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Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens

P. H. S. Kwakman, L. Boer, C. P. Ruyter-Spira, T. Creemers-Molenaar, J. P. F. G. Helsper, C. M. J. E. Vandenbroucke-Grauls, S. A. J. Zaat, A. A. Velde

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Abstract: Purpose. Honey has potent activity against both antibiotic-sensitive and -resistant bacteria and is an interesting agent for topical antimicrobial application to wounds. As honey is diluted by wound exudate, rapid bactericidal activity up to high dilution is a prerequisite for its successful application.

Methods. We investigated the kinetics of killing of antibiotic-resistant bacteria by RS honey, the source for production of Revamil® medical-grade honey, and we aimed to enhance the rapid bactericidal activity of RS honey by enrichment with its endogenous compounds or addition of antimicrobial peptides.

Results. RS honey killed antibiotic-resistant isolates of *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Enterococcus faecium* and *Burkholderia cepacia* within 2 hours, but lacked such rapid activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase-producing (ESBL) *Escherichia coli*. It was not feasible to enhance the rapid activity of RS honey by enrichment with endogenous compounds, but RS honey enriched with 75 µM of the synthetic peptide Bactericidal Peptide 2 (BP2) showed rapid bactericidal activity against all species tested, including MRSA and ESBL *E. coli*, at up to 10-20-fold dilution.

Conclusions. RS honey enriched with BP2 rapidly killed all bacteria tested and had a broader spectrum of bactericidal activity than either BP2 or honey alone.

Response to Reviewers: Reviewer #1:

This is an interesting paper, but I believe that the abstract does not adequately reflect the content of your paper and needs a complete revision. Surely the purpose was to evaluate the antibacterial effects of a medical grade honey that had been enriched with antimicrobial peptides? Also your methods as described in the abstract suggest that you only tested RS honey without supplements.

AUTHOR: abstract has been adjusted; more emphasis on enrichment with antimicrobial peptides (e.g. pag. 3, line 9-10).

On page 6 in the methods and materials MRSE is not included.

AUTHOR: MRSE is now described in the M&M section.

On page 7 was TSB prepared according the manufacturer's instructions and further diluted 3% (w/v)? or simply made up according to the manufacturer's instructions, with 1/10 dilution of TSB subsequently used to wash harvested cells?

AUTHOR: TSB was prepared according to the manufacturers' instructions, used full strength to grow the bacteria, and used in a final concentration of 1% (v/v) in 10 mM phosphate buffer pH 7.0. This is corrected in the text (pag. 7, line 17-20).

On page 8, I suggest that "incubations" is changed to "samples from the incubated flasks".

AUTHOR: "Incubations" has been changed to "suspensions".

Also in line 50 of page 8 and on page 9 line 2 insert (v/v) after 20% and 40% respectively.

AUTHOR: text is adjusted.

Throughout this paper terms such as killing and killed appear rather extreme. Normally terms such as kinetics of inhibition and reducing populations to undetectable levels would be considered to be more suitable.

AUTHOR: we measured bactericidal, not inhibiting activity. When appropriate, 'killing' has been modified to 'reduced to undetectable levels'.

On page 14 the statement that hydrogen peroxide is frequently used in wound cleansing should either be qualified by a suitable citation or modified. My understanding is that it is no longer used extensively in wound care.

AUTHOR: hydrogen peroxide is still used in clinical practice, but indeed not recommended in guidelines due to concerns regarding toxicity/delayed wound healing. This is adjusted in the text (pag. 15, line 13-15).

The discussion might have included other methods of detecting hydrogen peroxide, for example: Bang LM, Buntting C, Molan PC (2003) The effects of dilution rate on hydrogen peroxide production in honey and its implications for wound healing *J. Altern. Complement. Med.* 9 (21): 267-273 Brudzynski K. (2006).Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. *Canadian Journal of Microbiology* 52 (12):1228-1237.

AUTHOR: with respect to production of hydrogen peroxide by glucose oxidase in diluted honey, we now added citations referring to White et al. (1963) and Bang et al. (2003) (pag. 15, line 4). In our opinion the study of Brudzynski lacks sufficient novelty with respect to this subject to justify citation.

Furthermore, the effects of honey in inhibiting *B. cepacia* have been reported in Cooper, R.A., Wigley, P., & Burton, N.F (2000) Susceptibility of multiresistant strains of *Burkholderia cepacia* to honey. *Letters in Applied Microbiology*, 31 20-24. Reference to this work would be relevant

AUTHOR: this reference is indeed relevant and has been included in the manuscript (pag. 17, line 15).

Reviewer #2: General

The aim of this study should be clearly stated. It appears that the aim of the work was to look at the effectiveness of RS honey over time and to see if its activity could be enhanced with various additives. Although it is not clear why this may be of benefit. There is no discussion of why one wouldn't simply use the peptides or why adding them to the honey would be worthwhile.

AUTHOR: peptide-enriched honey has a broader spectrum of bactericidal activity than either agent alone. The aim is stated more clearly in the abstract (pag. 3, line 9-10) and the benefit of combining honey with peptides is discussed more elaborate the revised manuscript (pag. 17, lines 13-17 and lines 19-21).

It does not appear that using RS honey as a wound dressing would be particularly advantageous compared to other commercially available, medically registered honeys as it seems to have relatively low antibacterial activity. Comparing RS to other medical honeys would be of benefit to determine if this is the case.

AUTHOR: Revamil is unique with respect to its method of production. It is produced under controlled conditions in greenhouses, resulting in low variation in antibacterial activity compared to other medical grade honeys.

Activity of honey is mostly measured in growth-inhibition assays, but in our opinion measuring the bactericidal activity is a more stringent approach and is highly clinically relevant. Comparison of activity of different honeys indeed is relevant, but will require the use of one test system, since the growth-inhibiting and bactericidal activity of honeys cannot be compared directly. Comparison of bactericidal activity of RS and other honeys was however not the purpose of the present work.

It looks as though the activity is predominantly due to the osmotic effects of this honey - but it is difficult to tell as no sugar controls were included and this would have been useful.

AUTHOR: in the current manuscript we focus on the kinetics of bactericidal activity of RS honey, irrespective of the components that contribute to this activity. In the introduction we refer to our recent publication in The FASEB Journal, which reported the full dissection of the mechanism of bactericidal activity of RS honey. In that study we clearly demonstrated that several factors other than sugar contribute substantially to the bactericidal activity of honey.

The manuscript is quite long and there is quite a bit of repetition in the Results and Discussion sections (see below)

"Broad spectrum" should generally be "broad-spectrum"

AUTHOR: 'broad spectrum' has been changed to 'broad-spectrum' throughout the text.

Introduction

Line 36 - The variation usually seen in the activity of different types of honey is predominantly due to the floral source of the honey. What is the floral source of this honey? This is important if this honey is intended for medicinal use.

AUTHOR: different honeys show large variation in activity, which is not solely a result of floral origin; despite the identical floral source, different batches of Manuka honey show a very large variation in activity. As mentioned above, RS honey is different from regular honeys in that it is produced under standardized conditions. Details of this production platform are proprietary information of the manufacturer. Since the honey has been granted CE-certification for application on wounds there is no problem regarding its medical use.

As the antibacterial peptides feature extensively in this work it would be worth briefly introducing them in this section.

AUTHOR: a paragraph briefly describing the activity of antimicrobial peptides has been added to the 'Introduction' section (pag. 5, line 4-13).

Methods

Line 41 - how were the bacterial strains identified and stored?

AUTHOR: details regarding bacterial isolates have been added (pag. 6, line 21-23). Strains were identified and stored according to general medical microbiological practice. We consider this common knowledge among the readership of EJCMIID, and did not add this to the text.

Results

Quite a bit of material that would be more appropriate for either the Introduction or Discussion sections is included here. While this may be appropriate for this journal it adds unnecessary length to this manuscript, making it repetitive. E.g. page 10 lines 29 -34, 54-58; page 11 lines 5-9, 18-31; page 12 lines 16-24, etc. Some of the figure legends contain too much information, in particular explicitly stating results that are clearly shown in the figures is redundant.

AUTHOR: unnecessary repetitive phrases in results section and in figure legends were removed (e.g. phrases on page 11 lines 5-9 in the original manuscript are removed and the legends of fig. 5 & 6 are now condensed).

It would have been worth including a sugar control in these experiments as the concentrations of RS honey needed to inhibit the test organisms seem quite high compared to those one would expect from other medical grade honeys (see further comment in Discussion section).

AUTHOR: discussed in reply to the 'General' comments of this reviewer.

Page 11, line 43 - from figure 5 it does not look like there is a substantial improvement in activity against VREF, although it does look like there is one for MRSE. Is the figure mislabelled? This statement is also made in the Discussion.

AUTHOR: data for VREF and MRSE were indeed mixed up, and is now corrected, thank you.

Page 12, line 38 - the controls with just honey seem to have produced different results from those in the previous experiment when comparing figures 5 and 6.

AUTHOR: in figure 5 & 6, the LC99.9 (the lowest concentration that results in at least 1000-fold reduction in numbers of CFU) is indicated for 2-fold dilution series of honey. Minor inter-experiment variation can thus result in an apparent 2-fold change in activity when survival of bacteria is near the cut-off for the LC99.9. Indeed, the differences in activity of honey in figure 5 & 6 never exceed one dilution step.

Page 12, line 43 - same comment as for Page 11, line 43 above, are MRSE and VREF mixed up?

AUTHOR: data for VREF and MRSE were indeed mixed up, and is now corrected, thank you.

Discussion

Much of the Discussion is a repeat of the Results adding unnecessary length to the manuscript. The whole section could be more concise. For example, the MGO paragraph is rather long considering MGO was not tested here.

AUTHOR: we have condensed the paragraph on MGO, and also removed unnecessary repetition.

It would be of interest to name the organisms referred to in reference 13, i.e. which "other bacteria" (page 13 line 34) does RS honey have rapid activity against?

AUTHOR: the 'other bacteria' refers to results of the current manuscript, and not to reference 13 as assumed by the reviewer. The text has been modified for clarity, and the bacteria are now named explicitly (pag. 14, lines 10-14).

No sugar controls were included and the RS honey activity appears be predominantly due to the osmotic effect of the sugars. This honey does not appear to have been selected based on its floral source, as other medical grade honeys generally are, so RS may not have an inherently high level of activity.

AUTHOR: the reviewer has indicated this point previously; see our reply in the 'General' comments section of this reviewer.

Page 15 line 39 - "Because of the lack of rapid bactericidal activity against MGO in Manuka honey" needs a reference. Also this is the first time Manuka is mentioned, for those not well acquainted with the field of antibacterial honey some explanation as to the relevance of discussing it here would be helpful.

AUTHOR: Manuka honey is now briefly described in the introduction (pag. 4, lines 13-17). The statement regarding lack of rapid activity of MGO has been removed in the act of condensation of the paragraph on MGO in the Discussion section, so no reference is required.

Page 16 line 14 - the abbreviation AMP is introduced without definition

AUTHOR: this abbreviation was indeed not clarified in the Discussion section, this is now corrected.

Page 17 line 7 - it is not appropriate to say that *B. cepacia* is "highly" susceptible to the RS honey. Quite high concentrations were used here, see sugars comment above.

AUTHOR: we have modified this phrase, now stating that *B. cepacia* is relatively susceptible to honey compared to other bacteria (pag. 17, lines 13-15).

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1 Title page

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3 Title:

4 Medical-grade honey enriched with antimicrobial peptides has enhanced activity
5 against antibiotic-resistant pathogens

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21 Keywords:

22 Antibacterial agents

23 Antimicrobial peptides

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1 honey

2 MRSA

3 E. coli ESBL

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5 Running title: Enrichment of medical-grade honey

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4 1 **ABSTRACT**
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9 3 **Purpose.** Honey has potent activity against both antibiotic-sensitive and -resistant
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11 4 bacteria and is an interesting agent for topical antimicrobial application to wounds.
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14 5 As honey is diluted by wound exudate, rapid bactericidal activity up to high dilution
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16 6 is a prerequisite for its successful application.
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18 7 **Methods.** We investigated the kinetics of killing of antibiotic-resistant bacteria by
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20 8 RS honey, the source for production of Revamil[®] medical-grade honey, and we
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22 9 aimed to enhance the rapid bactericidal activity of RS honey by enrichment with its
23
24 10 endogenous compounds or addition of antimicrobial peptides.
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27 11 **Results.** RS honey killed antibiotic-resistant isolates of *Pseudomonas aeruginosa*,
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29 12 *Staphylococcus epidermidis*, *Enterococcus faecium* and *Burkholderia cepacia*
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31 13 within 2 hours, but lacked such rapid activity against methicillin-resistant
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33 14 *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase-
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35 15 producing (ESBL) *Escherichia coli*. It was not feasible to enhance the rapid activity
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37 16 of RS honey by enrichment with endogenous compounds, but RS honey enriched
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39 17 with 75 µM of the synthetic peptide Bactericidal Peptide 2 (BP2) showed rapid
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41 18 bactericidal activity against all species tested, including MRSA and ESBL *E. coli*,
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43 19 at up to 10-20-fold dilution.
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46 20 **Conclusions.** RS honey enriched with BP2 rapidly killed all bacteria tested and
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48 21 had a broader spectrum of bactericidal activity than either BP2 or honey alone.
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4 **1 INTRODUCTION**
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9 3 Antibiotic-resistant bacteria pose a very serious threat to public health. For all
10 4 antibiotic classes, including the major last resort drugs, resistance is increasing
11 5 worldwide [1,2]. Even more alarming, very few new antibiotics are being developed
12 6 [1,3], so alternative antimicrobial strategies are urgently needed.
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19 7 The potent *in vitro* activity of honey against antibiotic-resistant bacteria [4]
20 8 and its successful application in treatment of chronic wound infections not
21 9 responding to antibiotic therapy [5] resulted in a revival of the interest in honey as
22 10 antibacterial agent [6-8]. Important prerequisites for application of honey as
23 11 antimicrobial agent are reproducible and rapid bactericidal activity [9] and
24 12 knowledge of its mechanism of action.
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33 13 honeys collected from the natural environment including Manuka honey,
34 14 which is used for production of most currently available medical-grade honeys,
35 15 show large variation in antibacterial activity [10,11]. Manuka honey can contain
36 16 very high levels of MGO, which is regarded the major antibacterial compound in
37 17 this honey [12,13]. The honey used as a source for Revamil[®] medical-grade honey
38 18 (RS honey) is produced under standardized conditions in greenhouses, and is
39 19 sterilized by gamma-irradiation to kill potentially present bacterial spores. Gamma
40 20 irradiation is known not to affect honey bactericidal activity [14]. Revamil[®] has
41 21 broad-spectrum, batch-to-batch reproducible bactericidal activity *in vitro*. It has
42 22 been shown that it can strongly diminish microbial colonization of the human skin
43 23 [15]. We have recently identified all major bactericidal factors in RS honey, i.e. its
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1 high sugar concentration, H₂O₂, low pH, methylglyoxal (MGO) and the cationic
2 antimicrobial peptide bee defensin-1 [16].

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4 Antimicrobial peptides are known for their potent, rapid broad-spectrum
5 microbicidal activity. Their supposed mechanism of action is direct targeting of
6 microbial membranes [17], although AMPs may also have intracellular targets
7 [18,19]. A cationic domain of these peptides specifically interacts with the
8 negatively charged outer surfaces of micro-organisms and a hydrophobic domain
9 is required for membrane perturbation or penetration, either causing membrane
10 disruption or translocation into the cell [20,21]. Some bacteria have evolved
11 mechanisms to reduce their outer surface negative charge to reduce susceptibility
12 to AMPs [22]. Since this involves complex biosynthetic pathways, the risk for rapid
13 resistance-development against AMPs is considered low [22].

14 In the current study we show that RS honey has potent bactericidal activity,
15 but this requires prolonged exposure of the target organisms. RS honey lacks rapid
16 bactericidal activity against several important antibiotic-resistant wound pathogens,
17 including methicillin-resistant *Staphylococcus aureus*. We were not able to
18 augment the bactericidal activity of RS honey by enrichment with endogenous
19 honey bactericidal compounds, but addition of the synthetic antimicrobial peptide
20 BP2 did result in broad-spectrum rapid bactericidal activity.

21

1 MATERIALS AND METHODS

3 Honey

4 Unprocessed Revamil source (RS) honey was kindly provided by Bfactory Health
5 Products (Rhenen, The Netherlands).

7 Peptides

8 BP2 (GKWKLFKAFKKFLKILAC) and LL-37 were synthesized at Pepscan
9 Systems (Lelystad, The Netherlands) using solid-phase Fmoc (9-
10 fluorenylmethoxycarbonyl) chemistry with a free amine at the N-terminus and a
11 free amide at the C-terminus. Peptides were HPLC-purified and purity (>95%) and
12 mass were confirmed by ionspray mass spectrometry. The lack of disulphide
13 formation between free cysteines of BP2 was confirmed by Q-TOF/MS analysis.

15 Microorganisms

16 Bactericidal activity was assessed against clinical isolates of methicillin-resistant
17 *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis*
18 (MRSE), vancomycin-resistant *Enterococcus faecium* (VREF), extended-spectrum
19 beta-lactamase producing *Escherichia coli* (*E. coli* ESBL) and *Pseudomonas*
20 *aeruginosa* ESBL and against the *Burkholderia cepacia* ATCC 25416 type strain.
21 Oxacillin susceptibility of *S. aureus* and *S. epidermidis* and vancomycin
22 susceptibility of *E. faecium* were determined by Etest (AB Biodisk) according to the
23 manufacturers' instructions. ESBLs were identified as described by Al Naiemi [23].

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2 **Determination of H₂O₂ concentration in honey**

3 Hydrogen peroxide concentrations in honey were determined quantitatively using a
4 modification of a method described by White & Subers [24]. Undiluted and ten-fold
5 diluted samples of honey (40 µl) were mixed in wells of microtiterplates with 135 µl
6 reagent, consisting of 50 µg/ml o-dianisidine (Sigma) and 20 µg/ml horseradish
7 peroxidase type IV (Sigma) in 10 mM phosphate buffer pH 6.5. o-Dianisidine was
8 freshly prepared as a 1 mg/ml stock in demineralized water and peroxidase was
9 diluted from a 10 mg/ml stock in 10 mM phosphate buffer pH 6.5 stored at -20 °C.
10 After 5 min. of incubation at room temperature, reactions were stopped by addition
11 of 120 µl 6 M H₂SO₄ and absorption at 540 nm was measured. Hydrogen peroxide
12 concentrations were calculated using a calibration curve of 2-fold serial dilutions of
13 H₂O₂ ranging from 2200 to 2.1 µM.

14

15 **Liquid bactericidal assay**

16 Bactericidal activity was quantitatively assessed in low protein binding
17 polypropylene microtiterplates (Costar Corning). Bacteria from logarithmic phase
18 cultures in trypticase soy broth (TSB; BD Difco) were washed twice with incubation
19 buffer containing 10 mM phosphate buffer pH 7.0 supplemented with 1% (v/v) TSB
20 and were suspended at a concentration of 5x10⁶ CFU/ml, based on optical density.
21 A 50% (v/v) stock solution of honey was freshly prepared in incubation buffer. For
22 enrichment with antimicrobial peptides, an aliquot of 1.2 mM LL-37 or BP2 stock
23 solutions was added to 50% honey solutions to obtain 37.5 µM of peptide, thus

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1 corresponding to enrichment of undiluted honey with 75 µM of the respective
2 peptides. Eighty microliter of diluted honey was mixed with 20 µl of a bacterial
3 inoculum containing 5×10^6 CFU/ml, and plates were incubated at 37°C on a rotary
4 shaker at 150 rpm. At indicated time points, duplicate 10 µl aliquots of undiluted
5 and 10-fold serially diluted suspensions were plated on blood agar. The dilutions
6 were prepared in incubation buffer containing 0.025% sodium
7 polyanetholsulphonate (SPS; Sigma), which neutralizes cationic bactericidal
8 components [25]. Bacterial survival was quantified after overnight growth at 37 °C.
9 The detection level of this assay is 100 CFU/ml.

10 To determine the LC99.9 values of LL-37 and BP2, 25 µl aliquots of 2-fold serially
11 diluted peptide in incubation buffer were prepared in polypropylene microtiterplates
12 (Costar Corning) and to each of the wells 25 µl of a bacterial suspension
13 containing 2×10^6 CFU/ml was added. After 2 h of incubation on a rotary shaker at
14 150 rpm at 37°C, triplicate 10 µl aliquots were plated on blood agar plates. The
15 plates were inspected for growth after 24 h. LC99.9 was defined as the lowest
16 concentration of peptide which killed >99.9% of the inoculum of 10^6 CFU/ml after 2
17 h.

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19 **Partial purification of bee defensin-1**

20 We previously demonstrated that bee defensin-1 is the only bactericidal
21 factor in the >5 kDa fraction of RS honey [16]. To prepare a >5 kDa fraction, 15 ml
22 of 20% (v/v) honey was centrifuged in a 5 kDa molecular weight cut-off Amicon
23 Ultra-15 tube (Millipore) at 4000 x g for 45 min. at room temperature. The >5 kDa

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1 retentate was subsequently washed three times in the filter tube with 15 ml of
2 demineralized water and concentrated to 0.3 ml.

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4 1 **RESULTS**
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9 3 **Kinetics of bactericidal activity of RS honey**
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11 4 We determined the kinetics of bactericidal activity of different dilutions of RS
12 5 honey against various antibiotic-resistant pathogens. RS honey at a concentration
13 6 of 40% (v/v) reduced survival of methicillin-resistant *Staphylococcus epidermidis*
14 7 (MRSE), vancomycin-resistant *Enterococcus faecium* (VREF), enhanced-spectrum
15 8 beta-lactamase-producing (ESBL) *Pseudomonas aeruginosa* and *Burkholderia*
16 9 *cepacia* to undetectable levels within 2 hours, while similar activity against
17 10 methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL *Escherichia coli*
18 11 required 6 hours of incubation (Fig. 1). RS honey at a concentration of 20% killed
19 12 *B. cepacia* within 4 hours of incubation, while activity against all other bacteria
20 13 required 24 hours of incubation (Fig. 1). RS honey diluted to 10% killed MRSA and
21 14 MRSE after 24 hours but lacked activity against all other bacteria tested (Fig. 1).
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38 15 Even in undiluted RS honey, survival of MRSA was not affected within 2
39 16 hours (supplemental Fig. 2) of incubation and numbers of CFU of *E. coli* ESBL
40 17 were only reduced by 2.3-log. After 24 hours incubation in undiluted honey,
41 18 survival of *E. coli* was reduced to undetectable levels but numbers of CFU of
42 19 MRSA were only reduced by 1-log (supplemental Fig. 2). In summary, RS honey
43 20 did not rapidly kill MRSA and *E. coli* ESBL, and rapid activity against all other
44 21 species tested was abolished upon dilution of the honey to $\leq 20\%$.
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H₂O₂ concentration required for rapid killing of MRSA

Since MRSA and *E. coli* ESBL were not rapidly killed by RS honey, we assessed whether the endogenous concentrations of bactericidal compounds in RS honey might be too low for rapid activity. Dilution of RS honey to 30% was optimal for H₂O₂ accumulation, resulting in maximal concentrations of 22.5 ± 1.3 µg/ml and 148.4 ± 27.8 µg/ml H₂O₂ after 2 and 24 hours, respectively (Fig. 3a) In incubations not containing honey but only H₂O₂, 3200 µg/ml H₂O₂ was required to kill MRSA within 2 hours (Fig. 3b). This is 142 times the concentration of H₂O₂ that maximally accumulated in RS honey after a similar 2 hours incubation period, indicating that the H₂O₂ concentration in honey is far too low for rapid activity against MRSA. In view of possible toxicity (see discussion), it is not feasible to increase the concentration of H₂O₂ in honey to the level required for rapid killing. For similar reasons we did not assess enrichment of RS honey with MGO.

Bee defensin-1 concentration required for rapid killing of MRSA

Next, we assessed the contribution of bee defensin-1 to rapid bactericidal activity of RS honey. At the concentration present in undiluted RS honey, bee defensin-1 effectively killed *Bacillus subtilis*, a highly bee defensin-1-susceptible organism [16], within 2 hours (Fig. 4a). However, this peptide had no substantial activity against MRSA, *E. coli* ESBL or VREF (Fig. 4a). Even at an 8-fold higher concentration, bee defensin-1 reduced numbers of CFU of MRSA after 2 hours by

1 only 1-log (Fig. 4b), indicating that it also was not feasible to enhance the rapid
2 bactericidal activity of RS honey by increasing the concentration of bee defensin-1.

3 4 5 **Enhanced rapid bactericidal activity of LL-37-enriched honey**

6 We subsequently assessed the potential of LL-37, a broad-spectrum human
7 antimicrobial peptide expressed in neutrophils and various epithelial cells [26,27],
8 to enhance the rapid bactericidal activity of honey. RS honey was enriched with
9 tenfold excess (75 μ M) of the concentration LL-37 required to reduce survival of
10 MRSA to undetectable levels (Fig. 5a). The lethal concentration of LL-37 for
11 99.9% of the inocula (LC99.9) of MRSA and *E. coli* in 2 hour incubations were 7.5
12 and 1.9 μ M, respectively (Fig. 5a). Enrichment of RS honey with LL-37
13 substantially improved the activity against *E. coli* ESBL and VREF but not against
14 other bacteria tested (Fig. 5b). LL-37-enriched honey retained bactericidal activity
15 against *E. coli* ESBL and VREF up to 20-fold dilution, which was a major
16 improvement compared to non-enriched honey (Fig. 5b). The LC99.9 of LL-37 in
17 honey however was 4-fold higher than in incubation buffer (Fig. 5b), indicating that
18 honey inhibited LL-37. Inhibition was even more clear from the tests with MRSA,
19 MRSE and *P. aeruginosa*. Enrichment with LL-37 did not substantially improve the
20 activity of honey while LL-37 alone effectively killed these bacteria (Fig. 5b).

21 22 23 **Enhanced bactericidal activity of BP2-enriched honey**

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1 The synthetic antimicrobial peptide Bactericidal Peptide 2 (BP2) has potent
2 activity in physiological salt concentrations and in plasma, and is effective *in vivo* in
3 a mouse model of biomaterial-associated infection[28]. The LC99.9 concentrations
4 of BP2 for MRSA and *E. coli* in 2 hour incubations were 1.9 and 3.8 μ M,
5 respectively (Fig. 6a). RS honey enriched with tenfold excess (75 μ M) of the
6 concentration BP2 required to reduce survival of MRSA to undetectable levels (Fig.
7 6a) retained bactericidal activity against all bacteria tested up to 20-fold dilution,
8 except for *P. aeruginosa* (Fig. 6b), which was killed by up to a 10-fold dilution of
9 this enriched RS honey (Fig. 6B). Non-enriched RS honey lacked rapid bactericidal
10 activity for all bacteria tested when diluted more than 2.5-fold.

11 The activity of BP2 against *E. coli* ESBL and VREF was not inhibited in
12 honey (Fig. 6b). Killing of MRSA, MRSE and *P. aeruginosa* ESBL required 2-4 fold
13 higher concentrations of BP2 in honey than in buffer (Fig. 6b). This indicates a
14 slight reduction of BP2 activity in honey, but markedly less than the observed
15 inhibition of LL-37 in honey. We conclude that enrichment with 75 μ M BP2
16 markedly enhanced the rapid bactericidal activity of RS honey.

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4 1 **DISCUSSION**
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10 The potent activity against antibiotic-resistant pathogenic bacteria makes
11 honey an interesting agent to treat topical infections not responding to antibiotics.
12 Ideally, honey used for such applications should have rapid and broad-spectrum
13 bactericidal activity. In addition, honey should remain active upon dilution, since
14 honey will rapidly be diluted at the wound interface due to its hygroscopic
15 characteristics and the presence of wound exudate.
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23 9 RS honey has reproducible, broad-spectrum bactericidal activity *in vitro* and
24 effectively reduces microbial colonization of human skin [15]. Our present results,
25 however, show that the activity of this honey against the major wound-infecting
26 pathogens MRSA and *E. coli* ESBL is not rapid. Honey does have rapid activity
27 against *P. aeruginosa*, *E. faecium* and *S. epidermidis* but this activity is lost when
28 RS honey becomes diluted. Of note, *B. cepacia*, an otherwise notoriously antibiotic
29 resistant pathogen, proved to be the most honey-susceptible organism.
30 Enhancement of activity of RS honey with its endogenous microbicidal compounds
31 appeared not to be feasible, but addition of the cationic antimicrobial peptide BP2
32 did increase the activity to the desired levels.
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50 20 We recently identified all bactericidal factors in RS honey using an approach
51 of successive neutralization of individual factors combined with activity-guided
52 isolation of factors responsible for residual bactericidal activity [16]. Thus, we
53 determined that the high sugar concentration, H₂O₂ production, MGO, the low pH
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1 and bee defensin-1 were responsible for bactericidal activity of RS honey. In order
2 to enhance the rapid bactericidal activity of RS honey, we first assessed the
3 potential of H₂O₂ and of bee defensin-1. Upon dilution of honey, H₂O₂ is produced
4 by the glucose oxidase enzyme from the bees [24,29]. Production of H₂O₂ was
5 highest in RS honey diluted to 30%, in which 22.5 and 148 µg/ml accumulated
6 after 2 and 24 hours, respectively. In a study with 90 different honeys, 12 ± 19
7 µg/ml hydrogen peroxide (range 0 - 72 µg/ml) accumulated in honey diluted to 20%
8 (w/v) after 4 hours [24], indicating that RS honey produces relatively high levels of
9 H₂O₂.

10 MRSA is highly susceptible to H₂O₂ -mediated killing by RS honey upon
11 incubation for 24 hours [16]. Killing of MRSA within 2 hours by H₂O₂ in absence of
12 honey required a concentration as high as 3200 µg/ml H₂O₂ (corresponding to a
13 0.32% (w/v) solution). Wound cleansing with a 3% (w/v) solution of H₂O₂ has been
14 a clinical practice, but at this concentration H₂O₂ is toxic to human cells and skin
15 tissue and tissue exposure can result in delayed wound healing [30-32] . Although
16 the concentration of H₂O₂ required to kill MRSA was about 10-fold lower than the
17 concentration used for wound cleansing, we did not consider increasing the levels
18 of H₂O₂ as a possibility to improve the rapid bactericidal activity of RS honey.

19 Bee defensin-1 (also referred to as royalisin [33]) is a 51-residue
20 antimicrobial peptide identified in honey bee hemolymph, royal jelly and in honey
21 [16,33,34]. Because of its complicated folding with three intramolecular cysteine
22 bonds, synthetic production of bee defensin-1 is not possible and recombinant
23 production would be highly challenging. Bee defensin-1 effectively kills *B. subtilis*

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1 but lacks activity against all other bacteria tested at a concentration equivalent to
2 that in undiluted honey. Even at an 8-fold higher concentration, bee defensin-1
3 only slightly reduced survival of MRSA. The narrow spectrum of its bactericidal
4 activity renders bee defensin-1 unsuited for enhancement of the bactericidal
5 activity of honey.

6 MGO is present in RS honey at a relatively low concentration (0.25 mM)
7 compared to the concentrations reported for Manuka honey (up to 16.1 mM) [12].

8 MGO is a reactive metabolite that can exert toxic effects by direct inhibition of
9 enzymes, by genomic modifications resulting in carcinogenesis and by protein
10 modifications resulting in the formation of advanced glycation endproducts [35].

11 The latter are believed to be main determinants for pathological effects related to
12 diabetes [36,37]. Because of the concerns regarding potential toxicity of MGO we
13 did not pursue to augment the activity of RS honey with this compound.

14 Other honey bactericidal factors are the high sugar concentration and low
15 pH. Honey is a super-saturated sugar solution, so it is not possible to further
16 increase its sugar concentration. RS honey has a pH of 3.2, which is at the lower
17 end of the pH range found for honeys (3.2 - 4.5) [16]. Even such a low pH only
18 contributed to the activity against *B. subtilis* after 24 hours of incubation, and not to
19 the activity against other bacterial species [16]. Therefore, the sugar concentration
20 and low pH were not suited as factors for enhancement of the bactericidal activity
21 of honey.

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1 In contrast to bee defensin-1, most antimicrobial peptide (AMPs) have
2 broad-spectrum bactericidal activity. The human α -helical AMP LL-37 is one of the
3 best characterized AMPs [38-41]. Despite its potent activity in incubation buffer,
4 LL-37 was strongly inhibited in the presence of honey. BP2 is a synthetic AMP with
5 very rapid broad-spectrum microbicidal activity, which is retained in plasma and in
6 physiological salt solution [28]. BP2 also effectively kills *S. epidermidis in vivo*, in a
7 murine model of biomaterial-associated infection [28], indicating its potential for
8 clinical application. The activity of BP2 against *E. coli* ESBL and VREF was not
9 inhibited in honey, and activity against MRSA, MRSE and *P. aeruginosa* ESBL
10 required only slightly higher (2-4 fold) concentrations in honey than in buffer. Thus,
11 BP2 certainly was suited for enrichment of honey.

12 *B. cepacia* is notorious for its intrinsic resistance against antibiotics [42,43] and
13 AMPs [44] and is indeed not susceptible to LL-37 [44] or to BP2 [28]. Our results
14 demonstrate however that *B. cepacia* is relatively susceptible to honey compared
15 to other tested bacteria, which is in accordance with findings of Cooper *et al.* [45].
16 Not surprisingly, addition of BP2 to RS honey did not enhance the bactericidal
17 activity against *B. cepacia* (not shown).

18 In summary, we were able to enhance the bactericidal activity of honey by
19 enrichment with the antimicrobial peptide BP2. BP2-enriched RS honey had rapid
20 bactericidal activity up to a high dilution against all bacteria tested and had a
21 broader spectrum of bactericidal activity than either agent alone. This offers
22 prospects for development of clinically applicable honey-based antimicrobials with
23 rapid and broad-range microbicidal activity.

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3 of Economic Affairs.

5 **Conflict of interest**

6 None to declare

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4 1 **FIGURE LEGENDS**
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11 4 **Fig. 1 Kinetics of killing of various antibiotic-resistant bacteria by RS honey.**

12 5 Bacteria were incubated in honey diluted to 40% (squares), 20% (triangles) and
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14 6 10% (diamonds). At indicated time points survival was determined quantitatively.
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21 8 **Fig. 2 Bactericidal activity of undiluted RS honey.** Bactericidal activity of

22 9 undiluted honey against MRSA (white bars) and *E. coli* ESBL (grey bars). Inocula
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24 10 of approximately 10^5 CFU in 4 microliter were added to 0.5 ml undiluted honey. At
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26 11 indicated time points, samples were diluted with one volume of incubation buffer
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29 12 and subsequently survival was quantified as described for the liquid bactericidal
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38 15 **Fig. 3 Concentration of H₂O₂ required for activity against MRSA.** (A)

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41 16 Production of H₂O₂ in diluted RS honey. Accumulation of H₂O₂ in indicated
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43 17 concentrations RS honey was assessed at 2 hours (squares) or 24 hours
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45 18 (triangles) after dilution. (B) Bactericidal activity of solutions containing only H₂O₂.
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48 19 Survival of MRSA was assessed after 2 hours (white bars) or 24 hours (grey bars)
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51 20 of incubation with the indicated concentrations of H₂O₂.
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55 22 **Fig. 4 Bactericidal activity of bee defensin-1.** Bactericidal activity of bee

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58 23 defensin-1 was assessed using the >5 kDa fraction of RS honey in which bee
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1 defensin-1 is the only antibacterial factor present [16]. (A) Bactericidal activity of
2 bee defensin-1 against various bacteria was assessed after 2 hours (grey bars) or
3 24 hours (hatched bars) of incubation at the concentration of this peptide as
4 present in undiluted RS honey. As a control, bacteria were incubated for 2 hours in
5 incubation buffer (white bars). (B) Bactericidal activity of concentrated bee
6 defensin-1 against MRSA after 2 hours of incubation. Incubation in buffer was used
7 as a control.

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9 **Fig. 5 Efficacy of LL-37 to enhance rapid bactericidal activity of RS honey.** (A)

10 Survival of MRSA (squares) and *E. coli* ESBL (triangles) after 2 hours of incubation
11 in indicated concentrations of LL-37 in incubation buffer. (B) Bactericidal activity of
12 LL-37 enriched RS honey. Indicated microorganisms were incubated for 2 hours in
13 2-fold serial dilutions of 75 μ M LL-37 (white bars), RS honey alone (grey bars) or
14 RS honey enriched with 75 μ M LL-37 (black bars). The highest dilutions of these
15 preparations killing at least 99.9% of the inocula are indicated. Mean \pm SEM values
16 of independent triplicate incubations are shown.

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19 **Fig. 6 Efficacy of BP2 to enhance rapid bactericidal activity of RS honey.** (A)

20 Survival of MRSA (squares) and *E. coli* ESBL (triangles) after 2 hours of incubation
21 in indicated concentrations of BP2 in incubation buffer. (B) Bactericidal activity of
22 BP2-enriched RS honey. Indicated microorganisms were incubated for 2 hours in
23 2-fold serial dilutions of 75 μ M BP2 (white bars), RS honey alone (grey bars) or RS

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1 honey enriched with 75 μ M BP2 (black bars). The highest dilutions of these
2 preparations killing at least 99.9% of the inocula are indicated. Mean \pm SEM values
3 of independent triplicate incubations are shown.

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1 Staphylococcus epidermidis experimental biomaterial-associated infection
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Figure 1

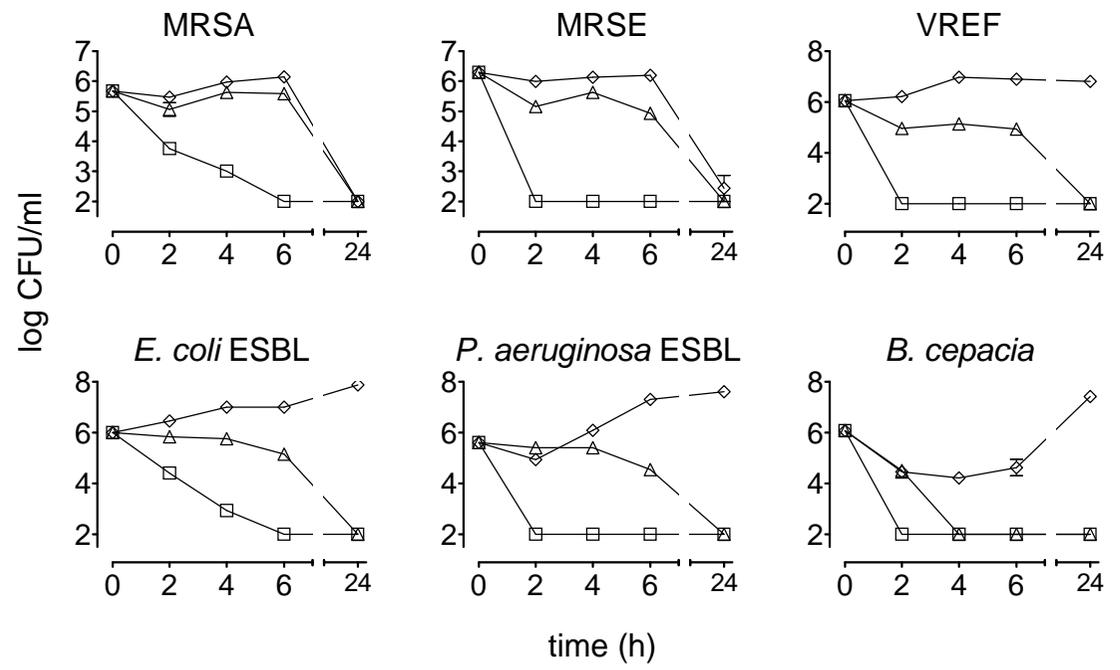


Figure 2

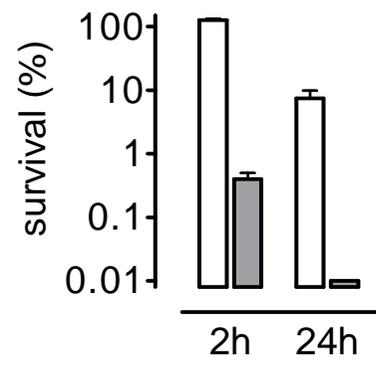


Figure 3

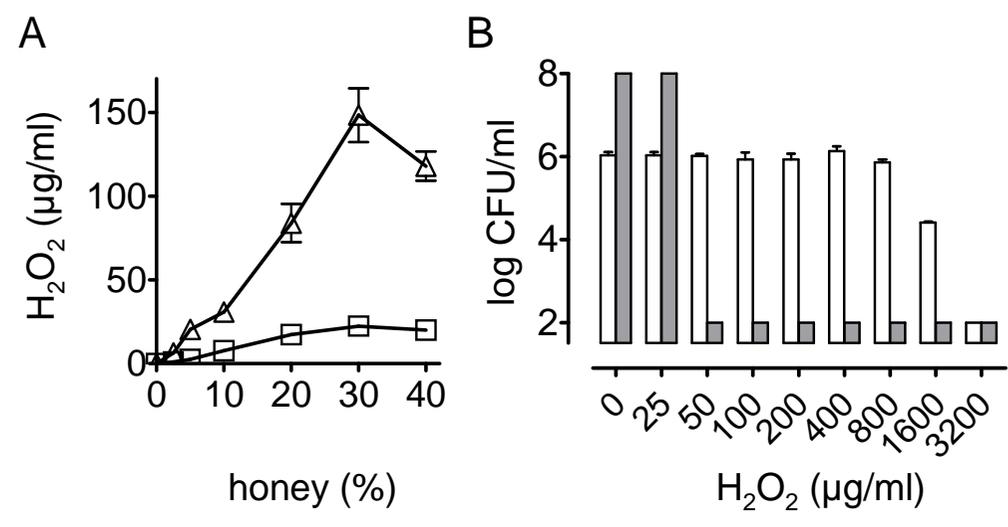


Figure 4

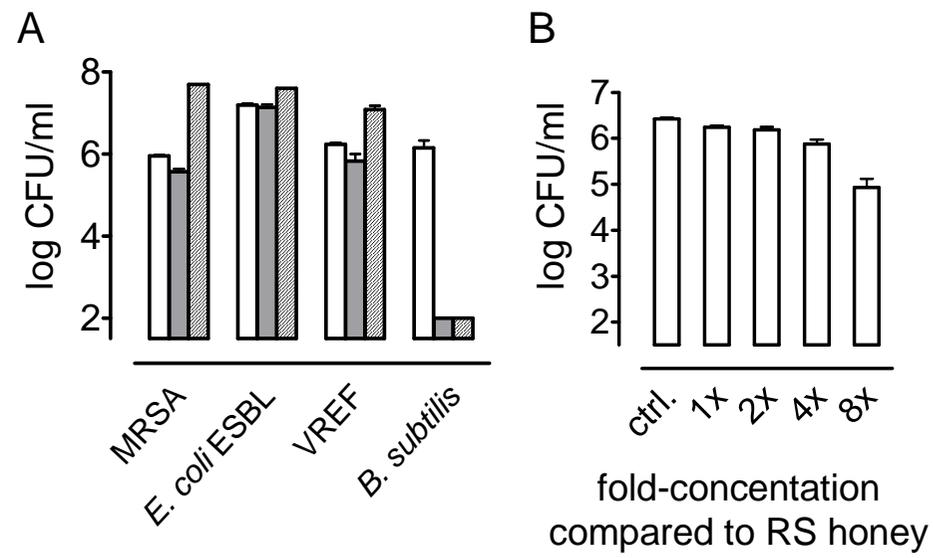


Figure 5

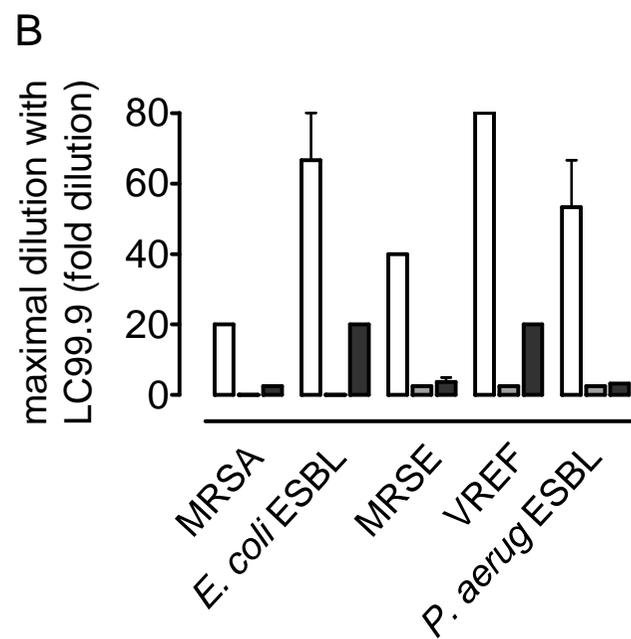
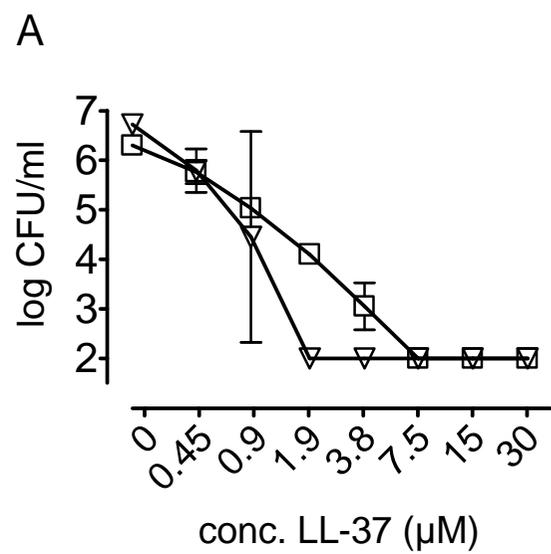


Figure 6

