1 **Anti-biofilm efficacy of a medieval treatment for bacterial infection requires**

2 **the combination of multiple ingredients**.

3

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24 Conceived the study: CL, FH

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32

33 **Abstract**

34   Novel antimicrobials are urgently needed to combat the increasing occurrence of drug-resistant

35   bacteria and to overcome the inherent difficulties in treating biofilm-associated infections. Research

36   into natural antimicrobials could provide candidates to fill the antibiotic discovery gap, and the study

37   of plants and other natural materials used in historical infection remedies may enable further

38   discoveries of natural products with useful antimicrobial activity. We previously reconstructed a

39   1,000-year-old remedy containing onion, garlic, wine, and bile salts, which is known as 'Bald's

40   eyesalve', and showed it to have promising antibacterial activity. In this paper, we have found this

41   remedy has bactericidal activity against a range of Gram-negative and Gram-positive wound

42   pathogens in planktonic culture and, crucially, that this activity is maintained against *Acinetobacter*

43   *baumannii, Stenotrophomonas maltophilia, Staphylococcus aureus*, *Staphylococcus epidermidis* and

44   *Streptococcus pyogenes* in a model of soft-tissue wound biofilm. While the presence of garlic in the

45   mixture is sufficient to explain activity against planktonic cultures, garlic alone has no activity against

46   biofilms. We have found the potent anti-biofilm activity of Bald's eyesalve cannot be attributed to a

47   single ingredient and requires the combination of all ingredients to achieve full activity. Our work

48   highlights the need to explore not only single compounds but also mixtures of natural products for

49   treating biofilm infections. These results also underline the importance of working with biofilm models

50   when exploring natural products for the anti-biofilm pipeline.

51

52 **Importance**

53   Bacteria can live in two ways, as individual planktonic cells or as a multicellular biofilm. Biofilm helps

54   protect bacteria from antibiotics and makes them much harder to treat. Both the biofilm lifestyle and

55   the evolution of antibiotic resistance mean we urgently need new drugs to treat infections. Here, we

56   show that a medieval remedy made from onion, garlic, wine, and bile can kill a range of problematic

57   bacteria grown both planktonically and as biofilms. A single component of the remedy – allicin, derived

58   from garlic – is sufficient to kill planktonic bacteria. However, garlic or allicin alone do nothing against

59   bacteria when they form a biofilm. All four ingredients are needed to fully kill bacterial biofilm

60   communities, hinting that these ingredients work together to kill the bacteria. This suggests that

61   future discovery of antibiotics from natural products could be enhanced by studying combinations of

62   ingredients, rather than single plants or compounds.

63

64   **Introduction**

65   Widespread multidrug resistance of once-susceptible pathogens, combined with a lack of

66   success in developing novel antimicrobials, has resulted in a looming crisis. Antimicrobial

67   resistance leads to problematic infections, threatens the success of routine surgery and

68   cancer treatments (1), and is estimated to kill 10 million people per year by 2050 (2,3). One

69   particularly troublesome area is biofilm-associated infection. Biofilm infections are estimated

70   to cost the UK's National Health Service over a billion pounds every year, with this cost only

71   set to increase (4,5). Biofilms are communities of bacteria that produce a protective

72   extracellular matrix and are especially persistent (6). Biofilm eradication often requires 100-

73   1,000 times higher antibiotic concentrations to achieve clearance than the same bacteria

74   growing planktonically (as individual free-floating cells)(7). *In vivo*, biofilms may essentially be

75   completely impervious to antibiotic treatment (8).

76   The path to overcoming biofilm infections requires a multifaceted response, including the

77   discovery and clinical deployment of novel antimicrobials. This search for novel candidates

78   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·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·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 μg·ml^{-1} to 0.5 μg·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 (μg·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

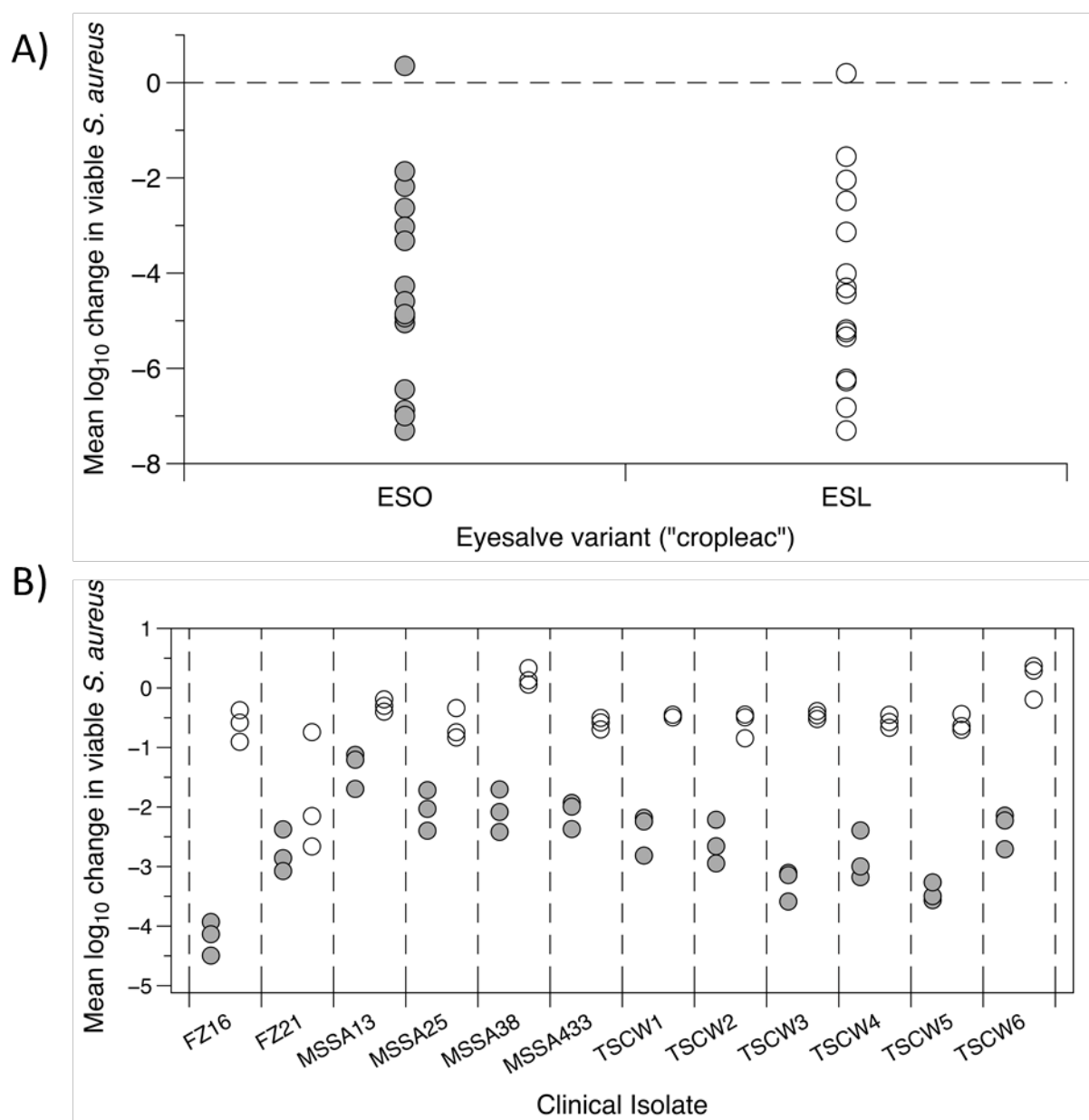252   drop, and 28 of these causing > 6-log drop, all relative to control biofilms treated with sterile

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

258   biofilms of *S. aureus* Newman were grown in a model of a soft tissue wound, then treated with either

259   sterile water or Bald's eyesalve (n = 3-5 replicates per treatment) for 24 hours before recovering

260   bacteria for CFU counts. The mean log change in viable bacteria in treated vs control wounds was

261   calculated for each preparation and no significant difference was seen (paired *t*-test t_{14}= 0.025, *p* =

262   0.981). Raw data are supplied in the Data Supplement. (B) Six carriage isolates of *S. aureus*, and six

263   isolates from a chronic post-surgical wound (detailed in Table 1), were grown in the synthetic wound

264   biofilm model and treated with ESO, ESL or water (n=3 per treatment). Each strain grew to different

265   densities in untreated wounds (range approx. 10^6–10^8). The $\log_{10}$ drop in bacteria associated with

266   treatment was calculated for each treated wound relative to the mean CFU in the three untreated

267   wounds. Log drop data was analysed by ANOVA which revealed significant differences in log drop

268   between eyesalve variants ($F_{1,48}$ = 732, *p* < 0.001) and strains ($F_{11,48}$ = 14.4, *p* < 0.001) and a significant

269   strain-dependent difference in the magnitude of the effect of eyesalve variant (strain*variant

270   interaction $F_{11,48}$ = 8.66, *p* < 0.001). The family-wise error rate was calculated from the ANOVA table

271   and used to conduct planned contrasts of ESO vs ESL treatment effects for each strain using *t*-tests. ESO

272   caused a larger reduction in viable cells than ESL for all strains (all *p* < 0.001). Raw data and R scripts are

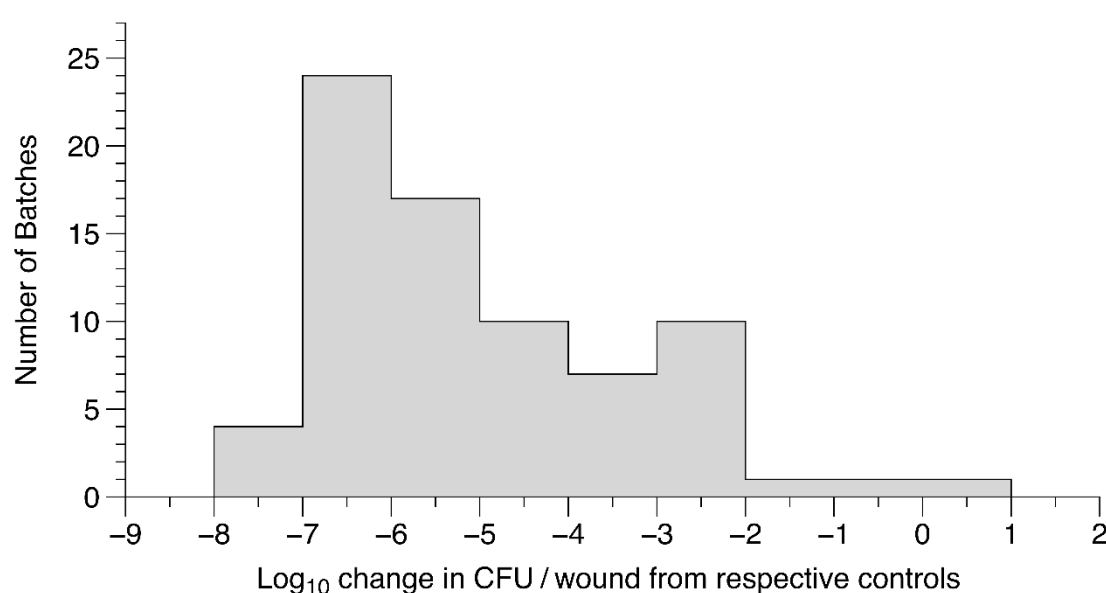273   supplied in the Data Supplement.

274

275

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.
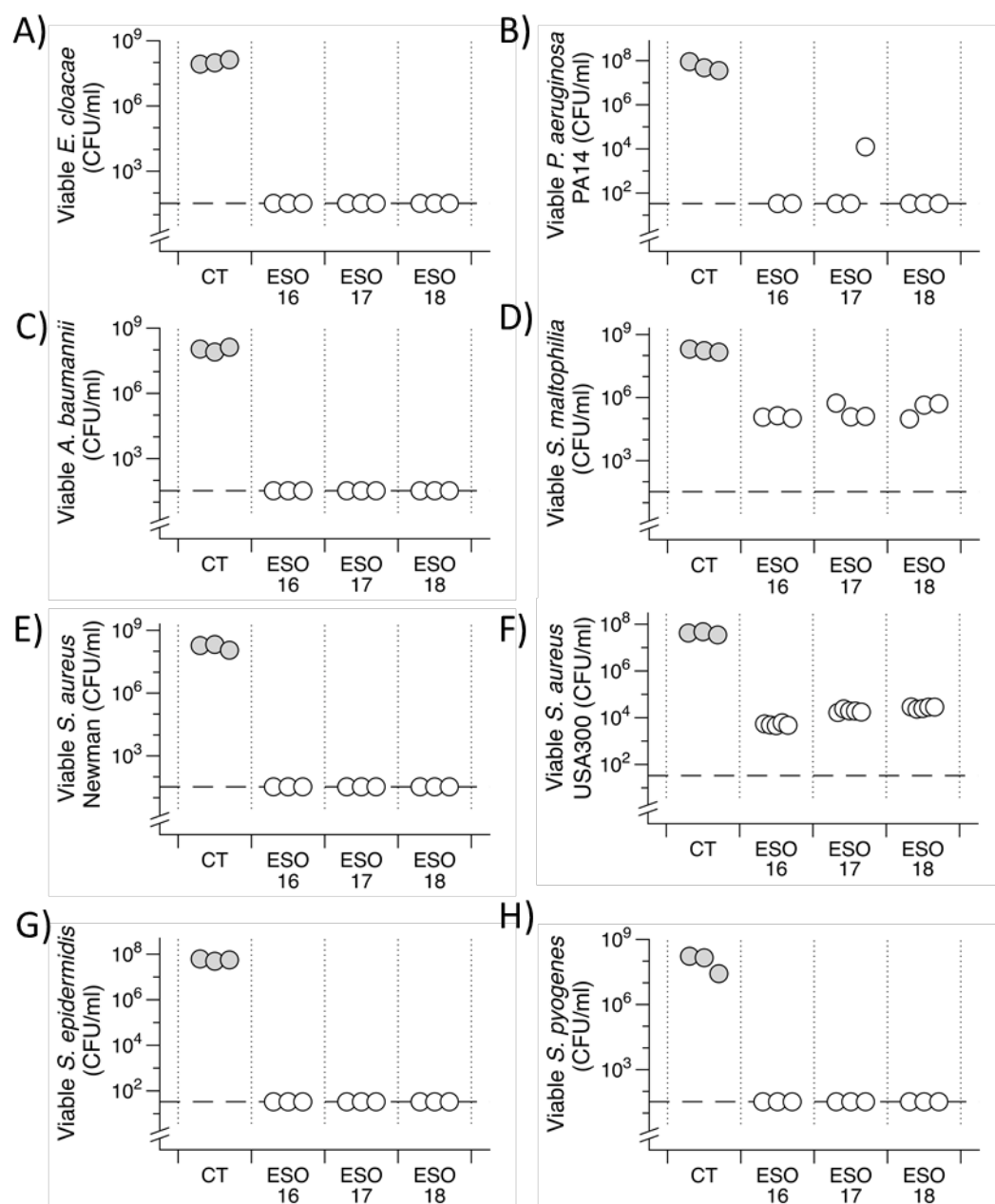
Figure 3. **Bald's eyesalve (ESO) activity against eight example strains of bacteria that commonly cause chronic wound infections.** Planktonic cultures were grown for 6 hours in synthetic wound fluid. Cultures were treated with ESO (batches 16-18) or sterile water (CT) to a final concentration of 33% v/v (n = 3-5 replicates per treatment). After 18 hours bacteria were recovered for CFU counts. The dashed line represents the limit of detection by plating. For *S. maltophila* and *S. aureus* USA300, where we did not observe complete killing, we used ANOVA to determine that the CFU recovered from ESO-treated wounds was significantly different from 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

299   transformed to meet the assumptions of linear modelling. A Dunnett's test was conducted to compare the CFU

300   of the three batches with the CFU of the controls. All 3 batches of ESO were significantly different from their

301   respective controls for both isolates (all $p < 0.001$). Raw data and R scripts are supplied in the Data Supplement.

302

303   Mature biofilms of the above isolates were grown in the synthetic wound model and treated

304   with the same batches of eyesalve used for planktonic killing experiments. A 2-6-log drop in

305   viable cells was observed for the Gram-positives *S. aureus* Newman, *S. aureus* USA300,

306   *S. epidermidis, S. pyogenes* and the Gram-negative *A. baumannii* (Figure 4). No or inconsistent

307   killing was observed for *P. aeruginosa*, *E. cloacae* and *S. maltophilia* biofilms (Figure S3).
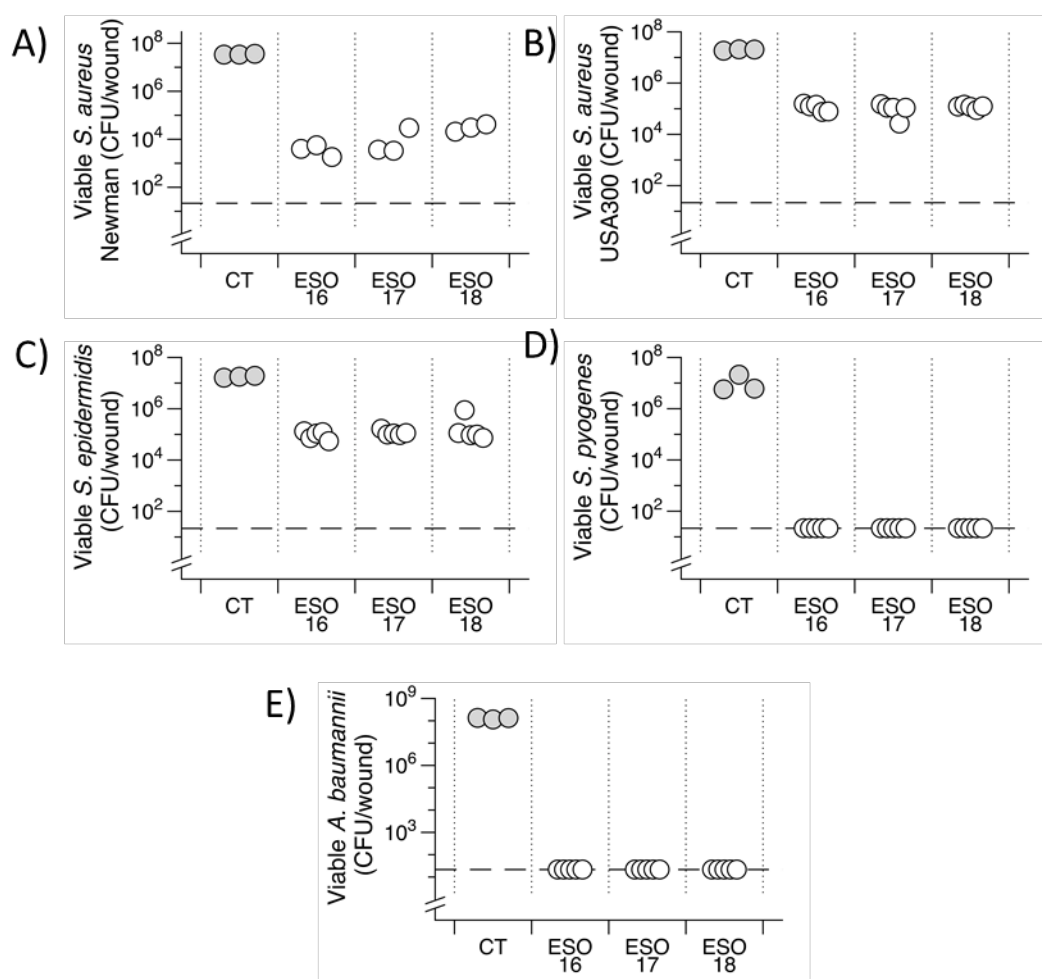
308

309

310   **Figure 4. Bald's eyesalve (ESO) anti-biofilm activity.** Mature biofilms of various isolates were grown in a model

311   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
	Garlic	50	50	50	50	50	>50	>50	>50
	Wine	<0.78	6.25	3.13	12.5	1.56	6.25	25	50
	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.

375
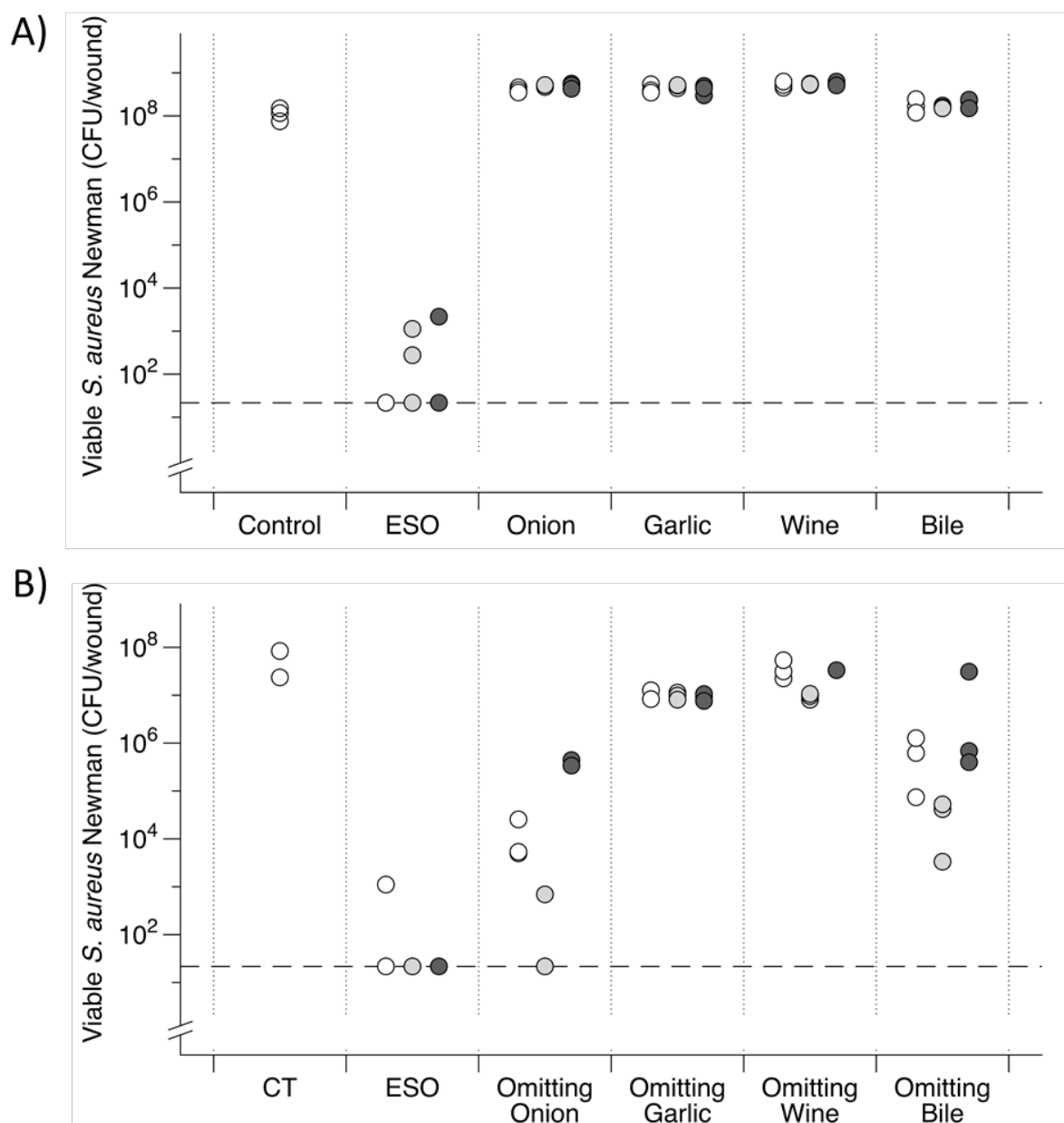
Figure 5. **Contribution of each ingredient to Bald's eyesalve (ESO) anti-biofilm activity.** Mature biofilms of *S. aureus* Newman were grown in a model of a soft tissue wound, then treated with either water (CT) or treatment for 24 hours, before recovering bacteria for CFU counts. The dashed lines 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 μg·ml^{-1}. Therefore, the estimated concentration of allicin in their respective

416   MICs would be in the range 11 - 39 μg·ml^{-1}. This is much lower than the MIC obtained for

417   purified allicin, which was 62.5 μg·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) μg·ml^{-1}	11 - 39 μg·ml^{-1}	Eradicated biofilm (>5)	142 μg·ml^{-1}
Garlic only	358 - 434 (389 ± 19) μg·ml^{-1}	45 - 54 μg·ml^{-1}	No activity (0)	160 μg·ml^{-1}
Allicin Standard		62.5 μg·ml^{-1}	Partial activity (2.6)	167 μg·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
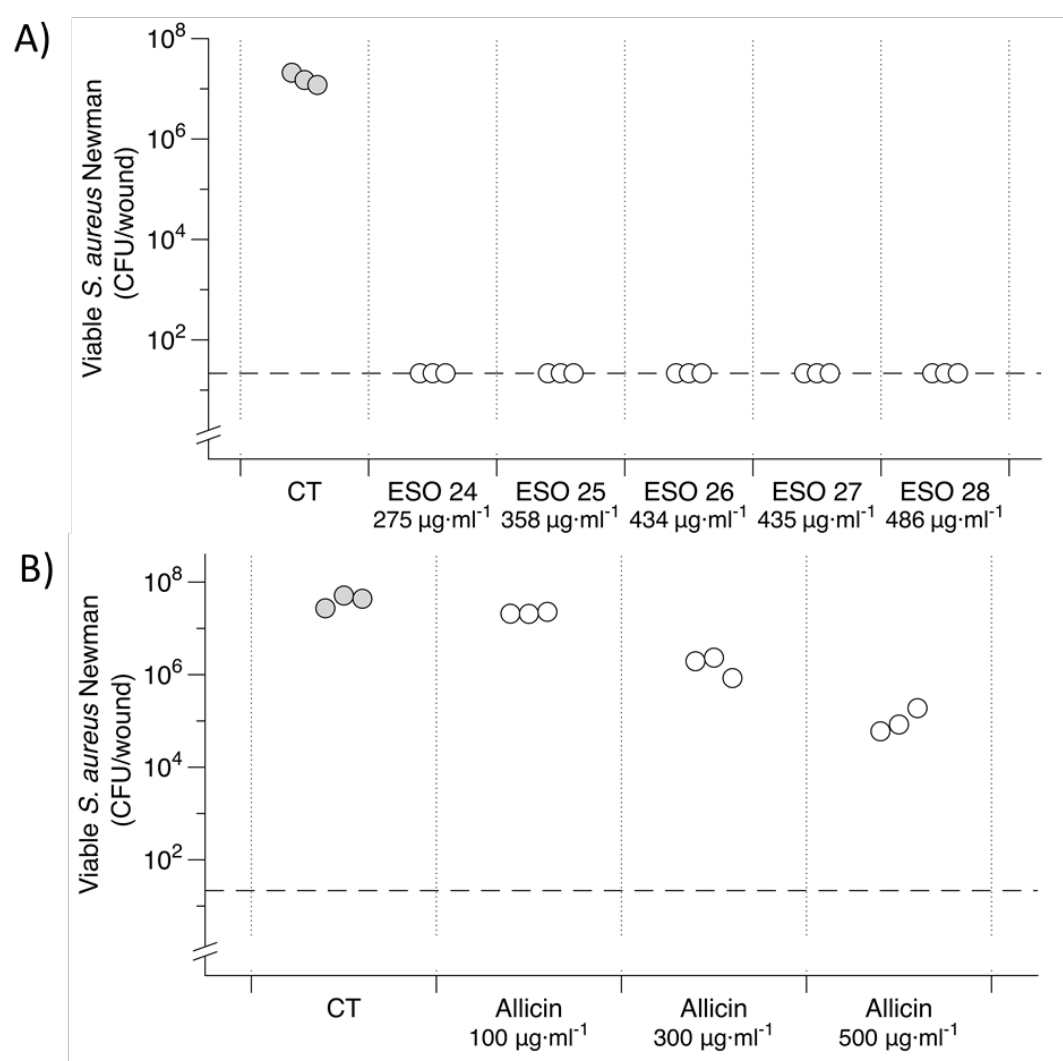
434   bacteria or its activity against planktonic cultures.

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 µg·ml^{-1} and 500 µg·ml^{-1}

444   were significantly different ($p < 0.001$), 100 µg·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

Acknowledgements

598

599 **Supplementary Figures**

600

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

602

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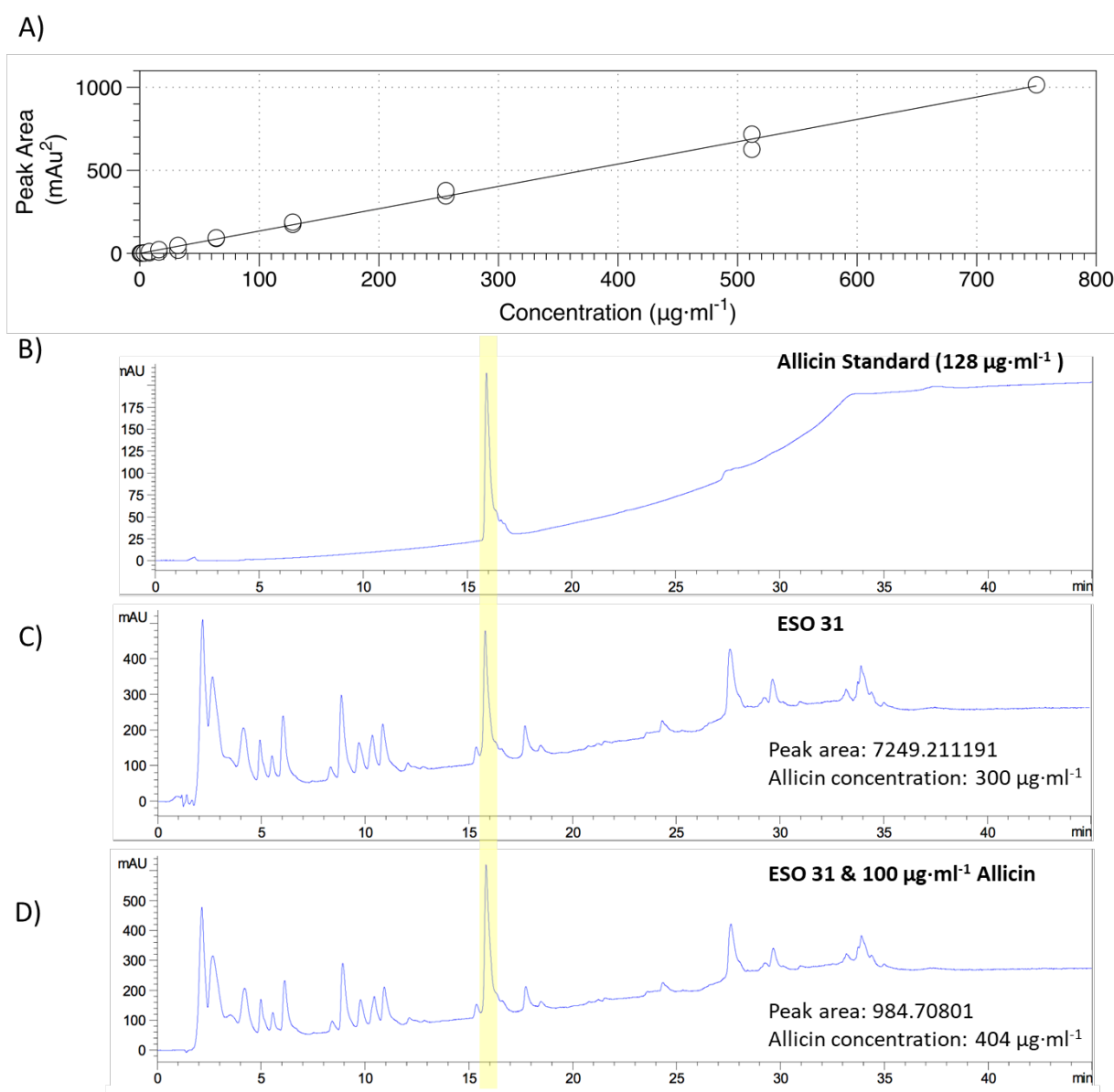
604 **Figure S2. Confirmation of allicin in Bald's eyesalve.** All chromatograms were run against a 0-95% methanol

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.
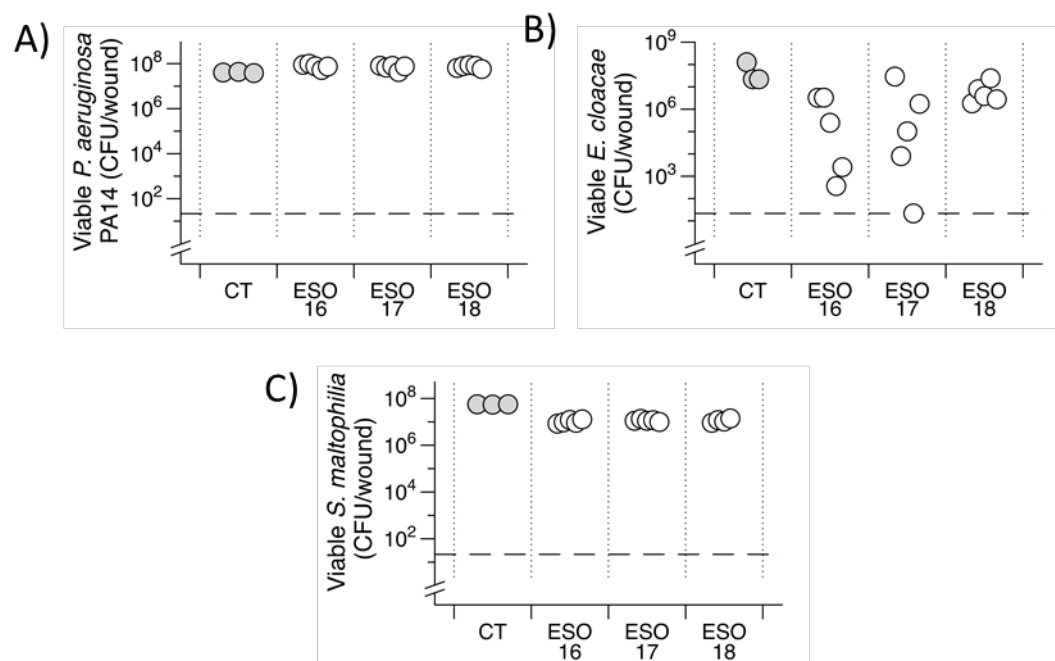
612

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Figure S3. 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 0.5 vols sterile water (control, CT) or 0.5 vols of ESO for 24h before recovering bacteria for CFU counts (n = 3-5 replicates per treatment). The dashed line represents the limit of detection by plating. Data could not be transformed to fit assumptions of parametric tests, therefore, Kruskall-Wallis tests were used to determined that the CFU recovered from ESO-treated wounds was not significantly different from control wounds for *P. aeruginosa* PA14 (X^2 = 6.88, df = 3, *p* value = 0.076), *S. 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 scripts are supplied in the Data Supplement.

622

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