A 1,000-year-old antimicrobial remedy with anti-Staphylococcal activity

Running title: A 1,000-year-old anti-Staphylococcal remedy

Authors: Freya Harrison[1]*, Aled E. L. Roberts[1], Rebecca Gabrilska[2], Kendra P. Rumbaugh[2], Christina Lee[3] & Stephen P. Diggle[1]*

Affiliations:

[1]Centre for Biomolecular Sciences, School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK.

[2] Department of Surgery, Texas Tech University Health Sciences Center, School of Medicine, 3601 4th Street, Lubbock, TX 79430, USA.

[3] School of English & Centre for the Study of the Viking Age, Trent Building, University of Nottingham, University Park, Nottingham NG7 2RD, UK.

*Correspondence to: freya.andersdottir@gmail.com or steve.diggle@nottingham.ac.uk.

Abstract: Plant-derived compounds and other natural substances are a rich potential source of compounds that kill or attenuate pathogens that are resistant to current antibiotics. Medieval societies used a range of these natural substances to treat conditions clearly recognizable to the modern eye as microbial infections, and there has been much debate over the likely efficacy of these treatments. Our interdisciplinary team, comprising researchers from both sciences and humanities, identified and reconstructed a potential remedy for *Staphylococcus aureus* infection from a 10th Century Anglo-Saxon Leechbook. The remedy repeatedly killed established *S. aureus* biofilms in an *in vitro* model of soft tissue infection and killed methicillin-resistance *S. aureus* (MRSA) in a mouse chronic wound model. While the remedy contained several ingredients that are individually known to have some antibacterial activity, full efficacy required the combined action of several ingredients, highlighting the scholarship of pre-modern doctors and the potential of ancient texts as a source of new antimicrobial agents.

Importance: While the antibiotic potential of some materials used in historical medicine has been demonstrated, empirical tests of entire remedies are scarce. This is an important omission, because the efficacy of "ancientbiotics" could rely on the combined activity of their various ingredients. This would lead us to underestimate their efficacy and, by extension, the scholarship of pre-modern doctors. It could also help us to understand why some natural compounds that show antibacterial promise in the laboratory fail to yield positive results in clinical trials. We have reconstructed a 1,000-year-old "antibiotic" which kills the bacteria it was designed to treat, and shown that this activity relies on combined activity of several antimicrobial ingredients. Our results highlight (a) the scholarship and rational methodology of pre-modern medical professionals and (b) the untapped potential of pre-modern remedies for yielding novel therapeutics at a time when new antibiotics are desperately needed.

Introduction

Antibiotic resistance is a clear and present danger to human health, and there are worryingly few new antibiotics in the developmental pipeline (1). A rich source of new antimicrobials potentially resides in medieval and early modern medical texts: microbial infection has been a constant presence throughout human history, and manuscript evidence shows that early modern and pre-modern societies used a range of natural compounds to treat conditions that are clearly recognizable as microbial infections. Some substances used in pre-modern treatments for infection (e.g. extracts from garlic (2-5)), show antimicrobial or virulence-reducing effects under certain conditions, but experiments that test the antibiotic activities of entire historical remedies are few and far between. This is an important omission, because the efficacy of these "ancientbiotics" could rely on the combined activity of their various ingredients. This would lead us to underestimate their efficacy and, by extension, the scholarship of pre-modern doctors. It could also help to explain why *in vitro* antibacterial activity of individual compounds does not always reliably translate into *in vivo* antimicrobial potential (e.g. garlic, (6)).

Bald's Leechbook (7) (Materials & Methods), is an English medical text from the Anglo-Saxon period. The manuscript was written in the 10th Century and contains remedies for various ailments, including clearly recognizable microbial infections. Medieval medicine has generally been dismissed as backwards or superstitious, but recent scholars have suggested that among the remedies there may be methods and recipes that indicate a more factual application (8-10). One of Bald's remedies, a salve for a 'wen' or lump in the eye (Fig. 1), is particularly interesting to the modern microbiologist. It aims to treat a condition caused by bacterial infection, and contains ingredients with the potential for antimicrobial activity. The recipe instructs the reader to crush garlic and a second *Allium* species (whose translation into modern English is ambiguous), combine these with wine and oxgall (bovine bile), and leave the mixture to stand in a brass or bronze vessel for nine days and nights.

The 'wen' is most likely a stye: an infection of an eyelash follicle. Today, most styes are caused by the Gram-positive bacterium *Staphylococcus aureus* (11), which also causes other severe and persistent infections of various tissues. The spread of methicillin-resistant (MRSA) strains has exacerbated this problem (11, 12). MRSA is particularly associated with chronic wound infections, which affect increasing numbers of people and are often highly resistant to treatment (11, 13).

The ingredients combined to treat this infection appear promising to the modern microbiologist. *Allium* species produce a range of antimicrobial compounds (3). Ajoene, which is found in garlic (*A. sativum*), and allicin (found in garlic, ramsons (*A. ursinum*) and spring onions (*A. fistulosum*), may prevent biofilm formation by *S. aureus* and other bacterial species (4) and interfere with quorum sensing and virulence in *Pseudomonas aeruginosa* (5). Other antimicrobial compounds produced by *Allium* species include flavonoids such as quercetin, kaempferol and their derivatives, found in leek (*A. ampeloprasum* / *A. porrum*) and onion (*A. cepa*) (3), and the antimicrobial peptide Ace-AMP1, so far only characterized in onion (14). Bile may also be antibacterial: it is generally thought to limit bacterial overgrowth in the host small intestine (15). Wine

may act as a source of plant-derived antimicrobial small molecules or simply as a solvent for the extraction of compounds from the plant matter in the recipe. Finally, it is possible that copper salts may leach from the vessel in which the eyesalve is prepared. Copper surfaces prevent bacterial growth (16) and host-derived copper plays a role in immune defense (17). Interestingly, there is evidence that *Allium*-derived compounds may act synergistically with other antimicrobial agents, including copper (18) and tobramycin (2, 5).

We therefore sought to test the effect of Bald's eyesalve on *S. aureus*, and to determine whether any antibacterial activity could be attributed to a single ingredient, or was reliant on combining the ingredients according to the instructions laid down by 'Bald'.

Results

Reconstruction of Bald's eyesalve

We reconstructed four independent batches of the recipe (A-D, see Materials & Methods). For each batch, we made two variants of the recipe, one using onion (ES-O) and one using leek (ES-L) for the ambiguous *Allium* sp. We chose these because they both produce antimicrobial flavonoids that are not found in garlic, and onion also produces an antimicrobial peptide (3, 19). We also made preparations of the individual ingredients at approximately the same concentrations as they are found in the recipe (Batches A,B), versions of the recipe with single ingredients omitted (Batch C), and a version of the recipe which was tested before and after the nine-day waiting period (Batch D). All of these were prepared in glass bottles, to which we added squares of brass sheet to simulate the brass/bronze vessel. The complete recipes were a clear, slightly acidic (pH 4.6-4.7) brownish liquid. The full recipes were apparently self-sterilizing: we cultured bacteria from aliquots of the leek and oxgall preparations, but not from aliquots of ES-O or ES-L (Data S1).

Bald's eyesalve kills planktonic and biofilm cultures of *S. aureus*

As shown in Fig. 2A, both ES-O and ES-L prevented growth of planktonic cultures of *S. aureus* growing aerobically at 37°C in synthetic wound fluid (SWF, (20)). Aliquots of cultures were plated out at the end of the incubation period and no colonies observed (detection limit: 30 colony-forming units). Further, 5 µl of each culture was transferred to 1 ml fresh SWF and incubated overnight at 37°C but no growth was observed, showing that Bald's eyesalve was bactericidal, rather than simply bacteriostatic, in these conditions.

However, during infection, bacteria often exist as dense, antibiotic-resistant biofilms in a matrix of self-excreted polysaccharides and soft tissue from the host (11, 13, 21). We therefore grew *S. aureus* for 24 hours at 37°C in a synthetic model of soft tissue infection comprising SWF solidified with collagen (20). We exposed the resulting mature biofilms to batches A and B of ES-O or ES-L, or preparations of the individual ingredients, for a further 24 hours (Fig. 2B). A generalized linear model revealed a significant effect of treatment (χ^2_7 1.5e+10, $p < 0.001$): post-hoc Tukey tests showed that only ES-O and ES-L were significantly different from the control treatment ($p < 0.001$), causing marked reductions in the number of viable cells recovered from synthetic wounds. None of the individual ingredients alone had a significant effect on viable cell counts. There was also a significant effect of batch (χ^2_1 4.2e+9, $p < 0.001$) and a significant in-

teraction between batch and treatment (χ^2_7 2.0e+9, $p < 0.001$): this was because the full recipes from batch B were slightly more bactericidal than batch A, and the effects of the individual ingredient preparations differed between batches. The full recipes retained bactericidal activity after thirty days of storage at 4°C (Fig. S1).

Bald's eyesalve is only effective when the recipe is followed carefully
We then made a third batch (C) of ES-O and ES-L, along with variants in which individual ingredients were systematically omitted. These were tested against *S. aureus* in the synthetic wound biofilm model. A generalized linear model revealed a significant effect of treatment (χ^2_{11} 3.1e+9, $p < 0.001$). We used Dunnett's Test to test contrasts of interest. First, we contrasted each recipe with the control. Both ES-O and ES-L caused a significant reduction in viable cell numbers ($t_{21} = 6.38$, $p < 0.001$; $t_{21} = 6.25$, $p < 0.001$); this was comparable to results for batches A and B (Fig. 3). When onion/leek was dropped from the common recipe base, partial bactericidal activity was still observed ($t_{21} = 3.38$, $p = 0.015$). Bactericidal activity was also retained when brass or oxgall was dropped from either ES-O ($t_{21} = 6.00$, $p < 0.001$; $t_{21} = 3.80$, $p = 0.005$) or ES-L ($t_{21} = 7.08$, $p < 0.001$; $t_{21} = 5.38$, $p < 0.001$). Dropping either garlic or wine from ES-O or ES-L completely removed bactericidal activity: viable cell counts were not significantly different form the control ($p \geq 0.166$). We then compared the modified recipes that retained bactericidal activity with their parent recipes. The bactericidal power of the common recipe base when onion/leek was omitted was significantly impaired compared with either ES-O or ES-L ($t_6 = 9.307$, $p = 0.005$ and $t_6 = 18.2$, $p < 0.001$, respectively). Omitting brass from either ES-O or ES-L had no effect on their bactericidal activities ($t_{10} = 0.684$, $p = 0.897$ and $t_{10} = 0.685$, $p = 0.896$, respectively. Omitting oxgall decreased the bactericidal activity of ES-O ($t_{10} = 4.66$, $p = 0.001$) but not ES-L ($t_{10} = 0.710$, $p = 0.885$).

The combination of garlic, wine and the other *Allium* species thus appears crucial for the full efficacy of Bald's eyesalve and, when onion is used in place of leek, oxgall is also necessary for full activity. Brass may be dispensable, but it is possible that its presence may facilitate activity against other bacterial species; additionally, using a brass pot may have allowed the Anglo-Saxon physician to start work with an uncontaminated vessel, as the copper content could prevent bacteria colonizing its surface.

Batch D of Bald's eyesalve was used to test whether the nine-day waiting period was important. ES-O and ES-L, with a water control, were tested in the synthetic wound biofilm model (a) immediately after preparation and (b) after nine days (Fig. 4). ANOVA revealed significant effects of treatment ($F_{2,24} = 110.9$, $p < 0.001$), time (immediate/after nine days; $F_{1,24} = 410.2$, $p < 0.001$) and their interaction ($F_{2,24} = 44.3$, $p < 0.001$). Post-hoc Tukey tests showed that while both fresh and nine-day-old ES-O and ES-L killed cells (comparisons vs. respective control, $p \leq 0.037$), the number of viable cells left after treatment with either version of the eyesalve was lower when the eyesalve had been left to stand for nine days prior to use (both $p < 0.001$). ES-O and ES-L caused a two-log drop in viable *S. aureus* cells if used immediately after preparation, but a four- to five-log drop if used after nine days (Fig. 4, consistent with data in Fig. 2B, Fig. 3).

Bald's eyesalve kills MRSA in chronically-infected animal tissue

All the ingredients in Bald's eyesalve are edible, and the likelihood of copper accumulating to levels toxic to mammalian cells seems low. Therefore, we tested its efficacy against *S. aureus* in a mouse model of chronic wound infection (22) using a thoroughly modern pathogen: methicillin-resistant *S. aureus* (MRSA) (12). Mice were administered wounds as previously described (22) and infected with MRSA. Four days post-infection, wounds were excised and submerged in ES-O, ES-L (Batch B), sterile saline or the clinical last-line antibiotic vancomycin (100 µg ml^{-1}) for four hours. As shown in Fig. 5, vancomycin did not cause significant reductions in viable bacteria during this relatively very short exposure time (contrast of least-squares mean adjusted for experimental block; $t_{16} = 1.45$, $p = 0.491$). However, ES-O and ES-L caused statistically significant drops in the numbers of viable cells recovered from wounds (approximately tenfold reduction; $t_{16} = 3.94$, $p = 0.006$ and $t_{16} = 3.66$, $p = 0.010$ respectively).. Batches A and B of ES-O and ES-L were also tested in the mouse wound model after three months of storage at 4°C, but without the vancomycin comparison (Fig. S2): in this experiment, ES-L, but not ES-O, caused a statistically significant drop in the numbers of viable cells recovered from wounds (approximately tenfold reduction). Thus, while there is some inter-batch variability in activity, Bald's eyesalve shows potential as an anti-Staphylococcal agent *in vivo*.

Discussion

In summary, we have reconstituted a 1,000-year-old remedy for bacterial infection and shown that it kills the most common cause of the infection it was designed to treat. Bald's eyesalve eliminates *S. aureus* in planktonic culture and reduces viable cell numbers by several orders of magnitude in a synthetic model of established biofilm infection. This effect depends upon the combination of several ingredients and upon the nine-day storage period specified in the recipe. Further, a highly antibiotic resistant strain of *S. aureus*, MRSA, is susceptible to Bald's eyesalve when infected tissue from a mouse chronic wound model is exposed to the recipe. In this model, significant bactericidal activity was observed after a brief period of exposure during which the current last-line clinical antibiotic (vancomycin) does not kill significant numbers of bacteria. Our findings contrast with a previous attempt to test Bald's eyesalve by Brennessel *et al*., (23), who found it ineffective against *S. aureus* in disk diffusion assays; however, these authors did not specify the methods of preparation and do not give quantitative results nor details of replication, so we do not know exactly how their tests were conducted.

Future work will explore the mechanism(s) by which Bald's eyesalve kills *S. aureus*. We note that several of the ingredients may damage biological membranes. Bile salts act as surfactants (15), the Ace-AMP1 antimicrobial peptide present in onion is thought to have lipid-binding activity (19), and saponins (present in a wide variety of *Allium* spp. can bind sterols present in biological membranes (3). Other antimicrobial compounds present in *Allium* spp. have been shown to act as enzyme inhibitors (allicin and flavonoids: (3)). Wine may be a source of further plant-derived antimicrobial small molecules, or it may simply act as a solvent for the extraction of compounds from the other ingredients in the recipe – with the nine-day waiting period providing time for molecule extraction.

It is interesting that copper was entirely dispensable for anti-Staphylococcal activity in our synthetic wound model. Copper has been shown to have broad-spectrum antibacterial activity (16, 17) and we would expect copper salts to accumulate in our preparations: we observed copper compounds forming on the surface of the brass squares used to simulate the preparation vessel, and Brennessel et al (who used an actual brass pot), found that copper salts accumulated to high levels in their version of Bald's eyesalve (M Drout, personal communication). It is possible that we simply did not have a large enough copper surface to allow copper to accumulate to biologically active levels in our recipe. Alternatively, the copper vessel itself may have allowed the Anglo-Saxon physician to begin their work with an uncontaminated pot due to the resistance of copper surfaces to bacterial colonization. A third possibility is that copper is entirely dispensable: copper alloy bowls of the Anglo-Saxon period can be highly decorated (even inlaid with silver) and appear as grave goods in high status burials as a marker of feasting power (24), and perhaps the more humble vessels may have been a marker of the physician's professional status.

The combined activity of molecules derived from the different ingredients might explain why the activity of Bald's eyesalve is greater than the sum of its parts: the mixture may attack the bacteria on several fronts. This is an important consideration for future work on natural antimicrobial compounds, as strong *in vitro* activity of individual compounds has not always translated into positive *in vivo* results (6). Alternatively, novel compounds may be formed as molecules from different ingredients react over the nine-day storage period specified by the recipe. Future research into antibiotics derived from natural materials would benefit from considering potential combinatorial activity of different ingredients, and pre-modern medical texts provide an excellent starting point for this strategy. Such work will require extensive collaborative research involving historians, linguists, microbiologists and analytical/medicinal chemists.

When we describe Bald's eyesalve as being "designed" to treat eye infection, we do not use the term lightly. There has been considerable debate about levels of scholarship and scientific method among early medieval medical practitioners (8, 9, 25). This may be partly due because of an almost complete absence of theoretical material, and the fact that the surviving corpus of Anglo-Saxon medicine seems to be compilations of excerpts and treatises (26). Further, many recipes - such as the getting a virgin to get water from an eastwards-flowing spring for a cyst 'which pains the heart' from the mid-eleventh century *Lacnunga* - look rather dubious as a remedy. Medicine, as some scholars have claimed, was a craft and not a science in early medieval Europe (26), and there was a lack of explanatory frameworks. Texts, such as *Bald's Leechbook*, were most likely composed in a monastic environment, and in many text sources there is a conflation of spiritual and practical healing. More credence has been given to later medieval texts, associated with the School of Salerno and the growth of university-based training (27).

However, our finding that the *combination* of ingredients used is crucial for bactericidal activity supports the hypothesis that this "ancientbiotic" was systematically constructed based on empirical knowledge. The fact that Anglo-Saxon recipes do not state detailed amounts of each component requires the practitioners to have had some knowledge about how much of each ingredient to use. It is also notable that numerous 'alternative' recipes are often given for a condition – indicating that a trained physician could adapt treatments when necessary. If medieval physicians really did use observation and experience to design effective antimicrobial medicines, then this

predates the generally accepted date for the adoption of a rational scientific method (formation of the Royal Society in the mid 17th century) and the modern age of antibacterial medicine (Lister's use of carbolic acid in the late 19th century) by several hundred years.

Materials and Methods

Bacterial strains and culture conditions. We used a standard laboratory wild-type strain of *S. aureus* (Newman) for initial *in vitro* tests of antibacterial activity. For the mouse chronic wound model, we used *S. aureus* Mu50 (ATCC number: 700699). Synthetic wound fluid (50% w/v fetal bovine serum, 50% peptone water, (20)) was used as the growth medium for all experiments. All cultures were grown aerobically at 37°C.

Bald's eyesalve. The recipe (Fig. 1) calls for equal amounts ("*begea emfela*") of the *Allium* species and equal amounts of wine and oxgall, but it is not clear whether these two pairs of quantities are equal to each other, nor whether ingredients were measured by weight or volume. We decided to combine equal amounts by volume of all of the recipe ingredients. For the complete eyesalve recipes, 25 ml peeled, finely-chopped garlic bulb was mixed with either 25 ml peeled, finely-chopped yellow onion or 25 ml finely-chopped leek (leaves) and these ingredients were crushed with a mortar and pestle for two minutes (the recipe asks for it to be pounded well: "*gecnuwe wel tosomne*"). Vegetables were purchased fresh from a greengrocer. 25 ml wine (Pennard Organic Wines) was added to the crushed alliums; we used an organic English white wine produced by a vineyard situated seven miles from the village of Panborough in Somerset: a charter of AD956 tells us that the Anglo-Saxon monastery of St Mary's Abbey in Glastonbury held land rights to a vineyard at Panborough (28). We then added 25ml bovine bile salts (Fluka / Sigma Aldrich) dissolved in water at a concentration of 87mg ml^{-1}; this is the natural concentration of bile salts in the bovine gall bladder (source: Sigma Aldrich technical support). The mixture of alliums, wine and oxgall was placed in a sterile 250 ml Duran bottle and, following the reasoning of Brennessel *et al.* (23), nine clean and sterile 15 mm squares of 0.51 mm brass 260 sheet (Hypertriton Inc, Canada) were added to the bottle to simulate the copper alloy vessel ("*arfæt*"). The lid was closed and the bottle wrapped in foil (as the original vessel would not have been transparent and we did not know if any ingredients were light sensitive). The recipes, denoted ES-O for the version containing onion and ES-L for the version containing leek, were then left refrigerated at 4°C for nine days. While the Anglo-Saxons did not have refrigerators, neither did they have centrally-heated buildings: we reasoned that the daily fluctuations in ambient temperature in the lab caused by heating during working hours were probably less realistic than a constant cool temperature.

To test the antibacterial effects of the individual ingredients of the recipe, when we made batches A and B we also combined 6 ml of each ingredient (vegetables were finely chopped and crushed as described above) with 17 ml distilled water in a foil-wrapped glass bottle containing two 15 mm squares of the brass sheet and left at 4°C for nine days. This is an approximately scaled-down version of the recipe given the constraints imposed by measuring chopped vegetables accurately and by requiring a whole number of brass squares. To provide a control treatment, 23 ml distilled water and two 15 mm squares of the brass sheet were left at 4°C for nine days.

We repeated the preparation of full recipes and individual ingredients twice, using fresh ingredients each time, to produce two independent batches of the eyesalve/individual ingredient prepa-

rations (A and B). We later repeated the preparation a third time (batch C), making complete ES-O and ES-L recipes, plus versions of the recipes that were each missing one ingredient, using the scaled-down, 24 ml total volume that was used for the individual ingredient preparations for batches A and B.

The recipe calls for the mixture to be strained and "purified well" ("*hlyttre wel*") before use or further storage. We debated how to simulate this and whether we should sterilize the mixtures. We decided that any microbes present in the ingredients are part of the "natural" state of the medicine and would not have been removed by filtering through cloth (probably linen). Therefore, we briefly centrifuged each recipe/ingredient to pellet the solid material, and filtered the remaining liquid through a filter small enough to remove solid particles of vegetable matter, but large enough to let bacterial cells through (5 µm). Because the oxgall does not fully dissolve in the mixture, the finished eyesalve contains a fine white precipitate (especially noticeable when taken directly from cold storage). All versions of the eyesalve or its ingredients were thus briefly vortexed before use to ensure a homogenous suspension.

Batch D of the complete recipes was made as above but in two sub-batches from the same ingredients. One sub-batch was made without brass, shaken for ten seconds and then filtered and used in experiments immediately. Brass was added to the other sub-batch and this was stored for nine days at 4°C following the original recipe.

Growth and testing of planktonic cultures in synthetic wound fluid. Cultures of *S. aureus* were grown for 6 hours (mid-log phase) in SWF at 37°C on an orbital shaker and 200 µl aliquots of this culture (containing 10^4-10^5 cells) were transferred to twelve wells of a 96-well microtitre plate. 100 µl sterile distilled water, ES-O or ES-L (Batch A only) were added to four wells each. Cultures were incubated at 37°C in a microplate spectrophotometer (Tecan Infinite) for 18 hours with periodic shaking; every 15 minutes the cultures were shaken for a few seconds and the OD_{600} of each well was measured. Because the eyesalve recipes were colored, we standardised the OD readings by subtracting the OD for each well at time = zero.

Culturing and treatment of synthetic wound infections. *S. aureus* was streaked onto LB agar and incubated aerobically at 37°C for 24 hours. Multiple colonies were pooled to inoculate starter cultures in SWF. These were incubated aerobically at 37°C on an orbital shaker for 6 hours. Cultures were then diluted to an OD_{600} of 0.05 in SWF and 100 µl added to wells of 24-well cell culture plates containing synthetic wound medium. Synthetic wounds comprised 400 µl polymerized collagen (2 mg ml^{-1} collagen, 0.01% acetic acid, 60% v/v SWF, 0.01 M sodium hydroxide). For experiments on Batches A and B of the eyesalve, additional synthetic wounds were mock-inoculated with 100 µl sterile distilled water instead of *S. aureus* to reveal any growth of bacteria resident in the eyesalve or its component ingredients. Synthetic wounds were incubated aerobically at 37°C for 24 hours to allow establishment of bacterial biofilms within the collagen matrix.

Batches A and B of the full recipes and individual ingredient preparations were tested as follows. For *S. aureus*-infected wounds and the no-infection control, five replica synthetic wounds were overlaid with 200 µl of each complete recipe or individual ingredients. A further five infected and non-infected synthetic wounds were overlaid with 200µl sterile distilled water to provide

mock-treated controls. Treated synthetic wounds were then incubated aerobically at 37°C for 24 hours. The collagen was then depolymerized by treating each wound with 500 μl 0.5 mg ml^{-1} collagenase. An aliquot of each wound was serially diluted and plated on LB agar to enumerate the number of viable cells present.

For batch C (full recipes and recipes missing a single ingredient), this experiment was repeated but each preparation was tested in triplicate. For Batch D (full recipes before and after nine days storage), each preparation was tested in five replica wounds. For batches C and D, only a single no-infection control wound was used for each experiment; this was visually inspected to confirm the absence of contaminating bacterial growth and not analyzed further.

Mouse chronic wound infections. Adult female, Swiss Webster mice were administered wounds as previously described (22) and infected with approximately 10^5 CFU *S. aureus* Mu50. At four days post-infection, mice were euthanized and their wound tissue was extracted and cut into 3 equal pieces, which were weighed. Wound sections were then submerged in 300 μl sterile saline, ES-O or ES-L for 4 hours, after which they were rinsed in sterile saline and homogenized. Wound homogenates were then serially diluted and plated on Mueller Hinton Agar with 4% NaCl and 6 μg/mL oxacillin (MRSA Screen Agar) to determine CFU/g tissue. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (Protocol Number: 07044) and an NC3Rs ARRIVE checklist (29) is available for this work (Data S2).

Statistical analyses. All data were analyzed with R (version 2.14.0 (R Foundation for Statistical Computing, http://www.R-project.org). Different models and their residuals were explored to find the most appropriate analysis for each data set. The *S. aureus* synthetic wound growth data sets for batches A-C of the recipes/ingredients were highly skewed; therefore, these data sets were analyzed using Generalized Linear Models with Poisson error. ANOVAs of log-transformed data were used to analyze (a) the 30-day re-test of batches A and B in synthetic wound infections; and (b) the test for an effect of the nine-day waiting period using batch D. In all analyses where both batches A and B were used, we tested for effects of treatment, batch and their interaction. Post-hoc comparisons were conducted using the *multcomp* package (30). For the *S. aureus* synthetic wound data for batch C, one replica wound treated with eyesalve minus onion/leek had to be dropped from the data set as it completely dried out during incubation. Data on MRSA viable cell counts in mouse chronic wounds were not suitable for analysis using general linear models and so were analyzed by fitting least squares means for each treatment adjusted for the effect of experimental block; Tukey tests were then used to test pairwise differences between treatments, with *p*-values adjusted for multiple comparisons. Growth curves of the *S. aureus* reporter strains were compared using the compare GrowthCurves function of the *statmod* package (version 1.4.21. http://CRAN.R-project.org/package=statmod). For each strain, growth curves from different treatments were compared using 1,000 permutations of the data and the Holm-Bonferroni correction for multiple comparisons.

References

1. **The Review on Antimicrobial Resistance.** 2014. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. Available at amr-review.org.
2. **Bjarnsholt, T., Jensen, P.O., Rasmussen, T.B., Christophersen, L., Calum, H., Hentzer, M., Hougen, H.P., Rygaard, J., Moser, C., Eberl, L., Hoiby, N., Givskov, M.** 2005. Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. Microbiology **151:**3873-3880.
3. **Lanzotti, V., Bonanomi, G., Scala, F.** 2013. What makes *Allium* species effective against pathogenic microbes? Phytochem. Rev. **12:**751-772.
4. **Nidadavolu, P., Amor, W., Tran, P.L., Dertien, J., Colmer-Hamood, J.A., Hamood, A.N.** 2012. Garlic ointment inhibits biofilm formation by bacterial pathogens from burn wounds. J. Med. Microbiol. **61:**662-671.
5. **Jakobsen, T.H., Van Gennip, M., Phipps, R.K., Shanmugham, M.S., Christensen, L.D., Alhede, M., Skindersoe, M.E., Rasmussen, T.B., Friedrich, K., Uthe, F., Jensen, P.ò., Moser, C., Nielsen, K.F., Eberl, L., Larsen, T.O., Tanner, D., Høiby, N., Bjarnsholt, T., Givskov, M.** 2012. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. Antimicrob. Agents Chemother. **56:**2314-2325.
6. **Smyth, A.R., Cifelli, P.M., Ortori, C.A., Righetti, K., Lewis, S., Erskine, P., Holland, E.D., Givskov, M., Williams, P., Cámara, M., Barrett, D.A., Knox, A.** 2010. Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis—a pilot randomized controlled trial. Pediatr. Pulmonol. **45:**356-362.
7. **Cockayne, O.** 1864-1866. Leechdoms, wortcunning and starcraft: being a collection of documents, for the most part never before printed, illustrating the history of science before the Norman Conquest. Rolls Series 35, 3 vols. Longman, Green, Longman, Roberts, and Green, London.
8. **Cameron, M.L.** 1993. Anglo-Saxon Medicine. Cambridge University Press.
9. **Horden, P.** 2000. What's wrong with early medieval medicine? Soc. Hist. Med. **24:**2-25.
10. **Meaney, A.** 2000. The practice of medicine in England about the year 1000. Soc. Hist. Med. **13:**221-237.
11. **Archer, N.K., Mazaitis, M.J., Costerton, J.W., Leid, J.G., Powers, M.E., Shirtliff, M.E.** 2011. *Staphylococcus aureus* biofilms. Virulence **2:**445-459.
12. **Kock, R., Becker, K., Cookson, B., van Gemert-Pijnen, J.E., Harbarth, S., Kluytmans, J., Mielke, M., Peters, G., Skov, R.L., Struelens, M.J., Tacconelli, E., Navarro Torne, A., Witte, W., Friedrich, A.W.** 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. Eurosurveillance **15:**19688.
13. **Kirketerp-Møller, K., Zulkowski, K., James, G.** 2011. Chronic Wound Colonization, Infection, and Biofilms. *In* Bjarnsholt T (ed.), Biofilm Infections. Springer.
14. **Soininen, T.H., Jukarainen, N., Soininen, P., Auriola, S.O.K., Julkunen-Tiitto, R., Oleszek, W., Stochmal, A., Karjalainen, R.O., Vepsäläinen, J.J.** 2014. Metabolite profiling of leek (*Allium porrum* L) cultivars by 1H NMR and HPLC–MS. Phytochem. Anal.

25:220-228.

15. **Hofmann, A.F., Eckmann, L.** 2006. How bile acids confer gut mucosal protection against bacteria. Proc. Natl. Acad. Sci. USA **103:**4333-4334.

16. **Grass, G., Rensing, C., Solioz, M.** 2011. Metallic copper as an antimicrobial surface. Appl. Environ. Microbiol. **77:**1541-1547.

17. **Chaturvedi, K.S., Henderson, J.P.** 2014. Pathogenic adaptations to host-derived anti-bacterial copper. Frontiers Cellular Infect. Microbiol. **4:**3.

18. **Ogita, A., Fujita, K., Taniguchi, M., Tanaka, T.** 2006. Dependence of synergistic fun-gicidal activity of Cu2+ and allicin, an allyl sulfur compound from garlic, on selective accumulation of the ion in the plasma membrane fraction via allicin-mediated phospho-lipid peroxidation. Planta Med. **72:**875-880.

19. **Tassin, S., Broekaert, W.F., Marion, D., Acland, D.P., Ptak, M., Vovelle, F., Sodano, P.** 1998. Solution structure of Ace-AMP, a potent antimicrobial protein extracted from onion seeds. structural analogies with plant nonspecific lipid transfer proteins. Biochem-istry **37:**3623-3637.

20. **Werthén, M., Henriksson, L., Jensen, P.Ø., Sternberg, C., Givskov, M., Bjarnsholt, T.** 2010. An *in vitro* model of bacterial infections in wounds and other soft tissues. AP-MIS **118:**156-164.

21. **Costerton, J., Stewart, P., Greenberg, E.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318 - 1322.

22. **Dalton, T., Dowd, S.E., Wolcott, R.D., Sun, Y., Watters, C., Griswold, J.A., Rumbaugh, K.P.** 2011. An *in vivo* polymicrobial biofilm wound infection model to study interspecies interactions. PLoS ONE **6:**e27317.

23. **Brennessel, B., Drout, M.D.C., Gravel, R.** 2005. A reassessment of the efficacy of An-glo-Saxon medicine. Anglo-Saxon England **34:**183-195.

24. **Lee, C.** 2007. Feasting the dead: food and drink in Anglo-Saxon burial rituals. Boydell & Brewer, Woodbridge.

25. **Grattan, J.H.G., Singer, C.** 1952. Anglo-Saxon magic and medicine : illustrated spe-cially from the semi-pagan text 'Lacnunga'. Oxford University Press, London.

26. **Liuzza, R.** 2011. Anglo-Saxon prognostics: an edition and translation of texts from Lon-don, British Library, MS Cotton Tiberius A.iii / edited with a translation by R.M. Liuzza. D.S. Brewer, Cambridge.

27. **Banham, D.** 2011. Dun, Oxa and Pliny the Great Physician: attribution and authority in Old English medical texts. Soc. Hist. Med. **24:**57-73.

28. **Hooke, D.** 1990. A note on the evidence for vineyards and orchards in Anglo-Saxon Eng-land. J. Wine Res. **1:**77-80.

29. **Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G.** 2010. Improv-ing bioscience research reporting: The ARRIVE guidelines for reporting animal research. PLoS Biol. **8:**e1000412 (see also www.nc3rs.org.uk).

30. **Hothorn, T., Bretz, F., Westfall, P.** 2008. Simultaneous inference in general parametric models. Biometrical J. **50:**346-363.

Figure legends

Figure 1. Bald's eyesalve. A facsimile of the recipe, taken from the manuscript known as Bald's Leechbook (London, British Library, Royal 12, D xvii). The original text reads as follows. "Þyrc eaȝsealf piþ pænne: ȝenim cropleac 7 ȝarleac beȝea emfela, ȝecnupe pel tosomne, ȝenim pin 7 fearres ȝeallen beȝean emfela ȝemenȝ piþ þy leaces, do þonne on arfæt læt standan niȝon niht on þæm arfæt aprinȝ þurh claþ 7 hlyttre pel, do on horn 7 ymb niht do mid feþre on eaȝe; se betsta læcedom." This is translated into modern English as follows. "Make an eyesalve against a wen: take equal amounts of *cropleac* [an *Allium* species] and garlic, pound well together, take equal amounts of wine and oxgall, mix with the Alliums, put this in a brass vessel, let [the mixture] stand for nine nights in the brass vessel, wring through a cloth and clarify well, put in a horn and at night apply to the eye with a feather; the best medicine."

Figure 2. Bald's eyesalve kills *S. aureus* in planktonic culture and in a synthetic wound biofilm model. (A) 100 µl of sterile distilled water (black lines), the onion variant of the eyesalve (ES-O, red lines) or the leek variant of the eyesalve (ES-L, blue lines) (both from Batch A) was added to 200 µl mid log-phase cultures (10^4-10^5 cells) of *S. aureus* in synthetic wound fluid (SWF) and culture optical density measured over 18 hours' incubation at 37°C. The means of four replica populations are shown, with the associated standard error (shaded intervals; too small to see for ES-O and ES-L). (B) 200 µl of ES-O or ES-L (batch A, filled circles and batch B, open circles) or the individual ingredient preparations was added to five one-day-old cultures of *S. aureus* growing at 37°C in a synthetic wound (400 µl synthetic wound fluid rendered semi-solid by adding 2 mg ml^{-1} collagen). The control treatment was sterile distilled water left to stand for nine days in the presence of brass, which was present in all other preparations to simulate the presence of a copper alloy vessel (see Material & methods). After 24 hours' further incubation, the collagen was dissolved to recover cells for agar plate counts.

Figure 3. The activity of Bald's eyesalve against *S. aureus* biofilms requires several ingredients.

200 µl of the onion variant of the eyesalve (ESO) or the leek variant of the eyesalve (ESL) (batch C) or preparations missing a single ingredient, were added to three one-day-old cultures of *S. aureus* in synthetic wounds and bactericidal activity quantified as previously described. Red bars show the full or reduced versions of ESO, blue bars show the full or reduced versions of ESL and the green bar shows a variant of the eyesalve with neither onion nor leek (ES). The control treatment (grey bar) was sterile distilled water. Individual ingredients are coded as O = onion, L = leek, X = oxgall, W = wine, G = garlic and B = brass. Reduced recipes were assessed as having full (F), partial (P) or no (N) activity using contrasts versus the control treatment and the appropriate complete recipe.

Figure 4. The activity of Bald's eyesalve against *S. aureus* biofilms requires the nine-day waiting period specified by the recipe. Immediately after preparation, 200 µl of the onion variant of the eyesalve (ES-O), the leek variant of the eyesalve (ES-L) (Batch D) or sterile distilled water was added to five one-day-old cultures of *S. aureus* growing at 37°C in a synthetic wounds and its bactericidal activity quantified as previously described (filled bars); the experiment was repeated using ES-O, ES-L (Batch D) after the nine-day waiting period specified by Bald (open bars). Asterisks denote treatments that were significantly different from the control. While the control cultures for the nine-day experiment grew to slightly lower densities than the control for the fresh eyesalve ($p = 0.005$), this difference was small compared with the differences observed for ES-O and ES-L (1-2 log vs. 4-5 log difference).

Figure 5. Bactericidal activity of Bald's eyesalve in a mouse chronic wound model of MRSA infection. Six adult female Swiss Webster mice were administered wounds and infected with c.10^5 CFU *S. aureus* Mu50. Four days post-infection, mice were euthanised. Wound tissue was extracted and cut into either three (n = 3 mice) or four (n = 3 mice) equal pieces, which were weighed, submerged in 300 µl sterile saline (one replicate from n = 6 mice), 100µg ml^{-1} vancomycin (one replicate from n = 3 mice), the onion variant of the eyesalve (ES-O; one replicate from n = 6 mice) or the leek variant of the eyeslve (ES-L; one replicate from n = 6 mice) (both ES-O and ES-L from Batch B) for four hours, then rinsed in sterile saline and homogenised. Viable bacteria were enumerated and this number standardised per gram of tissue. Asterisk denotes treatments that were significantly different from the control.

Figure S1. Bald's eyesalve retains anti-Staphylococcal activity over thirty days of storage. The onion (ES-O) and leek (ES-L) variants of the recipes (Batches A and B) retained bactericidal activity against *S. aureus* biofilms in synthetic wounds after 30 days of storage. (ANOVA: treatment $F_{2,24} = 217$, $p < 0.01$; batch $F_{1,24} = 0.16$, $p = 0.693$; interaction $F_{2,24} = 0.196$, $p = 0.823$).

Figure S2. Bald's eyesalve kills MRSA in a mouse chronic wound infection. Adult female Swiss Webster mice were administered wounds and infected with c.10^5 CFU *S. aureus* Mu50. Four days post-infection, mice were euthanised. Wound tissue was extracted and cut into three equal pieces, which were weighed, submerged in 300 µl sterile saline, ES-O or ES-L (Batches A and B, after three months of storage at 4°C) for four hours, then rinsed in sterile saline and homogenised. Viable bacteria were enumerated by plating. ES-L, but not ES-O, killed MRSA in wounds (ANOVA treatment $F_{2,12} = 6.17$, $p = 0.014$; post-hoc Tukey tests versus control ES-O $p = 0.069$, ES-L $p = 0.014$). There was no effect of eyesalve batch (batch $F_{1,12} = 0.443$, $p = 0.518$; interac-

tion $F_{2,12} = 0.583$, $p = 0.573$). The graph shows the mean of data from six mice per treatment (three for each batch of the eyesalve).

Data S1. Bacterial species cultured from recipes and ingredient preparations.

Recipe Base	Control	ESO	ESL	ES	ESO				ESL			
Minus	-	-	-	O/L	X	W	G	B	X	W	G	B
Activity	-	F	F	P	P	N	N	F	F	N	N	F

Y-axis: Total viable cells in artifical wound (bars show mean ± s.e.)