Thrombocidins and honey: mechanisms of action and optimization of antimicrobial activity

Kwakman, P.H.S.

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HOW HONEY KILLS BACTERIA

Paulus H.S. Kwakman[1], Anje A. te Velde [2], Leonie de Boer[1], Dave Speijer[3], Christina M.J.E. Vandenbroucke-Grauls[1,4], Sebastian A.J. Zaat[1]

[1]Department of Medical Microbiology, CINIMA (Center for Infection and Immunity Amsterdam), Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[2] Laboratory of experimental gastroenterology and hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[3] Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[4]Department of Medical Microbiology and Infectious Diseases, VU Medical Center, Amsterdam, The Netherlands

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ABSTRACT

With the rise in prevalence of antibiotic-resistant bacteria honey is increasingly valued for its antibacterial activity. In order to characterize all bactericidal factors in a medical-grade honey, we used a novel approach of successive neutralization of individual honey bactericidal factors. All bacteria tested, including *Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus*, extended-spectrum beta-lactamase producing *Escherichia coli*, ciprofloxacin-resistant *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus faecium* were killed by 10-20% (v/v) honey, whereas at least 40% (v/v) of a honey-equivalent sugar solution was required for similar activity. Honey accumulated up to 5.62 ± 0.54 mM H_2O_2 and contained 0.25 ± 0.01 mM methylglyoxal (MGO). After enzymatic neutralization of these two compounds, honey retained substantial activity. Using *B. subtilis* for activity-guided isolation of the additional antimicrobial factor(s), we discovered bee defensin-1 in honey. After combined neutralization of H_2O_2, MGO and bee defensin-1, 20% honey had only minimal activity left, and subsequent adjustment of the pH of this honey from 3.3 to 7.0 reduced the activity to that of sugar alone. Activity against all other bacteria tested depended on sugar, H_2O_2, MGO and bee defensin-1. Thus, we fully characterized the antibacterial activity of medical-grade honey.

INTRODUCTION

Honey has been renowned for its wound-healing properties since ancient times (1). At least part of its positive influence is attributed to antibacterial properties (2;3). With the advent of antibiotics, clinical application of honey was abandoned in modern Western medicine, though in many cultures it is still used (4). These days, however, abundant use of antibiotics has resulted in widespread resistance. With the development of novel antibiotics lagging behind (5), alternative antimicrobial strategies are urgently needed. The potent *in vitro* activity of honey against antibiotic-resistant bacteria (6;7) and its successful application in treatment of chronic wound infections not responding to antibiotic therapy (3), have attracted considerable attention (8-10).

The broad spectrum antibacterial activity of honey is multifactorial in nature. Hydrogen peroxide and high osmolarity – honey consists for approximately 80% (w/v) of sugars - are the only well-characterized antibacterial factors in honey (11). Recently, high concentrations of the antibacterial compound methylglyoxal (MGO) were found specifically in Manuka honey, derived from the Manuka tree (*Leptospermum scoparium*) (12;13). Until now no honey has ever been fully characterized, which hampers clinical application of honey.

Recently we determined that Revamil® medical-grade honey produced under standardized conditions in greenhouses, has potent, reproducible bactericidal activity (14). In the current study we identified all bactericidal factors in the honey used as source for this product (RS honey) and assessed their contribution to honey bactericidal activity.

To accomplish this we used a novel approach of successive neutralization of individual honey bactericidal factors combined with activity-guided identification of unknown factors.

MATERIALS AND METHODS

HONEY

Unprocessed Revamil® source (RS) honey was kindly provided by Bfactory Health Products (Rhenen, The Netherlands). RS honey has a density of 1.4 kg/l and contains 333 g/kg glucose, 385 g/kg fructose, 73 g/kg sucrose and 62 g/kg maltose. To study the contribution of the sugars to the bactericidal activity of honey, a solution with a sugar composition identical to that of the honey was prepared.

MICROORGANISMS

Bactericidal activity of honey was assessed against the laboratory strains *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* 42D, *Escherichia coli* ML-35p (15) and *Pseudomonas aeruginosa* PAO-1 (ATCC 15692), and against clinical isolates of methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VREF), extended-spectrum beta-lactamase-producing *E. coli* (*E. coli* ESBL) and ciprofloxacin-resistant *P. aeruginosa* (CRPA).

DETERMINATION OF H_2O_2 CONCENTRATION IN HONEY

Hydrogen peroxide concentrations in honey were determined quantitatively using a modification of a method described previously (16). Undiluted and ten-fold diluted samples of honey (40 µl) were mixed in wells of microtiterplates with 135 µl reagent, consisting of 50 µg/ml o-dianisidine (Sigma, St. Louis, MO, USA) and 20 µg/ml horseradish peroxidase type IV (Sigma) in 10 mM phosphate buffer pH 6.5. O-dianisidine and peroxidase solutions were freshly prepared from a 1 mg/ml stock in demineralized water and from a 10 mg/ml stock in 10 mM phosphate buffer pH 6.5, respectively. After 5 min. incubations at room temperature, reactions were stopped by 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 2-fold serial dilutions of H_2O_2 ranging from 2200 µM to 2.1 µM.

METHYLGLYOXAL (MGO) NEUTRALIZATION ASSAY

Reduced glutathione (Sigma) was added to diluted honey to a final concentration of 15 mM, and conversion of MGO to S-D-lactoyl-glutathione (SLG) was initiated by addition of 0.5 U/ml glyoxalase I (Sigma). The amount of MGO converted was determined using the extinction coefficient of SLG of 3.37 mM^{-1} at 240 nm (17). Thus, we determined that up to 10 mM of exogenous MGO added to 40% honey was completely converted, and that undiluted RS honey contained 0.25 ± 0.01 mM of MGO.

ANTI-BEE DEFENSIN-1 POLYCLONAL ANTIBODY

An affinity-purified polyclonal anti-bee defensin-1 antibody was purchased from Eurogentec (Seraing, Belgium). The N-terminal part of bee defensin-1 is hydrophobic and contains three disulphide bonds, whereas the hydrophilic C-terminal region lacks cysteine residues (18). Therefore, rabbits were immunized with a synthetic peptide corresponding to the C-terminus of bee defensin-1 (CRKTSFKDLWDKRF), and antibodies were subsequently affinity-purified using this peptide coupled to AF-Amino Toyopearl 650 M resin (Toso, Tokyo, Japan).

LIQUID BACTERICIDAL ASSAY

Bactericidal activity of honey was quantified in 100 µl volume liquid tests, in polypropylene microtiterplates (Costar Corning, New York, NY, USA). For each experiment, a 50% (v/v) stock solution of honey was freshly prepared in incubation buffer containing 10 mM phosphate buffer pH 7.0 supplemented with 0.03% (w/v) trypticase soy broth (TSB; BD Difco, Detroit, MI, USA). Bacteria from logarithmic phase cultures in TSB were washed twice with incubation buffer and suspended at a final concentration of $1x10^6$ CFU/ml, based on optical density. Plates were incubated at 37 °C on a rotary shaker at 150 rpm. At indicated time points, duplicate 10 µl aliquots of undiluted and 10-fold serially diluted incubations were plated on blood agar. Bacterial survival was quantified after overnight incubation at 37 °C. The detection level of this assay is 100 CFU/ml.

To assess the contribution of H_2O_2 to the bactericidal activity of honey, bovine liver catalase (Sigma) was added to a final concentration of 600 U/ml. A catalase stock solution was prepared according to the manufacturers' instructions in 50 mM phosphate buffer pH 7.0. Addition of 0.25% (v/v) of this catalase stock solution reduced the amount of H_2O_2 to undetectable levels at all honey concentrations tested and did not affect bacterial viability.

Sodium polyanetholsulfonate (SPS) (Sigma) was added to neutralize cationic bactericidal components (19) at a final concentration of 0.025% (w/v). The incubation buffer did not affect the pH of the concentrations of honey used in our experiments. A 1 M NaOH solution was used to titrate honey solutions to pH 7.0.

AGAR DIFFUSION ASSAY

To assess antibacterial activity of fractionated honey an agar diffusion assay was used (20). In brief, a *B. subtilis* inoculum suspension was prepared as described for the liquid bactericidal assay. Bacteria (10^7 CFU) were mixed with 20 ml nutrient-poor agar (0.03% (w/v) TSB in 10 mM sodium phosphate buffer, pH 7.0, with 1% low EEO agarose [Sigma]) of 45 °C, and immediately poured into 10x10-cm culture plates. Wells of 1 mm in diameter were punched

into the agarose, and 2.5 µl samples were added to the wells and allowed to diffuse into the agarose for 3 h at 37 °C. Subsequently, the agarose was overlayed with 20 ml of double-strength nutrient agarose (6% TSB, 1% Bacto-agar [BD Difco], 45 °C), and plates were incubated overnight at 37 °C. Clear zones around the wells indicated antibacterial activity.

ULTRAFILTRATION OF HONEY COMPONENTS

Fifteen ml of 20% honey was centrifuged in a 5 kDa molecular weight cut-off Amicon Ultra-15 tube (Millipore, Bedford, MA, USA) at 4000 x g for 45 min. at room temperature. The <5 kDa filtrate was collected, and the >5 kDa retentate was subsequently washed three times in the filter tube with 15 ml of demineralized water and concentrated to 0.4 ml.

BACTERIAL OVERLAY ASSAY

Native cationic proteins were separated by acid urea polyacrylamide gel electrophoresis (AU-PAGE) (21). Gels were either stained with PAGE-Blue (Fermentas, St. Leon-Rot, Germany) or washed 3 x 8 min. in 10 mM phosphate buffer pH 7.0 for a bacterial overlay assay. After washing, the gel was incubated for 3 hours on *B. subtilis*-inoculated nutrient-poor agarose (see 'agar diffusion assay'). After removal of the gel, the agarose was overlayed with double-strength nutrient agarose and treated as described for the agar diffusion assay.

IMMUNOBLOTTING

Proteins were separated by tris-tricine SDS-PAGE as described previously (22), and transfered onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were subsequently blocked with 5% nonfat dry milk (Bio-Rad, Veenendaal, The Netherlands) plus 0.5 M NaCl and 0.5% (v/v) tween-20 in 10 mM tris-HCl pH 7.5 (rinse buffer) for 1 h. Blocked membranes were incubated with affinity-purified anti-bee defensin-1 antibody at 1.4 µg/ml in Rinse buffer for 2 h. After incubation with primary antibody, membranes were washed 2x for 15 min. in rinse buffer, incubated with horseradish peroxidase–labeled goat-anti-rabbit secondary antibody (Jackson ImmunoResearch West Grove, PA, USA) at 0.4 µg/ml in rinse buffer for 1 h, and washed again for 10 min. in rinse buffer and 5 min. in PBS, respectively. The membrane was developed using a DAB liquid substrate kit (Sigma).

PURIFICATION OF ANTIBACTERIAL PEPTIDE FROM HONEY

An amount of >5 kDa honey retentate equivalent to 13 ml of honey was dissolved in loading buffer (3M urea in 5% acetic acid with methyl green as tracking dye) and loaded on a preparative acid-urea PAGE as described previously (21) with slight modifications. A cylindrical gel (3.7 cm in diameter, 6 cm in height) in a Model 491 Prep Cell (Bio-Rad) was

prepared, prerun at reversed polarity for 3 h at 150V in 5% acetic acid at 4 °C, and protein was electrophoresed at 40 mA with reversed polarity. Protein was eluted in 5% acetic acid at 0.5 ml/min. and collected in fractions of 2 ml. Fractions were assessed for protein composition by tris-tricine SDS-PAGE and for antibacterial activity by bacterial overlay assay. Fractions containing purified antibacterial protein were pooled, concentrated, dialyzed against 0.01% acetic acid in a 3.5 kDa molecular weight cut-off MINI Slide-A-Lyzer tube (Pierce, Rockford, IL, USA), freeze-dried and dissolved in demineralized water.

PROTEIN IDENTIFICATION BY V8 DIGESTION WITH SUBSEQUENT MS ANALYSIS

Duplicate fractions (estimated to contain about 2 µg of protein each) were adjusted to 50 mM sodium phosphate pH 7.9 and 5% (v/v) acetonitrile. Approximately 0.5 µg of Endoproteinase Glu-C (Fluka) was added per fraction and incubated at 25 °C overnight. The resulting peptide mixtures were purified and concentrated with the aid of C18 ziptips (Millipore) and eluted in 10 µl 90% (v/v) acetonitril, 1% (v/v) formic acid. The samples were checked for the presence of non-autodigest peptides with a reflectron MALDI-TOF mass spectrometer (M@LDI, Waters, Millford, MA, USA). Next, samples were analyzed with ESI-tandem mass spectrometry (MS/MS). Data were acquired with a QTof1 (Waters) coupled to an Ultimate nano-LC system (LC Packings Dionex, Sunnyvale, CA, USA). One microliter of peptide mixture was diluted in 10 µl of 0.1% TFA. The peptides of both samples were separated on a nano-analytical column (75 mm i.d. x 15 cm C18 PepMap, LC Packings Dionex) using a standard gradient of acetonitrile in 0.1% formic acid. The flow of 300 nl/min. was directly electrosprayed in the QTof1 operating in data-dependent MS and MS/MS mode. The resulting MS/MS spectra were analyzed with Mascot software (Matrix Science). In both fractions a doubly charged ion (VTCDLLSFKGQVND, mass 1537.8) with a sequence corresponding to the mature N-terminus of bee defensin-1 could be identified (MOWSE scores > 73).

RESULTS

Hydrogen peroxide is produced by the *Apis mellifera* (honey bee) glucose oxidase enzyme upon dilution of honey. RS honey diluted to 40% to 20% accumulated high levels of H_2O_2 24 hours after dilution, with a maximum of 5.62 ± 0.54 mM H_2O_2 formed in 30% honey (Fig. 1A). Addition of catalase reduced H_2O_2 to negligible levels (Fig. 1A) and markedly reduced the bactericidal activity against all bacteria tested, except *Bacillus subtilis* (Fig. 1B). However, H_2O_2-neutralized honey exerted stronger bactericidal activity than equivalent sugar solutions (Fig. 1B). This indicates that H_2O_2 is important for the bactericidal activity of honey, but that additional factors must also be present. As *B. subtilis* was the most susceptible bacterium for non-peroxide bactericidal activity, we used it for identification of additional bactericidal factors.

The honey bactericidal compound MGO can be converted into S-lactoylglutathione (SLG) by glyoxalase I, and this product can be measured spectrophotometrically. RS honey contained 0.25 ± 0.01 mM MGO. We aimed to apply glyoxalase I to neutralize the bactericidal activity

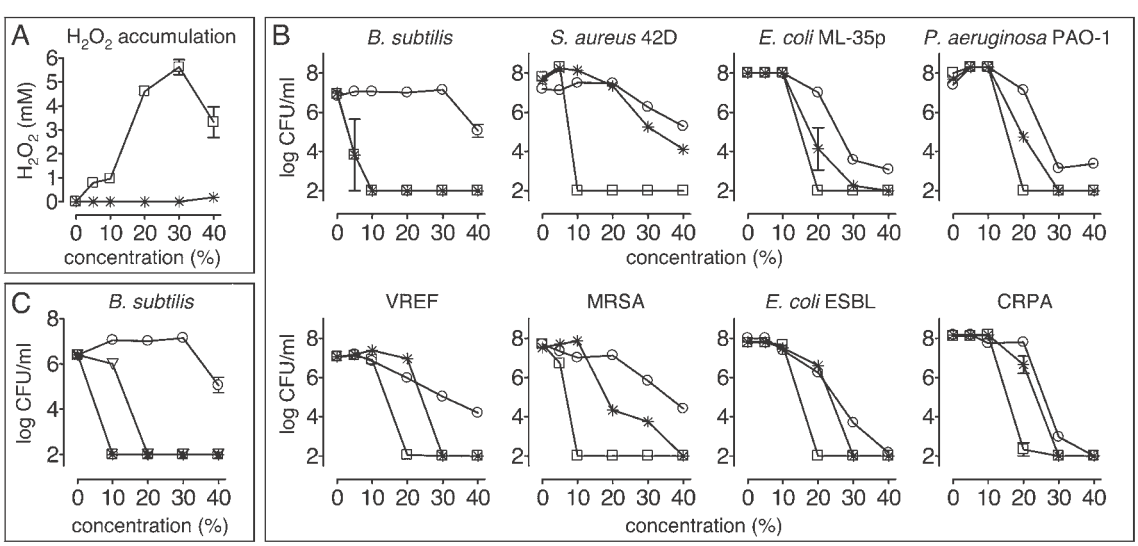

Figure 1. Contribution of H2O2, sugars and MGO to the bactericidal activity of honey after 24 hours. (A) Mean (± SEM) of hydrogen peroxide accumulation in different concentrations of honey, without (squares) or with catalase added (asterisks). (B) Bactericidal activity against indicated laboratory strains (top row) and against clinical isolates of vancomycin-resistant *Enterobacter faecium* (VREF), methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase producing *Escherichia coli* (*E. coli* ESBL) and ciprofloxacin-resistant *Pseudomonas aeruginosa* (CRPA) (bottom row). Bacteria were exposed to various concentrations of honey (squares), honey with catalase added (asterisks), or to honey-equivalent sugar solutions (circles). (C) Killing of *B. subtilis* by honey in incubation buffer without addition (squares), with catalase (asterisk), with glyoxalase (small filled circles) or with catalase and glyoxalase I (inverted triangles), added to neutralize H2O2 and MGO, respectively, or by a honey-equivalent sugar solution (circles). Numbers of CFU were log-transformed and shown as mean ± SEM.

of MGO in honey. This required that SLG, the reaction product of MGO, would be non-bactericidal. Indeed, the activity of up to 20 mM MGO was neutralized by conversion into SLG (Fig. S1), indicating that SLG up to high concentrations did not kill the bacteria. Neutralization of MGO or H_2O_2 alone did not alter bactericidal activity of RS honey, but simultaneous neutralization of MGO and H_2O_2 in 10% honey reduced the killing of *B. subtilis* by 4-logs (Fig. 1C). At higher concentrations of honey, the bactericidal activity was not affected by neutralization of H_2O_2 and MGO (Fig. 1C), indicating that still more factors were involved.

As a first step to characterize the unknown bactericidal factor(s), we size-fractionated honey by ultrafiltration with a 5 kDa molecular weight cut-off membrane. Unfractionated honey produced a small zone of complete bacterial growth inhibition and a larger zone with partial growth inhibition in an agar diffusion assay with *B. subtilis* (Fig. 2A). After ultrafiltration, the factor(s) that caused complete and partial bacterial growth inhibition were separated, and were present in the >5 kDa retentate and the <5 kDa filtrate, respectively (Fig. 2A).

Ion exchange chromatography of the retentate indicated a cationic nature of the antibacterial factor(s). Indeed, the polyanionic compound sodium polyanetholsulfonate (SPS) abolished the antibacterial activity of the retentate (Fig. 2B). Moreover, pepsin treatment also abolished this activity (Fig. 2B). Together, this implies that cationic antibacterial protein(s) were present. We separated cationic proteins in the retentate using a native acid-urea PAGE gel, and allowed the separated components to diffuse from this gel into a *B. subtilis*-inoculated agar to identify antibacterial proteins. This yielded a single zone of bacterial growth inhibition which corresponded to a protein band in a Coomassie-stained gel run in parallel (Fig. 2C). This protein was purified from a larger amount of retentate using preparative acid-urea PAGE (Fig. 2D), and identified by peptide mass analysis as bee defensin-1.

To specifically assess the contribution of bee defensin-1 to the bactericidal activity of honey, an anti-bee defensin-1 antibody was raised (Fig. 2E). Like SPS, this antibody negated all bactericidal activity of the >5 kDa retentate against *B. subtilis* (Fig. 3A). The <5 kDa filtrate had only minor bactericidal activity (Fig. 3A) but this was not due to cationic compounds since SPS failed to neutralize this activity (Fig. 3A). Thus, bee defensin-1 was the only cationic bactericidal compound present in RS honey.

Next, we assessed the contribution of bee defensin-1 to the bactericidal activity of non-fractionated honey against *B. subtilis*. As previously observed, ≥20% honey retained bactericidal activity when H_2O_2 and MGO were neutralized. Additional neutralization of bee defensin-1 strongly reduced the bactericidal activity of 20% honey, but did not affect the activity of 30% and 40% honey (Fig. 3B). So, bee defensin-1 contributed to the bactericidal activity of honey, but still other bactericidal factors were involved.

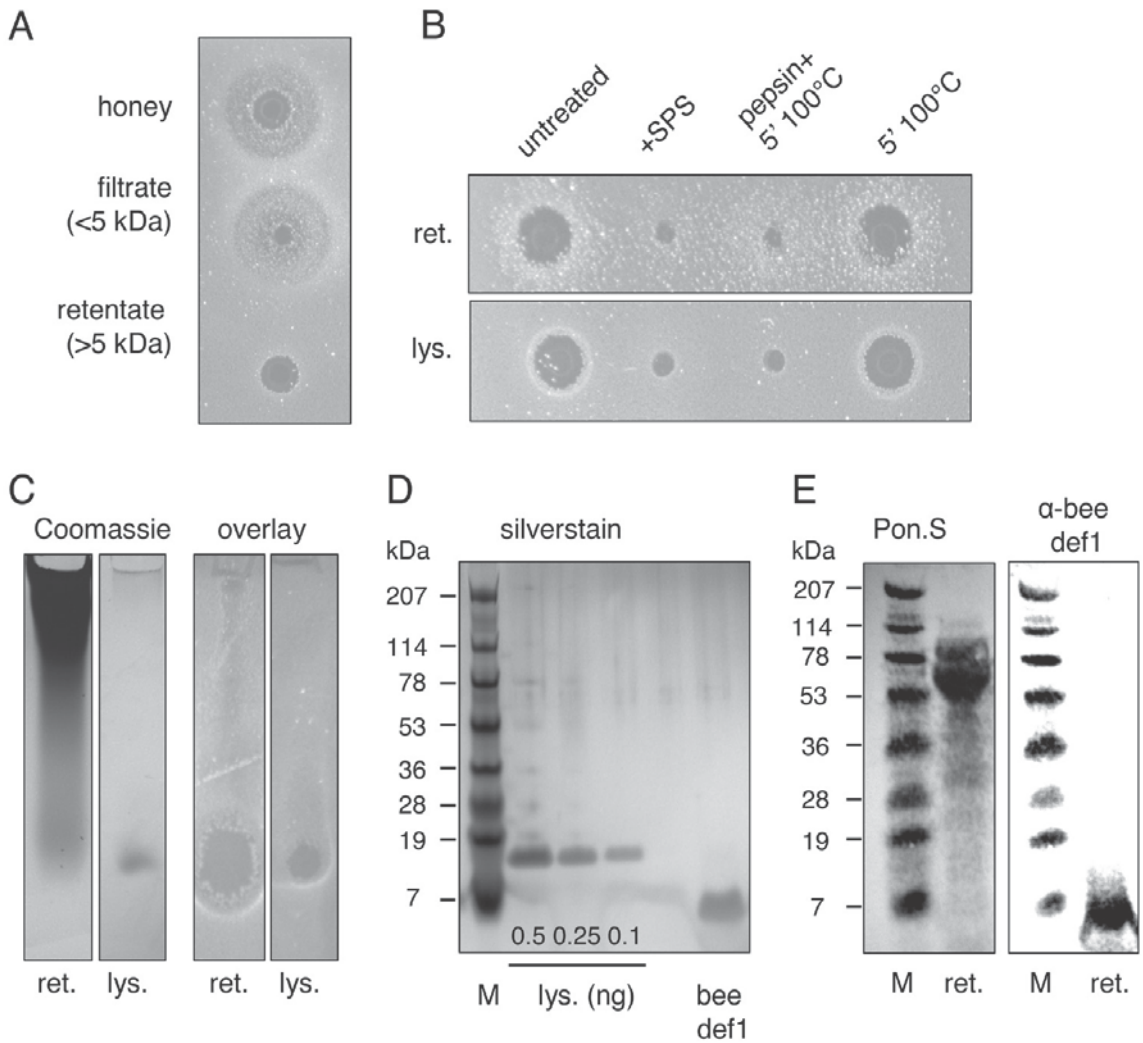

Figure 2. Identification of bee defensin-1 in honey. (A) Honey was fractionated by ultrafiltration using a 5 kDa molecular weight cut-off filter tube; antibacterial activity of 2.5 µl of 80% honey, and equivalent amounts of the <5 kDa filtrate and >5 kDa retentate were tested in an agar diffusion assay. (B) Retentate equivalent to 7.5 µl of undiluted honey was tested for the presence of cationic and proteinaceous antibacterial components. Activity of cationic components was neutralized by adding sodium polyanetholsulfonate (SPS), and protein was digested with pepsin, followed by 5 min. inactivation at 100°C. As control, incubation for 5 min. at 100°C without pepsin was performed. The activity in retentate (ret.) was compared with that of 0.2 µg hen egg white lysozyme (lys.). (C) To identify cationic antibacterial proteins in retentate, amounts of this fraction equivalent to 750 µl honey and 3 µg lysozyme as a reference, were run in duplicate sets on a single native acid-urea PAGE gel. One half of the gel was Coomassie-stained (left), the other was used for a bacterial overlay assay with *B. subtilis* (right). (D) Silverstained tris-tricine SDS-PAGE of different amounts of lysozyme and preparative acid-urea PAGE-purified bee defensin-1, separated by an empty lane. (E) Retentate separated on tris-tricine SDS-PAGE, blotted to nitrocellulose, stained with either Ponceau S ('Pon.S', left) or immunostained with anti-bee defensin-1 (right).

Honey has a low pH, mainly due to the conversion of glucose into hydrogen peroxide and gluconic acid by glucose oxidase. This low pH might also contribute to the bactericidal activity of honey (23). Titration of the pH of 40-10% RS honey from 3.4-3.5 to 7.0, combined with neutralization of H_2O_2, MGO and bee defensin-1, reduced the bactericidal activity of honey to a level identical to that of a honey-equivalent sugar solution (Fig. 3C). Thus, with this experiment, we succeeded in identifying all bactericidal factors in RS honey responsible for killing of *B. subtilis*.

The contribution of the identified bactericidal factors to activity against antibiotic-susceptible and -resistant strains of various species was tested with honey diluted to 20%, since this killed the entire inocula of all bacteria tested independent of sugar (Fig. 1). Simultaneous neutralization of H_2O_2, MGO and bee defensin-1 negated all activity (Fig. 4), showing that these were the major factors responsible for broad spectrum bactericidal activity of honey. We studied the contribution of the honey bactericidal factors in more detail by neutralizing the factors individually or combined. Neutralization of H_2O_2 alone strongly reduced the bactericidal activity against all bacteria tested except *B. subtilis* (Fig. 4). Neutralization of MGO alone strongly reduced killing of *Escherichia coli* and *Pseudomonas aeruginosa* strains (Fig. 4). Neutralization of bee defensin-1 alone reduced killing of vancomycin-resistant *Enterococcus faecium*, but not of the other bacteria tested (Fig. 4). When compared to neutralization of

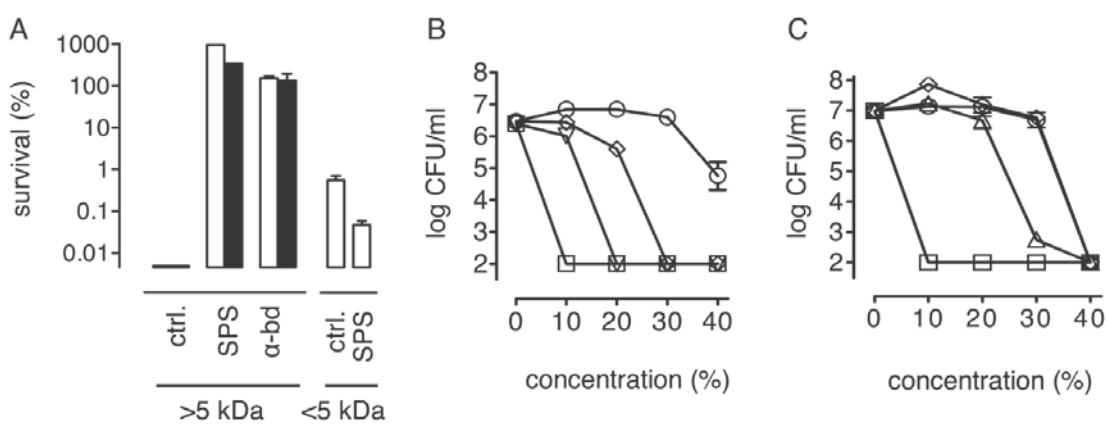

Figure 3. Roles of bee defensin-1 and pH in bactericidal activity of honey against *B. subtilis*. (A) The contribution of cationic components in general and of bee defensin-1 specifically, to bactericidal activity was tested by neutralization with SPS or with anti-bee defensin-1 antibody (α-bd), respectively, at concentrations of retentate equivalent to 20% honey (white bars) and 40% honey (gray bars); 'ctrl.' indicates survival without neutralization. (B) To assess the contribution of bee defensin-1 to bactericidal activity of unfractionated honey, *B. subtilis* was incubated in various concentrations of honey in incubation buffer (squares), or with catalase and glyoxalase I added either without (triangles), or with SPS (diamonds), or in a honey-equivalent sugar solution (circles). (C) To assess the contribution of the low pH to the bactericidal activity of honey, *B. subtilis* was incubated in various concentrations of honey in incubation buffer (squares), or with catalase, glyoxalase I and SPS added either without (triangles), or with neutralization to pH 7 (diamonds), or in a honey-equivalent sugar solution (circles). After 24 h, numbers of surviving bacteria were determined. Numbers of CFU were log-transformed and shown as mean ± SEM.

MGO alone, the additional neutralization of bee defensin-1 reduced killing of all bacteria tested, except extended-spectrum beta-lactamase producing *E. coli* (Fig. 4). In conclusion, H_2O_2, MGO and bee defensin-1 differentially contributed to the activity of honey against specific bacteria, and their combined presence was required for the broad spectrum activity.

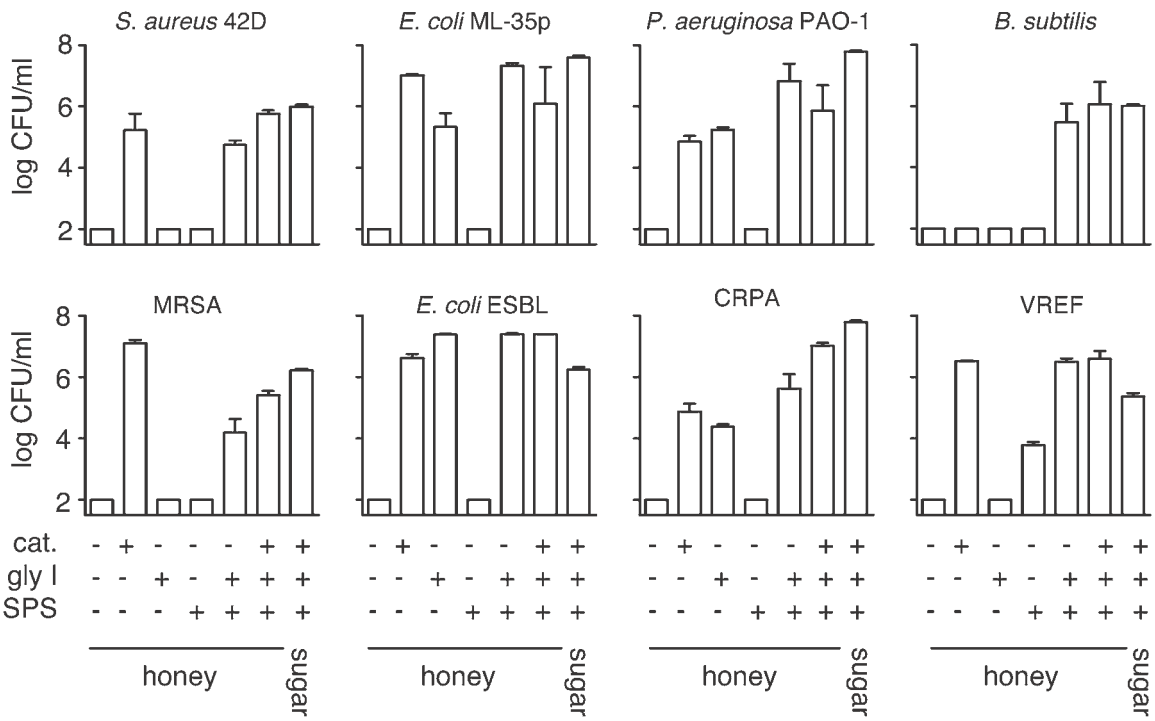

Figure 4. Effect of neutralization of H2O2, MGO and bee defensin-1 on bactericidal activity of honey. Hydrogen peroxide, MGO and bee defensin-1 were neutralized in 20% honey by adding catalase (cat.), glyoxalase I (gly I) and SPS, respectively. Bactericidal activity was tested against indicated laboratory strains (top row) and against clinical isolates of vancomycin-resistant *Enterobacter faecium* (VREF), methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase-producing *Escherichia coli* (E. coli ESBL) and ciprofloxacin-resistant *Pseudomonas aeruginosa* (CRPA) (bottom row). A sugar solution equivalent to 20% honey was used as reference. After 24 h, numbers of surviving bacteria were determined. Numbers of CFU were log-transformed and shown as mean ± SEM.

DISCUSSION

All bacterial species tested were susceptible to different combinations of bactericidal factors in honey, indicating that these bacteria were killed via distinct mechanisms. This clearly demonstrates the importance of the multifactorial nature of honey for its potent, broad spectrum bactericidal activity.

Some factors had overlapping activity. For instance, the activity of bee defensin-1 against most bacteria was only revealed after neutralization of MGO. This clearly demonstrates the importance of neutralizing known bactericidal factors in honey to reveal the presence of additional factors. Similarly, the contribution of the low pH for activity of honey against *B. subtilis* was only revealed when H_2O_2, MGO and bee defensin-1 were simultaneously neutralized.

In other situations, bactericidal activity depended on the combined presence of different factors. Thus, the activity of honey against *E. coli* and *P. aeruginosa* was markedly reduced by neutralization of either H_2O_2 or MGO. Alternatively, the activity of certain bactericidal factors likely is more potent in the context of honey than as pure substances. This is most clearly illustrated by the activity of MGO. When tested in a buffer, at least 0.3 mM MGO was required for activity against *B. subtilis* (Fig. S1). In contrast, as little as 0.05 mM MGO, the concentration in 20% RS honey, was sufficient to substantially contribute to the bactericidal activity. This suggests that the presence of the other bactericidal factors in honey enhanced the effect of MGO. It is not possible to quantify the contribution of the different factors to honey bactericidal activity since, as we have shown, these factors may have redundant activity, be mutually dependent, or have additive or synergistic activity depending on the bacterial species targeted.

We have demonstrated for the first time that honey contains an antimicrobial peptide, bee defensin-1, and that this peptide substantially contributes to the bactericidal activity. Bee defensin-1 was previously isolated from royal jelly (24), the major food source for bee queen larvae (and then referred to as 'royalisin'), and was identified in honey bee hemolymph (18). Royal jelly is produced by young worker bees and contains their hypopharyngeal and mandibular glands secretions (25;26). Bee defensin-1 mRNA has been identified in the hypopharyngeal gland of young worker bees (18), suggesting this gland is involved in production of bee defensin-1 found in royal jelly (24). When worker bees age, they become the major producers of honey. Major differences develop in morphology and protein expression of their hypopharyngeal glands (27;28), e.g. several important carbohydrate-metabolizing enzymes including glucose oxidase are expressed (29). The bees add the secretion from their hypopharyngeal glands to the collected nectar. The carbohydrate-metabolizing enzymes

then convert sucrose to glucose and fructose, and glucose oxidase converts the glucose to hydrogen peroxide and gluconic acid. These latter compounds presumably are involved in prevention of microbial spoilage of unripe honey (11). Since we have found bee defensin-1 in honey, this suggest that after the transition in hypopharyngeal gland function of the worker bees with age, the gland still produces bee defensin-1. This peptide therefore likely contributes to protection of both royal jelly and honey against microbial spoilage.

It remains to be established whether bee defensin-1 is also present in other honeys. In Manuka honey, no evidence was found for the presence of antimicrobial peptides (30). For several other honeys, protein(s) were reported to contribute to the antibacterial activity (31;32), but their identity remains unknown. Using our anti-bee defensin-1 antibody, we aim to assess the role of bee defensin-1 for the antibacterial activity of other honeys.

Previous studies regarding the effect of low pH to antibacterial activity of honey have yielded conflicting results (11). In our study, the contribution of the low pH for activity against *B. subtilis* was only revealed upon inactivation of all other bactericidal factors. So, in other studies which did not employ an approach of neutralization of bactericidal factors in honey, the contribution of the low pH of honey may easily have been overlooked.

Much effort has been put into identification of phenolic antibacterial components in honey (11). Several of these compounds have been isolated from honey but as they were tested at concentrations far exceeding those in honey no conclusions can be drawn regarding their contribution to honey bactericidal activity (11). Our data do not show a role of phenolic compounds in RS honey bactericidal activity.

Our approach of selectively neutralizing individual bactericidal factors present in a medical-grade honey allowed us to unravel the multifactorial bactericidal activity of a honey for the first time. We presently use the same approach to assess the contribution of these factors to activity of other honeys, and simultaneously to screen for novel bactericidal factors. Such honeys, or isolated components thereof, may serve as novel agents to prevent or treat infections, in particular those caused by antibiotic-resistant bacteria.

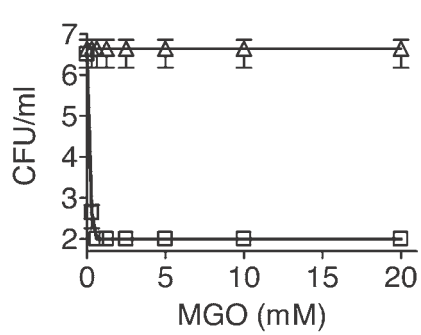

Figure S1. Bactericidal activity of MGO and SLG against B. subtilis.

Two-fold dilution series of MGO from 20 to 0.31 mM were pre-incubated with (triangles) or without (squares) glyoxalase I for 1 hour to enable conversion of MGO to SLG. Subsequently, activity against B. subtilis was assessed as described for the liquid bactericidal assay.

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