal (n = 3 replicates per treatment). Three independent batches of preparations are shown, corresponding to batches ESO 19, 20 and 21, shown in white, light grey and dark grey, respectively. Analysis of the data by ANOVA showed a significant effects of treatment ($F_{4,30} = 389.215$, $p < 0.001$) but no effect of batch ($F_{2,30} = 1.24$, $p = 0.305$) and no interaction between treatment and batch (i.e. the effect of

384 treatment did not depend on batch: $F_{8,30}$ = 0.930, p = 0.507). Data were square root transformed to meet the
385 assumptions of linear modelling. To compare each treatment group to the untreated controls, we then fitted a
386 new ANOVA to fit least-squares means and variances for viable CFU recovered after each treatment (excluding
387 controls), averaged over the three batches. These fitted means were then compared to the mean observed in
388 the untreated controls using a two-tailed, unpaired Welch's *t*-test, which takes into account the unequal sample
389 sizes and variances in control and treatment groups. The decrease in CFU on treatment with full ESO was
390 significant ($t_{2.93}$ = 9.18, p = 0.003). Garlic, onion and wine caused small but significant increases in viable CFU ($t_{2.93}$
391 = 9.05, p = 0.003, $t_{2.93}$ = 9.61, p = 0.003, $t_{2.93}$ = 11.1, p = 0.002), while bile had no significant effect on CFU ($t_{2.93}$ =
392 2.44, p = 0.094). B) ESO and batches with one ingredient omitted and replaced with water, to keep the
393 concentrations of remaining ingredients equal to those in the full recipe. Three independent batches of
394 preparations are shown, corresponding to ESO batches 22, 23 and 24, shown in white, light grey and dark grey,
395 respectively. ANOVA showed significant differences in CFU between treatments ($F_{4,26}$ = 162, p <0.001) and
396 between batches ($F_{2,26}$ = 18.7, p <0.001), and a significant interaction between treatment and batch ($F_{8,26}$ = 5.85,
397 p <0.001); the graph shows that this interaction is due to different batches showing larger or smaller differences
398 between the killing effects of the preparations, but that the rank order of preparations by CFU is the same in
399 each batch. Data were log-transformed to meet the assumptions of linear modelling. As only two control data
400 points were obtained for this experiment, one-sample *t*-tests were performed using least-squares mean CFU in
401 the treated wounds to the mean CFU in control wounds; the decrease in CFU vs control was statistically
402 significant for ESO and all dropouts ($p \leq 0.002$) except the wine dropout (p = 0.202). A Dunnett's test was
403 conducted to compare each dropout with the full ESO. All dropouts resulted in higher CFU than the full ESO ($p <$
404 0.001). Raw data and full statistical results are supplied in the Data Supplement.
405

406 ***The antibacterial activity of ESO is not simply due to its allicin content***

407 The study which attributed the planktonic activity of ESO to garlic further concluded that this
408 was specifically due to the presence of allicin (21), an organosulfur compound with well
409 known, potent antimicrobial activity *in vitro* (32–34). We used HPLC, calibrated against allicin
410 standards of known concentrations, to determine the allicin concentration in 12 batches of

411 fresh ESO. These batches were then assayed for MIC against *S. aureus* Newman in SWF. We
412 were then able to calculate the concentration of allicin present in the MIC of each batch and
413 compare this with the MIC of purified allicin.

414 As shown in Table 5, the allicin concentration in the 12 batches of ESO was found to be in the
415 range of 391 ± 19 $\mu g \cdot ml^{-1}$. Therefore, the estimated concentration of allicin in their respective
416 MICs would be in the range 11 - 39 $\mu g \cdot ml^{-1}$. This is much lower than the MIC obtained for
417 purified allicin, which was 62.5 $\mu g \cdot ml^{-1}$. Due to experimental error, two MIC test (ESO batches
418 28, 29) were lost; we did not repeat this due to the time delay between the initial and
419 repeated test potentially affecting concentrations of compounds present, and thus activity.

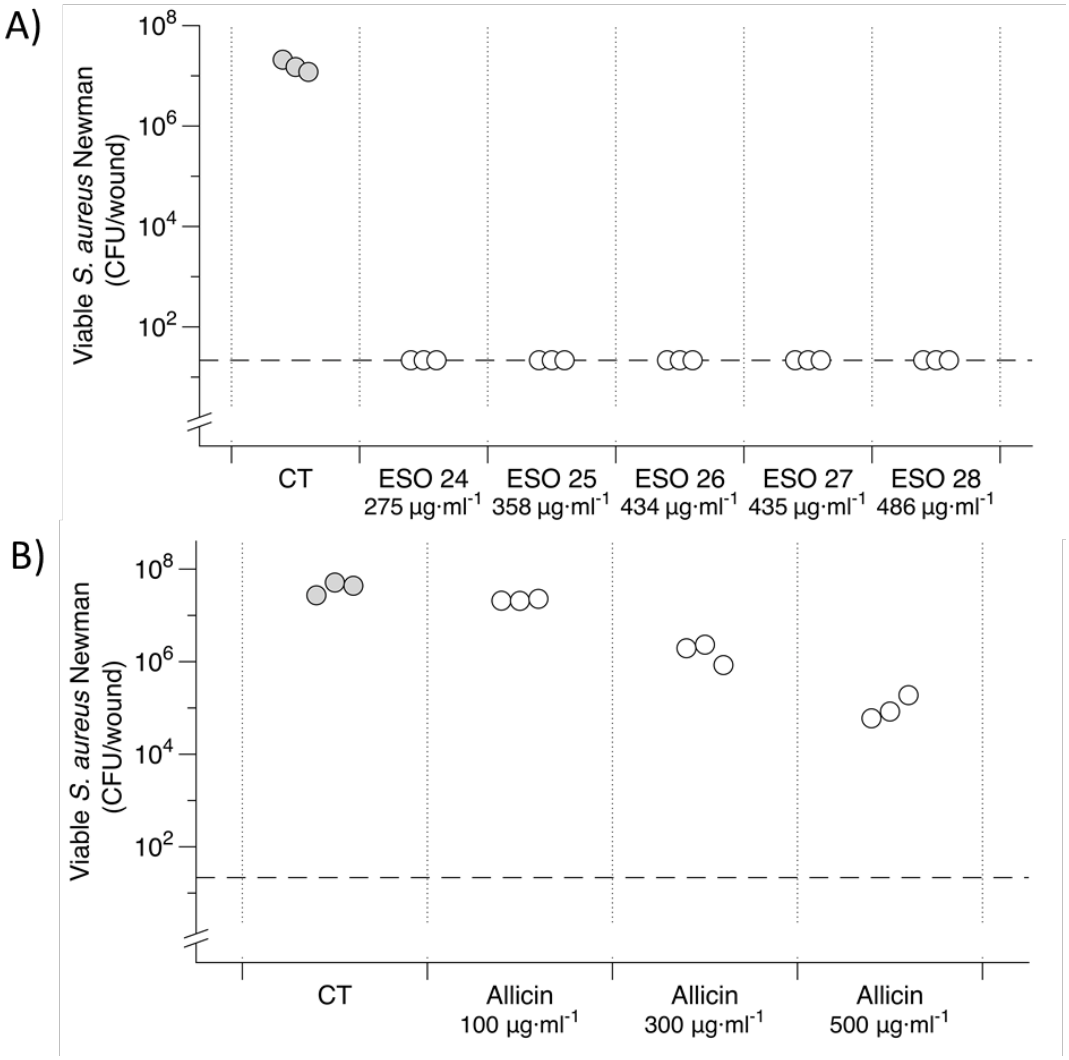
420 **Table 5.** Minimum inhibitory concentrations (MIC) were calculated for fresh Bald's eyesalve (n = 12; batches
421 19-30), garlic only preparations (n = 3) and external allicin standards (n = 3). Allicin concentrations for each batch
422 were quantified using HPLC and calculated using an allicin calibration curve. MICs were measured in synthetic
423 wound fluid. The allicin concentration in the MIC of each batch, and the allicin concentration present in the
424 biofilm treatment experiments, is calculated and the results of biofilm treatment experiments are provided for
425 reference. Raw data is supplied in the Data Supplement.

Treatment	Allicin concentration range (mean ± s.e.)	Allicin concentration in MIC	Biofilm activity (log drop from control)	Average predicted allicin concentration in biofilm treatment
ESO	275 - 486 (391 ± 19) $\mu g \cdot ml^{-1}$	11 - 39 $\mu g \cdot ml^{-1}$	Eradicated biofilm (>5)	142 $\mu g \cdot ml^{-1}$
Garlic only	358 - 434 (389 ± 19) $\mu g \cdot ml^{-1}$	45 - 54 $\mu g \cdot ml^{-1}$	No activity (0)	160 $\mu g \cdot ml^{-1}$
Allicin Standard		62.5 $\mu g \cdot ml^{-1}$	Partial activity (2.6)	167 $\mu g \cdot ml^{-1}$

426

427 All of these 12 batches eradicated mature *S. aureus* Newman biofilms in the synthetic wound
428 assay (see Data Supplement). The results of biofilm killing assays for five representative
429 batches are depicted in Figure 6A, alongside a biofilm killing assay conducted for preparations

430 of 100, 300 or 500 µg·ml^{-1} of purified allicin. Purified allicin at concentrations similar to those
431 found in ESO, had much less anti-biofilm activity (Figure 6B), and garlic preparations with the
432 same allicin concentration as ESO had no activity (data supplied in the supplementary data
433 file). Therefore, allicin alone does not explain either the activity of ESO against biofilm-grown
434 bacteria or its activity against planktonic cultures.



435

436 **Figure 6**. **Antimicrobial activity of Bald's eyesalve (ESO) and allicin standards.** Mature *S. aureus* Newman
437 biofilms were grown in a model of a soft tissue wound, then treated with either water (CT) or treatment for 24
438 hours, before recovering bacteria for CFU counts. A) Biofilms treated with 5 ESO batches, with their respective
439 allicin concentrations indicated. B) External allicin standards diluted in water. The dashed line represents the
440 limit of detection by plating. To statistically analyse, data were log transformed to meet the assumptions of

441 linear modelling. A one-way ANOVA was performed and found a significant difference between allicin
442 treatments and control cells ($F_{3,9}$ = 141, p < 0.001). A Dunnett's test was conducted to compare the CFU of the
443 three allicin concentrations with the CFU of the controls. Of the concentrations, 300 $\mu g \cdot ml^{-1}$ and 500 $\mu g \cdot ml^{-1}$
444 were significantly different (p < 0.001), 100 $\mu g \cdot ml^{-1}$ was not significantly different (p = 0.247). Raw data and full
445 statistical results are supplied in the Data Supplement.
446
447 **Discussion**

448 The activity of a range of natural products against microbes in simple *in vitro* assays (agar
449 diffusion, broth microdilution or simple surface-attached biofilm assays) demonstrates their
450 potential as a source of novel antibiotics (35–37). However, the standard approach of
451 purifying individual compounds from natural products rarely produces clinically useful
452 products and potent activity against planktonic bacteria in standard lab media rarely
453 translates to *in vivo* efficacy. This is an especially pressing problem in the case of biofilm
454 infections. Biofilms are much harder to treat due to reduced penetration of antibiotics
455 through the extracellular matrix and the enhanced tolerance of biofilm-grown cells to many
456 in-use antibiotics (6,12). Biofilm infections of wounds (e.g. burns, diabetic foot ulcers),
457 medical implants (e.g. artificial joints, catheters), the lungs (e.g. in cystic fibrosis) and other
458 body sites impose a major health and economic burden (38) and can be effectively
459 untreatable. Non-healing, infected foot ulcers, which can be a complication of diabetes,
460 provide an especially sobering example. Even if the infection is apparently successfully
461 treated, there is a high chance of recurrence and an estimated 50% of those affected die
462 within five years of ulcer development. Management of diabetic foot ulcers costs the UK's
463 NHS £650M per year (39).

464 Historical medical manuscripts often prescribe complex preparations of several ingredients
465 to treat infections. Thus, when considering natural products as a potential source of
466 anti-biofilm agents, we must consider the possibility that any efficacy they may possess could
467 rely on creating a cocktail of different products. Understanding the relationship between
468 combinations of natural products and antimicrobial activity may generate a novel way to
469 create new antibiotics from botanicals. Here, we confirm Bald's eyesalve as an example of an
470 "ancientbiotic" that requires the combination of all ingredients for potent activity against a
471 panel of clinically important bacterial strains.

472 Our research builds on previous work (20–22) to show that Bald's eyesalve can eradicate
473 planktonic cultures of a range of problematic Gram-positive and Gram-negative bacteria
474 including *P. aeruginosa*, *A. baumannii, E. cloacae, S. maltophilia, S. aureus*, *S. epidermidis* and
475 *S. pyogenes*. It is also able to cause a 4-log reduction in viable cell counts in planktonic cultures
476 of the especially problematic MRSA. Despite the widely understood problems of treating
477 biofilms (6,12), Bald's eyesalve was also able to significantly reduce viable cell counts in
478 biofilms of *S. epidermidis* and MRSA and was able to completely eradicate biofilms of *S. aureus*
479 Newman, *A. baumannii* and *S. pyogenes,* in an established soft-tissue wound model (23).
480 However, although there was promising planktonic activity against *P. aeruginosa*, *E. cloacae*
481 and *S. maltophilia,* variable or no activity was seen against biofilm cultures of these isolates
482 in the wound model. This highlights the importance of investigating the anti-biofilm activity
483 of candidate antibacterial agents, rather than extrapolating from the results of planktonic
484 assays. Bald's eyesalve shows great promise as an effective antimicrobial candidate, although
485 further development and combination with biofilm-degrading adjuvants may be necessary to
486 achieve activity against species such as *P. aeruginosa*.

487 Each of Bald's eyesalve ingredients has known antimicrobial properties or compounds (onion
488 and garlic: (34,37,40,41), bile: (42–44), wine: (45–47)). We explored the contribution of all
489 four ingredients to both planktonic and biofilm activity of Bald's eyesalve to build a picture of
490 their relative contributions. Planktonic activity appeared almost entirely attributable to garlic.
491 However, tests against *S. aureus* Newman biofilms, grown in a synthetic wound model,
492 showed garlic exhibited no antibacterial activity in this more clinically-relevant setting. In fact,
493 no preparation which omitted any one ingredient possessed full activity in the biofilm assay.
494 This confirms our previously published finding that Bald's eyesalve anti-biofilm activity is
495 contingent on the presence of all four ingredients (20).

496 Our results against planktonic cultures of *S. aureus* and *P. aeruginosa* align with a report by
497 Fuchs *et al.* 2018, that garlic alone, and specifically allicin, accounts for the majority of the
498 planktonic activity of Bald's remedy. Allicin is a defensive compound that is converted from
499 alliin by the enzyme alliinase, upon damage to the plant tissue. Allicin can kill a wide range of
500 both Gram-negative and Gram-positive bacteria *in vitro* (32) due to the thiosulfinate group (–
501 S(O)–S–) reacting with many cellular proteins in the pathogen (48). We therefore explored
502 the role of allicin in Bald's eyesalve in detail.

503 Crucially, we found that 12 batches of Bald's eyesalve were able to elicit much greater anti-
504 biofilm activity than purified allicin at comparable concentrations. Pure allicin at a
505 concentration of 500 $\mu g \cdot ml^{-1}$ was able to reduce viable cell numbers by 2-3 logs, whereas
506 Bald's eyesalve batches containing 275 - 486 $\mu g \cdot ml^{-1}$ allicin were able to cause a 6-7 log drop
507 and eradicate the biofilms. Even in planktonic tests, we found that the concentration of allicin
508 in the MIC of these batches of Bald's eyesalve was lower than the MIC of purified allicin.
509 Together, these results clearly illustrate that in addition to allicin, other ingredients in Bald's

510 eyesalve contribute to its activity. This highlights the importance of the combination of
511 ingredients.

512 The differences between our findings and those of Fuchs *et al.* are partly explained by the
513 contrasts between bacterial tolerance to killing (i) in MHB *versus* synthetic wound fluid; and
514 (ii) in planktonic culture *versus* established biofilms. This highlights the differences in
515 antibiotic susceptibility often seen in host-mimicking media versus standard MHB (18,19,49).
516 Further, the allicin concentrations found in our batches of Bald's eyesalve were lower than
517 that found in Fuchs *et al.* The highest allicin concentration we measured was 486 $\mu g \cdot ml^{-1}$,
518 compared with Fuchs *et al.*'s 836 $\mu g \cdot ml^{-1}$.

519 We think it unlikely that this is due to the differences in quantification methods used as both
520 are appropriate. We used HPLC, a method that has been widely used previously (33,50–53)
521 and is accepted to produce consistent and accurate results. Previous papers using HPLC have
522 detected allicin concentrations ranging from 1 $\mu g \cdot ml^{-1}$ to 2 $mg \cdot ml^{-1}$ (33,52), a range that the
523 results in this paper lie within. Fuchs *et al.* 2018 used quantitative NMR (qNMR), which too
524 has advantages and is being increasingly used in quantification of concentrations (54). It is
525 more likely that our Bald's eyesalve preparations really do have different allicin
526 concentrations. Alliin is released from storage vesicles and converted to allicin upon damage
527 to tissue: Fuchs *et al.,* 2018 used a food processor, in comparison to our pestle and mortar,
528 which may have resulted in greater damage to the tissue and therefore greater release of
529 alliin and more allicin created. Further, different garlic varieties are thought to have different
530 activities and compositions (55–59) and our research labs are based in different continents,
531 which presumably results in different garlic varieties and growth conditions. The higher allicin
532 concentration may explain the slightly increased planktonic activity seen in Fuchs *et al*. This

533 may also be due to their use of agitation in MIC assays, as agitation is known to lower the MIC
534 (60).

535 The step in the early Medieval remedy which specifies that the onion and garlic be ground
536 together would have been conducted manually – most likely with a pestle and mortar. We
537 have found that this process generates sub-bactericidal concentrations of allicin, which is
538 complemented or synergised by the presence of other ingredients to form a biofilm-killing
539 preparation. This demonstrates how contingent explorations of natural product
540 antimicrobials are on material preparation and testing conditions. This result is also
541 interesting because allicin can be toxic in high concentrations (61). Reducing the amount of
542 allicin present but maintaining full activity against bacterial biofilms might have been key to
543 producing a safe topical treatment. Further work is needed to elucidate the exact
544 combination of natural products responsible for the anti-biofilm activity.

545 Contrary to many papers in the literature (45,62,63), we found that wine possessed very
546 limited planktonic antimicrobial activity, and could not kill *S. aureus* in biofilms. Despite this,
547 its absence from the full remedy causes a large drop in activity against *S. aureus* biofilms.
548 Taken together, these observations suggest that the role of wine in the full recipe may be
549 more to do with its physical properties, perhaps its ethanol content and/or low pH. Ethanol is
550 a well-known extraction solvent and may allow better extraction of compounds from the
551 plant matter (64), or allow better diffusion through biofilms, while the lower pH may activate
552 pH-dependent compounds. The answer may lie in the combination of these properties, as the
553 activity of wine reported in the literature cannot be attributed exclusively to one aspect of it:
554 the physicochemical environment plays a large role in the activity of wine (65). Although pH
555 and ethanol concentrations corresponding to those found in various wines have minimal

556 effects on pathogens, without them the compounds within the wine have reduced activity
557 (65).

558 Similarly, individual preparations of onion and bile, prepared as they would be for Bald's
559 eyesalve, possessed no planktonic antibacterial activity. This is contrary to the literature that
560 has shown these ingredients to have activity against various strains (42,66). The most likely
561 cause for this disparity is the difference in the preparation or the dilution effect when
562 combined with other ingredients. However, the removal of onion or bile significantly reduced
563 the anti-biofilm activity against *S. aureus*, indicating the importance of their presence within
564 the whole remedy. They may be providing additional compounds necessary for the full killing
565 effect in biofilms or aiding penetration/activity of antibacterial compounds from other
566 ingredients.

567 Here, we have shown for the first time that Bald's eyesalve has anti-biofilm activity against
568 several clinically relevant bacteria, and we confirm our earlier work which concluded that this
569 activity is dependent on combining all the ingredients. Interestingly, statistical analysis of the
570 surviving portion of Bald's Leechbook (London, British Library, Royal MS 12 D XVII) showed
571 that garlic is combined with a second *Allium* species significantly more often than would be
572 expected given the frequency of use of either garlic or other *Allium* species across the book if
573 ingredients were combined randomly (67). Perhaps the patterns of ingredient combinations
574 used by pre-modern physicians do, in at least some cases, reflect a requirement for
575 combinatorial activity of several natural products to produce an efficacious antimicrobial
576 preparation.

577 Research into natural products is often focussed on isolating single compounds, however,
578 here we provide evidence that in doing so potent anti-biofilm mixtures can be overlooked.

579 Viewing natural products in this way has the potential to open a vast new source of
580 antimicrobials that can overcome the inherent difficulties of treating biofilm infections. In a
581 companion manuscript (22), we describe the low potential of Bald's eyesalve for producing
582 irritation or impeding wound healing. Future work will determine whether our results
583 translate into a candidate natural product cocktail for incorporation into wound ointments or
584 dressings. At present, we conclude by re-stating the exciting potential for pre-modern
585 European medical texts to contain antibacterial preparations of clinical interest.

586

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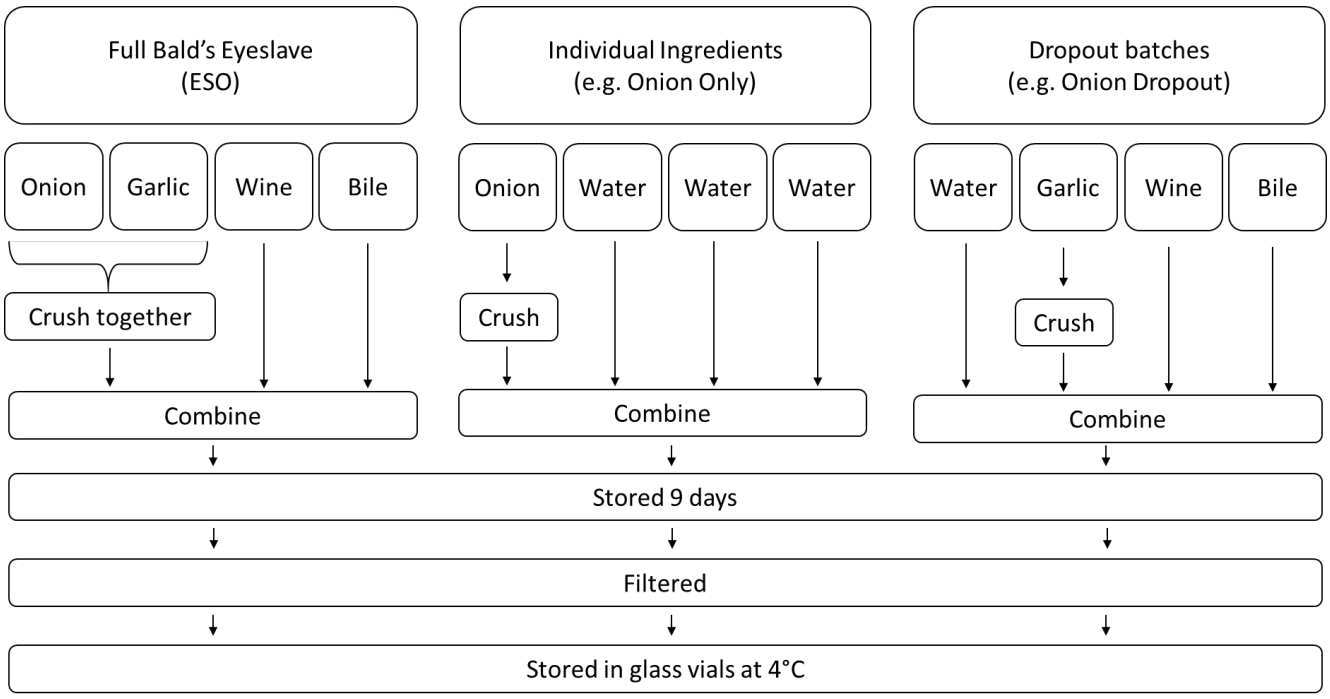
598

599 **Supplementary Figures**



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601 **Figure S1.** Schematic of the process to generate Bald's eyesalve and various batch variations.

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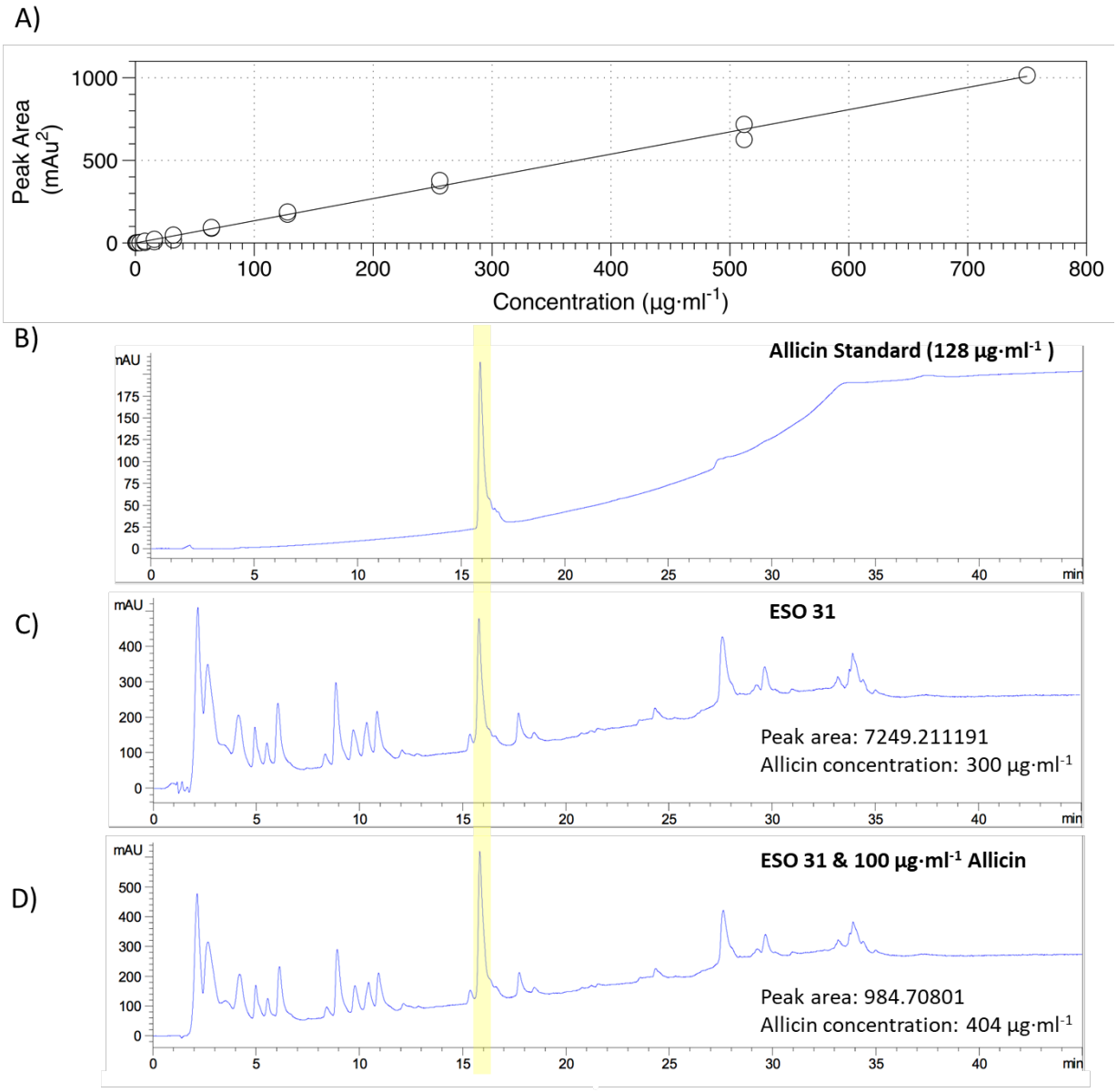
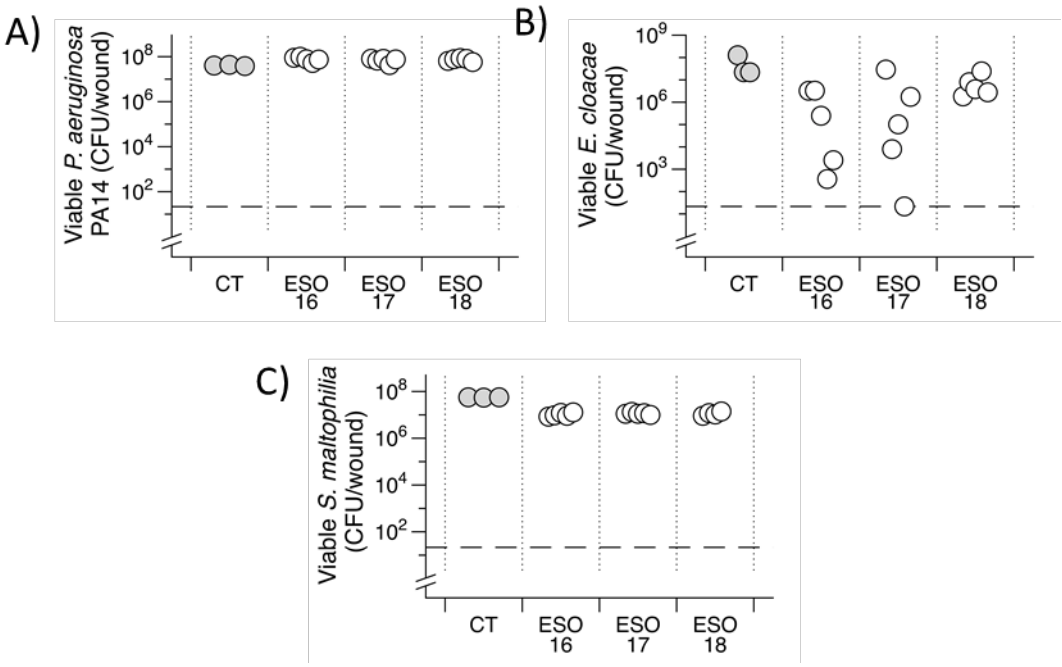


603

604 **Figure S2. Confirmation of allicin in Bald's eyesalve.** All chromatograms were run against a 0-95% methanol
605 gradient, with a flow rate of 1 ml·min^{-1} on HPLC Aligent 1200 series, at 210 nm UV wavelength. A) Allicin
606 calibration curve. External allicin standards, serially diluted in water were run and peak area was measured. A
607 linear relationship was found between HPLC peak area and allicin concentrations between 8 - 750 µg·ml^{-1} with
608 a correlation coefficient R^2 value > 0.99. The mid-range of the curve (8-512 µg·ml^{-1}) has 2 repeats. B) Exemplary
609 allicin external standard (128 µg·ml^{-1}) chromatogram, suspected allicin peak has a retention time of approx. 15
610 minutes. C-D) Bald's eyesalve fresh batch, ESO 31 without (C) and with (D) an additional 100 µg·ml^{-1} of allicin
611 standard; the differences in peak area are indicated. The highlighted region is the allicin peak.

612



613

614 **Figure S3. Bald's eyesalve (ESO) anti-biofilm activity.** Mature biofilms of various isolates were grown in a model
615 of a soft tissue wound, then treated with either 0.5 vols sterile water (control, CT) or 0.5 vols of ESO for 24h
616 before recovering bacteria for CFU counts (n = 3-5 replicates per treatment). The dashed line represents the
617 limit of detection by plating. Data could not be transformed to fit assumptions of parametric tests, therefore,
618 Kruskall-Wallis tests were used to determined that the CFU recovered from ESO-treated wounds was not
619 significantly different from control wounds for *P. aeruginosa* PA14 (X^2 = 6.88, df = 3, *p* value = 0.076), *S.*
620 *maltophilia* (X^2 = 7.63, df = 3, *p* value = 0.54) or *E. cloacae* (X^2 = 7.74, df = 3, *p* value = 0.051). Raw data and R
621 scripts are supplied in the Data Supplement.

622

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