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ARTICLE

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

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Abstract 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. We investigated the kinetics of the killing of antibiotic-resistant bacteria by RS honey, the source for the 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 the addition of antimicrobial peptides (AMPs). RS honey killed antibiotic-resistant isolates of *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Enterococcus faecium*, and *Burkholderia cepacia* within 2 h, but lacked such rapid activity against methicillin-resistant *S. aureus* (MRSA) and extended-spectrum beta-lactamase (ESBL)-producing *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. RS honey enriched with BP2 rapidly killed all bacteria tested and had a broader spectrum of bactericidal activity than either BP2 or honey alone.

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Introduction

Antibiotic-resistant bacteria pose a very serious threat to public health. For all antibiotic classes, including the major last-resort drugs, resistance is increasing worldwide [1, 2]. Even more alarming, very few new antibiotics are being developed [1, 3], so alternative antimicrobial strategies are urgently needed.

The potent in vitro activity of honey against antibiotic-resistant bacteria [4] and its successful application in the treatment of chronic wound infections not responding to antibiotic therapy [5] resulted in a revival of the interest in honey as an antibacterial agent [6–8]. Important prerequisites for the application of honey as an antimicrobial agent are reproducible and rapid bactericidal activity [9] and knowledge of its mechanism of action.

Honeys collected from the natural environment, including Manuka honey, which is used for the production of most currently available medical-grade honeys, show large variation in antibacterial activity [10, 11]. Manuka honey can contain very high levels of methylglyoxal (MGO), which is regarded

to be the major antibacterial compound in this honey [12, 13]. The honey used as a source for Revamil® medical-grade honey (RS honey) is produced under standardized conditions in greenhouses, and is sterilized by gamma irradiation to kill potentially present bacterial spores. Gamma irradiation is known not to affect honey bactericidal activity [14]. Revamil® has broad-spectrum, batch-to-batch reproducible bactericidal activity in vitro. It has been shown that it can strongly diminish microbial colonization of the human skin [15]. We have recently identified all major bactericidal factors in RS honey, i.e., its high sugar concentration, H_2O_2, low pH, MGO, and the cationic antimicrobial peptide (AMP) bee defensin-1 [16].

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

In the current study, we show that RS honey has potent bactericidal activity, but this requires prolonged exposure of the target organisms. RS honey lacks rapid bactericidal activity against several important antibiotic-resistant wound pathogens, including methicillin-resistant *Staphylococcus aureus*. We were not able to augment the bactericidal activity of RS honey by enrichment with endogenous honey bactericidal compounds, but addition of the synthetic AMP Bactericidal Peptide 2 (BP2) did result in broad-spectrum rapid bactericidal activity.

Materials and methods

Honey

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

Peptides

BP2 (GKWKLFKKAFKKFLKILAC) and LL-37 were synthesized at Pepscan Systems (Lelystad, The Netherlands) using solid-phase Fmoc (9-fluorenylmethoxycarbonyl) chemistry with a free amine at the N-terminus and a free amide at the C-terminus. Peptides were high-performance liquid chromatography (HPLC)-purified and the purity (>95%) and mass were confirmed by ion-spray mass spectrometry. The lack of disulfide formation between free cysteines of BP2 was confirmed by quadrupole time-of-flight/mass spectrometry (Q-TOF/MS) analysis.

Microorganisms

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

Determination of H_2O_2 concentration in honey

Hydrogen peroxide concentrations in honey were determined quantitatively using a modification of a method described by White and Subers [24]. Undiluted and ten-fold-diluted samples of honey (40 μl) were mixed in wells of microtiter plates with 135 μl reagent, consisting of 50 μg/ml o-dianisidine (Sigma) and 20 μg/ml horseradish peroxidase type IV (Sigma) in 10 mM phosphate buffer pH 6.5. o-Dianisidine was freshly prepared as a 1 mg/ml stock in demineralized water and peroxidase was diluted from a 10 mg/ml stock in 10 mM phosphate buffer pH 6.5 stored at −20°C. After 5 min of incubation at room temperature, reactions were stopped by the addition of 120 μl 6 M H_2SO_4 and absorption at 540 nm was measured. Hydrogen peroxide concentrations were calculated using a calibration curve of two-fold serial dilutions of H_2O_2 ranging from 2,200 to 2.1 μM.

Liquid bactericidal assay

Bactericidal activity was quantitatively assessed in low protein binding polypropylene microtiter plates (Costar Corning). Bacteria from logarithmic phase cultures in trypticase soy broth (TSB; BD Difco) were washed twice with incubation buffer containing 10 mM phosphate buffer pH 7.0 supplemented with 1% (v/v) TSB and were suspended at a concentration of 5×10^6 CFU/ml, based on optical density. A 50% (v/v) stock solution of honey was freshly prepared in incubation buffer. For enrichment with AMPs, an aliquot of 1.2 mM LL-37 or BP2 stock solutions

was added to 50% honey solutions to obtain 37.5 μM of peptide, thus, corresponding to the enrichment of undiluted honey with 75 μM of the respective peptides. Eighty microliters of diluted honey was mixed with 20 μl of a bacterial inoculum containing 5×10^6 CFU/ml, and the plates were incubated at 37°C on a rotary shaker at 150 rpm. At indicated time points, duplicate 10-μl aliquots of undiluted and ten-fold serially diluted suspensions were plated on blood agar. The dilutions were prepared in incubation buffer containing 0.025% sodium polyanethol sulfonate (SPS; Sigma), which neutralizes cationic bactericidal components [25]. Bacterial survival was quantified after overnight growth at 37°C. The detection level of this assay is 100 CFU/ml.

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

Partial purification of bee defensin-1

We previously demonstrated that bee defensin-1 is the only bactericidal factor in the >5-kDa fraction of RS honey [16]. To prepare a >5-kDa fraction, 15 ml of 20% (v/v) honey was centrifuged in a 5-kDa molecular weight cut-off Amicon Ultra-15 tube (Millipore) at 4,000g for 45 min at room temperature. The >5-kDa retentate was subsequently washed three times in the filter tube with 15 ml of demineralized water and concentrated to 0.3 ml.

Results

Kinetics of the bactericidal activity of RS honey

We determined the kinetics of the bactericidal activity of different dilutions of RS honey against various antibiotic-resistant pathogens. RS honey at a concentration of 40% (v/v) reduced the survival of MRSE, VREF, ESBL-producing *P. aeruginosa*, and *Burkholderia cepacia* to undetectable levels within 2 h, while similar activity against MRSA and ESBL *E. coli* required 6 h of incubation (Fig. 1). RS honey at a concentration of 20% killed *B. cepacia* within 4 h of incubation, while activity against all other bacteria required 24 h of incubation (Fig. 1). RS honey diluted to 10% killed MRSA and MRSE after 24 h, but lacked activity against all other bacteria tested (Fig. 1).

Even in undiluted RS honey, the survival of MRSA was not affected within 2 h (Fig. 2) of incubation and the numbers of CFU of *E. coli* ESBL were only reduced by 2.3-log. After 24 h incubation in undiluted honey, the survival of *E. coli* was reduced to undetectable levels, but the numbers of CFU of MRSA were only reduced by 1-log (Fig. 2). In summary, RS honey did not rapidly kill MRSA and *E. coli* ESBL, and rapid activity against all other species tested was abolished upon dilution of the honey to ≤20%.

H_2O_2 concentration required for the 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. The dilution of RS honey to 30% was optimal for H_2O_2 accumulation, resulting in maximal concentrations of 22.5±1.3 μg/ml and 148.4±27.8 μg/ml H_2O_2 after 2 and 24 h, respectively (Fig. 3a). In incubations not containing honey but only H_2O_2, 3,200 μg/ml H_2O_2 was required to kill MRSA within 2 h (Fig. 3b). This is 142 times the concentration of H_2O_2 that maximally accumulated in RS honey after a similar 2-h incubation period, indicating that the H_2O_2 concentration in honey is far too low for rapid activity against MRSA. In view of possible toxicity (see the Discussion section), it is not feasible to increase the concentration of H_2O_2 in honey to the level required for rapid killing. For similar reasons, we did not assess the enrichment of RS honey with MGO.

Bee defensin-1 concentration required for the rapid killing of MRSA

Next, we assessed the contribution of bee defensin-1 to the 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 h (Fig. 4a). However, this peptide had no substantial activity against MRSA, *E. coli* ESBL, or VREF (Fig. 4a). Even at an eight-fold higher concentration, bee defensin-1 reduced the numbers of CFU of MRSA after 2 h by only 1-log (Fig. 4b), indicating that it also was not feasible to enhance the rapid bactericidal activity of RS honey by increasing the concentration of bee defensin-1.

Enhanced rapid bactericidal activity of LL-37-enriched honey

We subsequently assessed the potential of LL-37, a broad-spectrum human AMP expressed in neutrophils and various

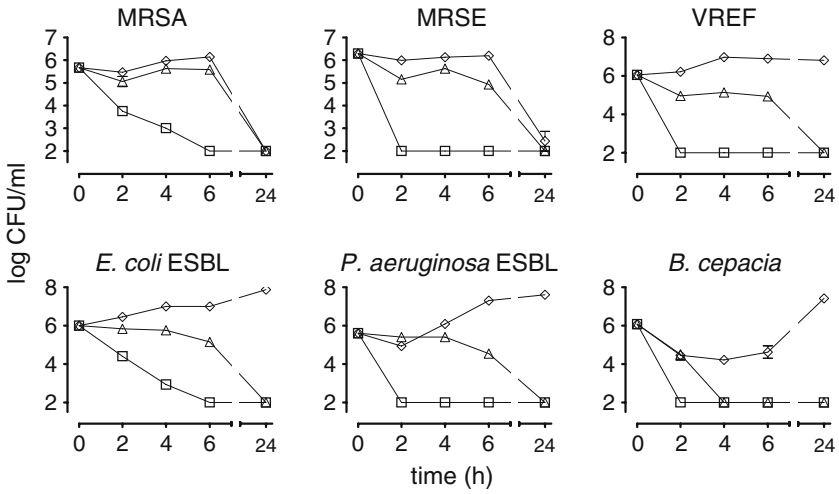

Fig. 1 Kinetics of the killing of various antibiotic-resistant bacteria by RS honey. Bacteria were incubated in honey diluted to 40% (*squares*), 20% (*triangles*), and 10% (*diamonds*). At the indicated time points, survival was determined quantitatively

epithelial cells [26, 27], to enhance the rapid bactericidal activity of honey. RS honey was enriched with ten-fold excess (75 μM) of the concentration of LL-37 required to reduce the survival of MRSA to undetectable levels (Fig. 5a). The lethal concentration of LL-37 for 99.9% of the inocula (LC99.9) of MRSA and *E. coli* in 2-h incubations were 7.5 and 1.9 μM, respectively (Fig. 5a). The enrichment of RS honey with LL-37 substantially improved the activity against *E. coli* ESBL and VREF, but not against other bacteria tested (Fig. 5b). LL-37-enriched honey retained bactericidal activity against *E. coli* ESBL and VREF up to 20-fold dilution, which was a major improvement compared to non-enriched honey (Fig. 5b). The LC99.9 of LL-37 in honey, however, was four-fold higher than in incubation buffer (Fig. 5b), indicating that honey inhibited LL-37. Inhibition was even more clear from the tests with MRSA, MRSE, and *P. aeruginosa*. Enrichment with LL-37 did not substantially improve the activity of honey, while LL-37 alone effectively killed these bacteria (Fig. 5b).

Enhanced bactericidal activity of BP2-enriched honey

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

The activity of BP2 against *E. coli* ESBL and VREF was not inhibited in honey (Fig. 6b). The killing of MRSA, MRSE, and *P. aeruginosa* ESBL required 2–4-fold higher

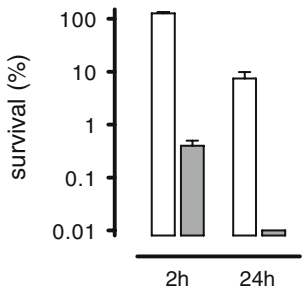

Fig. 2 Bactericidal activity of undiluted honey against methicillin-resistant *Staphylococcus aureus* (MRSA) (*white bars*) and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*gray bars*). Inocula of approximately 10^5 CFU in 4 μl were added to 0.5 ml undiluted honey. At the indicated time points, samples were diluted with one volume of incubation buffer and, subsequently, survival was quantified as described for the liquid bactericidal assay

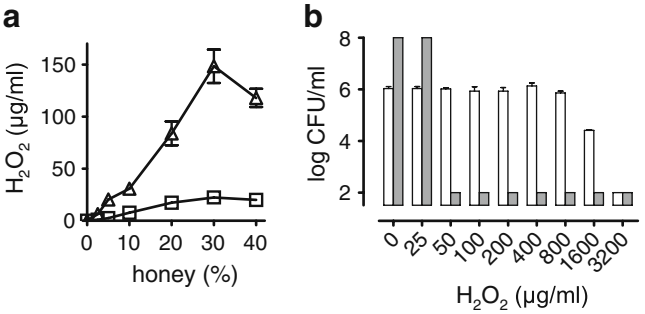

Fig. 3a, b Concentration of H_2O_2 required for activity against MRSA. **a** Production of H_2O_2 in diluted RS honey. The accumulation of H_2O_2 in indicated concentrations of RS honey was assessed at 2 h (*squares*) or 24 h (*triangles*) after dilution. **b** Bactericidal activity of solutions containing only H_2O_2. The survival of MRSA was assessed after 2 h (*white bars*) or 24 h (*gray bars*) of incubation with the indicated concentrations of H_2O_2

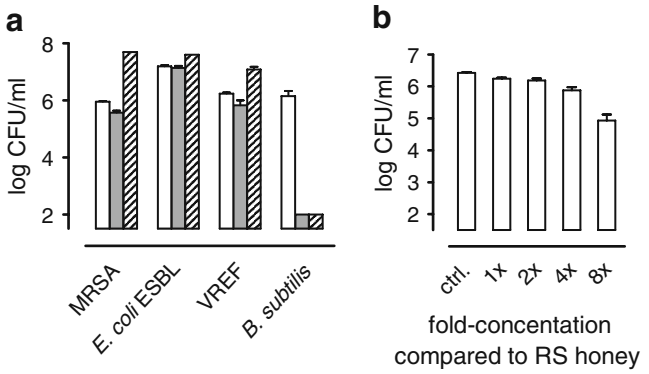

Fig. 4a, b The bactericidal activity of bee defensin-1 was assessed using the >5-kDa fraction of RS honey in which bee defensin-1 is the only antibacterial factor present [16]. **a** Bactericidal activity of bee defensin-1 against various bacteria was assessed after 2 h (*gray bars*) or 24 h (*hatched bars*) of incubation at the concentration of this peptide as present in undiluted RS honey. As a control, bacteria were incubated for 2 h in incubation buffer (*white bars*). **b** Bactericidal activity of concentrated bee defensin-1 against MRSA after 2 h of incubation. Incubation in buffer was used as a control

concentrations of BP2 in honey than in buffer (Fig. 6b). This indicates a slight reduction of BP2 activity in honey, but markedly less than the observed inhibition of LL-37 in honey. We conclude that enrichment with 75 μM BP2 markedly enhanced the rapid bactericidal activity of RS honey.

Discussion

The potent activity against antibiotic-resistant pathogenic bacteria makes honey an interesting agent to treat topical infections not responding to antibiotics. Ideally, honey used for such applications should have rapid and broad-spectrum bactericidal activity. In addition, honey should remain active upon dilution, since honey will be rapidly diluted at the wound interface due to its hygroscopic characteristics and the presence of wound exudate.

RS honey has reproducible, broad-spectrum bactericidal activity in vitro and effectively reduces the microbial colonization of human skin [15]. Our present results, however, show that the activity of this honey against the major wound-infecting pathogens MRSA and *E. coli* ESBL is not rapid. Honey does have rapid activity against *P. aeruginosa*, *E. faecium*, and *S. epidermidis*, but this activity is lost when RS honey becomes diluted. Of note, *B. cepacia*, an otherwise notoriously antibiotic-resistant pathogen, proved to be the most honey-susceptible organism. Enhancement of the activity of RS honey with its endogenous microbicidal compounds appeared not to be feasible, but addition of the cationic AMP BP2 did increase the activity to the desired levels.

We recently identified all bactericidal factors in RS honey using an approach of successive neutralization of individual factors combined with activity-guided isolation of factors responsible for residual bactericidal activity [16]. Thus, we determined that the high sugar concentration, H_2O_2 production, MGO, the low pH, and bee defensin-1 were responsible for the bactericidal activity of RS honey. In order to enhance the rapid bactericidal activity of RS honey, we first assessed the potential of H_2O_2 and of bee defensin-1. Upon the dilution of honey, H_2O_2 is produced by the glucose oxidase enzyme from the bees [24, 29]. The production of H_2O_2 was highest in RS honey diluted to 30%, in which 22.5 and 148 μg/ml accumulated after 2 and 24 h, respectively. In a study with 90 different honeys, 12±

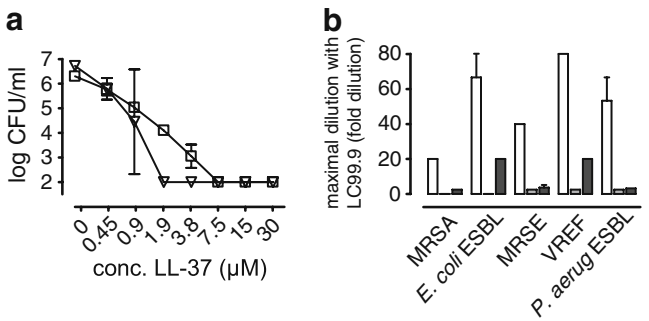

Fig. 5a, b Efficacy of LL-37 to enhance the rapid bactericidal activity of RS honey. **a** Survival of MRSA (*squares*) and *E. coli* ESBL (*triangles*) after 2 h of incubation in indicated concentrations of LL-37 in incubation buffer. **b** Bactericidal activity of LL-37-enriched RS honey. Indicated microorganisms were incubated for 2 h in two-fold serial dilutions of 75 μM LL-37 (*white bars*), RS honey alone (*gray bars*), or RS honey enriched with 75 μM LL-37 (*black bars*). The highest dilutions of these preparations killing at least 99.9% of the inocula are indicated. Mean±standard error of the mean (SEM) values of independent triplicate incubations are shown

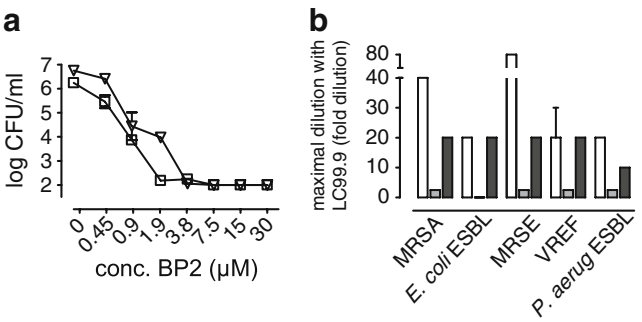

Fig. 6a, b Efficacy of bactericidal peptide 2 (BP2) to enhance the rapid bactericidal activity of RS honey. **a** Survival of MRSA (*squares*) and *E. coli* ESBL (*triangles*) after 2 h of incubation in indicated concentrations of BP2 in incubation buffer. **b** Bactericidal activity of BP2-enriched RS honey. Indicated microorganisms were incubated for 2 h in two-fold serial dilutions of 75 μM BP2 (*white bars*), RS honey alone (*gray bars*), or RS honey enriched with 75 μM BP2 (*black bars*). The highest dilutions of these preparations killing at least 99.9% of the inocula are indicated. Mean±SEM values of independent triplicate incubations are shown

19 μg/ml hydrogen peroxide (range 0–72 μg/ml) accumulated in honey diluted to 20% (w/v) after 4 h [24], indicating that RS honey produces relatively high levels of H_2O_2.

MRSA is highly susceptible to H_2O_2-mediated killing by RS honey upon incubation for 24 h [16]. The killing of MRSA within 2 h by H_2O_2 in the absence of honey required a concentration as high as 3,200 μg/ml H_2O_2 (corresponding to a 0.32% (w/v) solution). Wound cleansing with a 3% (w/v) solution of H_2O_2 has been a clinical practice, but at this concentration, H_2O_2 is toxic to human cells and skin tissue, and tissue exposure can result in delayed wound healing [30–32] . Although the concentration of H_2O_2 required to kill MRSA was about ten-fold lower than the concentration used for wound cleansing, we did not consider increasing the levels of H_2O_2 as a possibility to improve the rapid bactericidal activity of RS honey.

Bee defensin-1 (also referred to as royalisin [33]) is a 51-residue AMP identified in honey bee hemolymph, royal jelly, and in honey [16, 33, 34]. Because of its complicated folding with three intramolecular cysteine bonds, the synthetic production of bee defensin-1 is not possible and recombinant production would be highly challenging. Bee defensin-1 effectively kills *B. subtilis* but lacks activity against all other bacteria tested at a concentration equivalent to that in undiluted honey. Even at an eight-fold higher concentration, bee defensin-1 only slightly reduced the survival of MRSA. The narrow spectrum of its bactericidal activity renders bee defensin-1 unsuited for enhancement of the bactericidal activity of honey.

MGO is present in RS honey at a relatively low concentration (0.25 mM) compared to the concentrations reported for Manuka honey (up to 16.1 mM) [12]. MGO is a reactive metabolite that can exert toxic effects by the direct inhibition of enzymes, by genomic modifications resulting in carcinogenesis, and by protein modifications resulting in the formation of advanced glycation end products [35]. The latter are believed to be the main determinants for pathological effects related to diabetes [36, 37]. Because of the concerns regarding the potential toxicity of MGO, we did not pursue to augment the activity of RS honey with this compound.

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

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

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

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

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Conflict of interest None to declare.

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