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ORIGINAL ARTICLE

Lactic acid bacterial symbionts in honeybees – an unknown key to honey's antimicrobial and therapeutic activities

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Alternative antibiotic tools; Bioactive metabolites; Honey; Honeybees; Lactic acid bacteria; Symbiosis; Wound management

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Abstract

Could honeybees' most valuable contribution to mankind besides pollination services be alternative tools against infections? Today, due to the emerging antibiotic-resistant pathogens, we are facing a new era of searching for alternative tools against infections. Natural products such as honey have been applied against human's infections for millennia without sufficient scientific evidence. A unique lactic acid bacterial (LAB) microbiota was discovered by us, which is in symbiosis with honeybees and present in large amounts in fresh honey across the world. This work investigates if the LAB symbionts are the source to the unknown factors contributing to honey's properties. Hence, we tested the LAB *against* severe wound pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus* (VRE) among others. We demonstrate a strong antimicrobial activity from each symbiont and a synergistic effect, which counteracted all the tested pathogens. The mechanisms of action are partly shown by elucidating the production of active compounds such as proteins, fatty acids, anaesthetics, organic acids, volatiles and hydrogen peroxide. We show that the symbionts produce a myriad of active compounds that remain in variable amounts in mature honey. Further studies are now required to investigate if these symbionts have a potential in clinical applications as alternative tools against topical human and animal infections.

Introduction

Today, due to overuse of antibiotics and emerging antibiotic-resistant pathogens, we are facing a new era of searching for alternative tools against infectious diseases. Chronic wounds infected by pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella* and *Streptococcus pyogenes* are subjects for intensive research efforts because of the bacteria's ability to sustain antibiotic treatment and maintain chronic infections by biofilm production. In a previous study (1), 70% of all patients with wounds had critical colonisation or overt infection in their wounds. Antibiotic-resistant bacteria in wounds caused by frequent use of antibiotics are a threat to the Health Care Sector (2) and, now, researchers are searching for new antimicrobial weapons in natural products and unexplored ecological niches for alternative tools against infections (3,4). Symbionts in an ecological niche that are already shaped to defend their host by producing bioactive compounds are a relatively unexploited option (5,6).

Key Messages

- for centuries, honey has been used as a folk medicine for the treatment of upper respiratory tract infections and wounds. Today, many of its antimicrobial characteristics have been recognised; however, there are still unknown substances that contribute to this action
- it has been discovered that 13 LAB symbionts from the honey stomach of honeybees are found in large concentrations in fresh honey as well as having a wide spectrum of antimicrobial activity against various bee pathogens

and bacteria and yeasts from flowers. We hypothesise that many of the unknown healing and antimicrobial properties of honey are linked with these LAB symbionts
- our results show that these LAB are producing not only common metabolites such as formic acid and lactic acid but also a wide variety of other interesting metabolites such as benzene and 2-heptanone. We have also identified putative LAB proteins in different honey types, suggesting their importance in honey production and antimicrobial activity. Interestingly, we have shown that in combination and separately these LAB symbionts have antimicrobial activity against a variety of severe chronic wound pathogens
- in this study, we could confirm that LAB symbionts within honeybees are responsible for many of the antibacterial and therapeutic properties of honey. Our future aim is to develop new alternative tools in wound management against human and animal infections that are scientifically proven, well defined and standardised

Less than a decade ago, we discovered a large unexplored bacterial microbiota in symbiosis with honeybees and located in the honey stomach (7). The novel microbiota is entirely composed of approximately 40 lactic acid bacterial (LAB) strains with 13 taxonomically well-defined *Lactobacillus* (9 spp.) (8) and *Bifidobacterium* (4 spp.) species. LAB is a bacterial group functionally related by their ability to produce lactic acid during homo-or hetero-fermentative metabolism. In general, certain species within LAB may produce bioactive compounds such as organic acids, free fatty acids, ethanol, benzoate, enzymes, hydrogen peroxide, antimicrobial peptides and antibiotics (9,10). These qualities together result in a wide inhibitory spectrum against pathogens.

To our knowledge, this novel honeybee LAB microbiota is one of the greatest symbiotic flora ever found in a single organism (7,11,12). These LAB symbionts were shown to be similarly present and active within all honeybees (*Apis* spp.) and sampled stingless bees, and in their respective freshly harvest honey on all continents of the world (11–13). Besides their key role in honey production, our research shows that the symbionts have been evolutionarily shaped to work synergistically in order to defend bees against incoming microbial threats introduced by nectar foraging including several bacterial genera and yeast by producing different metabolites, peptides and proteins (11,14).

Honey is the best-known honeybee product and represents the only human food source created entirely by an insect. The medical effects of honey have been independently documented by many cultures throughout history (15). However, while honey has a number of applications by different cultures, it is most well-known for its actions against upper respiratory tract infections and in wound management (15,16). Honey's modes of action, besides its low pH and high osmolarity, are today explained by the hydrogen peroxide content as an action of peroxidase oxidase produced by the honeybee itself, the origin of the nectar by its different flavonoids and phenolic acid contents (17,18) and unidentified active compounds (19). Recently, other compounds have been shown, including methylglyoxal in *Leptospermum scoparium* (Manuka) honey (20), antibacterial peptides bee defensin-1 (21) and bioactive compounds that alter the expression of a specific protein in *S. aureus* (22). Furthermore, studies have shown that honey has an anti-inflammatory action in wounds (18,23). Although clinical reports have shown positive results when using honey in wound management and recent research has shown previously unknown bioactive compounds, the application of honey dressings still gives a low confidence for its use in therapeutic treatment in wound management (24), without a necessary scientific explanation of the source for those compounds and mechanisms of action behind honey's properties.

Every single member of the novel LAB microbiota is involved in varying concentrations in the process of nectar to become honey. However, from our research we now know that the 13 LAB species vary numerically in naturally harvested honey depending on the nectar source, honeybee health and presence of other microorganisms in the collected nectar (7,11,14,25). We noticed early that the LAB symbionts react in a synergistic matter and defend themselves by secreting a variety of active compounds that inhibit other microbial growth. These interesting numerical variations and varying production of active compounds appear to be a well-established symbiosis among bees, LAB symbionts, flowers' nectars and microbial threats that varies with season and honeybee health (11).

The massive presence of viable LAB (10^8 LAB/g fresh honey) (7,11) raised the hypothesis that these novel LAB with their active bio-products could be the reason for why honey has been regarded as an antimicrobial agent through human history. In this study, we explore the antimicrobial properties of LAB and their produced bioactive substances. We theorised that LAB substances produced during honey production should be present in freshly harvest honey and preserved in mature honey. Our future aim is to develop new alternative tools in wound management against human and animal infections that are scientifically proven, well defined and standardised.

Material and methods

Bacteria, media and honey

Lactic acid bacteria

The 13 LAB honeybee symbionts were previously isolated from the honey stomach of the honeybee species *Apis mellifera* around the world and identified to the strain level in our laboratory (7,8,11–13). The following bacteria were used in this study: *Lactobacillus helsingborgensis* Bma5, *Lactobacillus kimbladii* Hma2, *Lactobacillus mellis* Hon2, *Lactobacillus mellifer* Bin4, *Lactobacillus melliventris* Hma8, *Lactobacillus apis* Hma11, *Lactobacillus kullabergensis* Biut2, *Lactobacillus apinorum* Fhon13, *Lactobacillus kunkeei* Fhon2, *Bifidobacterium coryneforme* Bma6 and *Bifidobacterium*, Bin2, Hma3 and Bin7. LAB strains from the honey stomach of the dwarf honeybee *Apis andreniformis*, the giant honeybee *Apis laboriosa* and the stingless bee *Melipona beechii* previously isolated by us (11) were also tested in this study.

All LAB strains were cultivated anaerobically for 72 hours at 35°C on de Man, Rogosa & Sharpe (MRS) (Oxoid, Hampshire, UK) agar plates (1% agar, Oxoid) and broth supplemented with fructose (2%, Merck, Sollentuna, Sweden) and L-cysteine (0·1%, Sigma-Aldrich, Stockholm, Sweden), or in Pollen media, freshly collected bee pollen mixed with water and sterilised by autoclaving (26).

Human pathogens

Severe multidrug-resistant pathogens from chronic wound infections were tested at Prof. Rose Cooper's laboratory, UWIC (Cardiff, Wales, UK). The used pathogens shown in Table 1 were cultivated in nutrient broth (Oxoid) at 37°C for 24 hours prior to test in the antagonism assays.

Dual culture overlay assay

Antimicrobial activity was measured by using dual culture overlay assay as previously described (11), with few modifications. LAB separately or in combinations (Table 1) were put into a filter disc and placed on supplemented MRS agar plates followed by overnight incubation at 35°C. Wound pathogen cultures were mixed with a 10-ml soft nutrient agar (0·8%), holding a temperature of 42°C. Each mixture of soft agar was poured as an over layer on top of MRS plates with the overnight-cultivated LAB. The plates were incubated at 37°C for 24 hours. All the tests were performed in triplicate. Zone diameters were measured from the centre of the disc to the zone edge.

Honey types

Stored honeys were purchased from a local Swedish bee-keeper, which covered one summer season. These included honeys from the following flower nectars (bloom time in parenthesis): rapeseed (May), raspberry (June), linden (small-leaved lime, July), heather (August) and honeydew (pine, September). We purchased Manuka honey (Manuka factor +10) from a store in France (Comptoirs & Compagnies, Venelles, France). As we know that the LAB are present in large amounts in freshly harvest honey directly from the hive, we included freshly harvest rapeseed honey taken directly from one colony and stored it for 1 month 2 weeks.

Characterisation of LABs bioactive metabolites

Identification of bioactive metabolites produced by each of the 13 LAB strains originating from honeybees (*A. mellifera*) was conducted to uncover the mechanisms of action behind antimicrobial and therapeutic characteristics.

Organic acids

Reagents and bacteria. Organic acid standards: lactic acid (L+, 98%) (Sigma, St. Louis, MO), formic acid (~98%) (Sigma) and acetic acid (100%) (Merck, Darmstadt, Germany). Milli-Q ultrapure water (Merck) was used to dilute all standards and stock solutions. Deionised water was used to prepare the mobile phase. *Lactobacillus* Bma5, Hma2, Hon2, Bin4, Hma8, Biut2, Fhon2 and Fhon13 were cultured in MRS broth. For Fhon2 and Fhon13, the medium was enriched with 2% fructose (VWR, Poole, UK). *Bifidobacterium* Bma6, Bin2, Hma3 and Bin7 and *Lactobacillus* Hma11 were cultured in *Lactobacillus*-carrying medium (LCM) (27). The isolates were incubated in 15-ml tubes under anaerobic conditions for 24 hours at 35°C.

Equipment and chromatographic conditions. The HPLC analyses were carried out on an Elite LaChrom modular system composed of a high-pressure pump (L-2130) with built-in degasser, a column oven (L-2300), a diode array detector (L-2455) (Hitachi, Tokyo, Japan) and a manual sample injection valve (7725i, Rheodyne, Cotati, CA) with a 20-µl sample loop. EZChrom Elite (Version 3.2.1) software suite (Agilent Technologies, Kista, Sweden) was used for data acquisition and calculations. The HPLC column used was a Shodex RSpak KC-811 (6 µm, $300 \times 8{\cdot}0\,mm^2$ ID) (Showa Denko K.K., Kawasaki, Japan). The mobile phase consisted of 0·1% phosphoric acid. Elution was carried out under isocratic conditions with a flow rate of 1·0 ml/minute and a temperature of 40°C. Chromatograms of the UV absorbance were recorded at 210 nm (from a UV-spectra of 200–400 nm). The system was equilibrated for 30 minutes between each run.

Sample preparation. The medium containing the cultured LAB was vortexed (MS1, IKA, Taquara, Brazil) until the pellet had dissolved, then centrifuged for 10 minutes at 5100 g in a Sigma 2–5 centrifuge and the supernatants were passed through a 0·20-µm filter (Filtropur S, Sarstedt, Nümbrecht, Germany). The resulting liquid was injected directly into the HPLC without any previous dilution. Analyses were performed in triplicate for each bacterium.

Calibrations and calculations. Standard aqueous solutions of lactic acid (1·0%, v/v), formic acid (0·5%, v/v) and acetic acid (1·0%, v/v) were used to establish the mean individual retention time of each organic acid. Quantification was performed by the external standard method. Multilevel calibrations [concentration (%) versus peek area] with five loading levels in triplicates (true average) were used to construct the calibration curves, which were fitted using linear regression. Background levels of the corresponding medium for each of the bacterium were then subtracted before the calculation of final concentrations.

Free fatty acids (3-OH FAs)

The LAB species were grown in 5 ml supplemented MRS and 5 ml Pollen media until they reached their early stationary phase, after approximately 24 hours of incubation at 35°C (14). Three millilitres of the supernatant was filtered through a 0·20-µm filter (Sarstedt). The filtered supernatants were freeze-dried for 18 hours at −20°C prior to the GC–MS analysis.

The freeze-dried bacterial supernatants, and both stored and freshly harvest honey samples (200 mg) were analysed for 3-OH FAs. In brief, the preparations were heated in acid methanol, extracted with heptane and purified using silica gel columns. The hydroxy fatty acids, in the polar lipid fraction, were then subjected to derivatisation to form trimethylsilyl derivatives, and analysed by GC–MS/MS using an ion-trap

Table 1 Dual culture overlay assays with lactic acid bacterial (LAB) strains of bee origin against clinical isolates of pathogenic wound bacteria and yeast. The diameters of the inhibition zones are displayed in millimetres. Antibiotics commonly used against the same pathogens are depicted as controls.

Bee species origin	LAB strain	*Serratia narcescens* NJ19 5c	*Staphylococcus areus* FJ02	*Klebsiella aerogenes* Clmp R	*Citrobacter freundii* CR01 5A	*Staphylococcus areus* 74022 PR	*Staphylococcus areus* CR01	*Pseudomonas aeruginosa* LE08	*Enterobacter cloacae* JSB 5B	MRSA clinical isolate 18	*Klebsiella oxytoca* JSB 5B	*Escherichia coli* V517	*Candida albicans*	*Enterococcus faecalis* E12 VRE	*Acinetobacter* A23 Z32524
Apis mellifera	*L. kunkeei* Fhon2	42*	ND	24*	32‡	31*	ND	30†	38*	33	ND	40†	20‡	31*	55*
	L. apinorum Fhon13	8*	ND	10‡	18‡	20*	ND	0	0	18	ND	12‡	0	14*	13
	L. kimbladii Hma2	9*	ND	9‡	10‡	0	ND	10†	5	9†	ND	7‡	0	0	0
	L. melliventris Hma8	18	ND	9‡	15†	19*	ND	0	17*	16	ND	12†	0	8	18*
	L. helsingborgensis Bma5	11*	ND	10†	18‡	13‡	ND	0	11*	6†	ND	12†	0	0	6
	L. kullabergensis Biut2	7*	ND	7‡	8‡	16†	ND	0	7	12*	ND	10†	0	0	12†
	L. apis Hma11	0	ND	0	0	0	ND	0	0	0	ND	0	0	0	0
	L. mellifer Bin4	39*	ND	26*	32‡	22†	ND	29*	29*	32†	ND	30†	20‡	21	40*
	L. mellis Hon2	0	ND	0	0	0	ND	0	0	0	ND	0	0	0	0
	Bifidobacterium Bin2	18*	ND	15*	13†	9*	ND	10‡	21*	0	ND	19†	8‡	10	15*
	Bifidobacterium Bin7	22	ND	17*	22‡	14†	ND	15†	24*	15*	ND	28†	12‡	13	21*
	Bifidobacterium Hma3	26*	ND	16†	18‡	15†	ND	16†	21	19	ND	20†	8‡	15*	22*
	B. coryneforme Bma6	0	ND	0	6‡	9*	ND	0	0	0	ND	0	0	0	0
Apis laboriosa	All together§	40*	42*	40*	31†	28	39†	33†	40*	32*	36†	39†	12‡	25	32*
	L. kunkeei Lahm1to13	45*	ND	30*	32†	20†	ND	27†	38*	30	ND	46†	15‡	22	34*
	All together§	49*	45	30*	31†	24	41†	36†	43*	34	32†	45†	15‡	25	43*
	L. kunkeei Anhmro10	42*	ND	34*	35†	29†	ND	32†	42*	33	ND	44†	15‡	29	46*
Apis andreniformis	All together§	47*	40	38*	34†	33†	39†	32†	37*	31*	37†	40†	12‡	28	28*
	L. kunkeei Yubipro16	45*	ND	32*	36†	34†	ND	30†	45*	30	ND	46†	15‡	35*	62*
Melipona beechii	All together§	44*	40	37*	38†	28†	42†	37†	42*	33	32†	46†	15‡	25	45*
Antibiotics		V 9	V 19	F 29	Cx 28	Cl 19*	F 29†	V 19	F 29	Cn 19	C 31‡	Cn 21*	V 23	F 29*	A 0	A 19	C 0

MRSA, methicillin-resistant *Staphylococcus aureus*; ND, not determined; VRE, vancomycin-resistant *Enterococcus*. Used antibiotics: V, vancomycin, 30 μg; F, fusidic acid, 10 μg; Cx, cefuroxime sodium, 30 μg; Cl, chloramphenicol 30 μg; Cn, gentamicin, 10 μg; A, ampicillin, 10 μg; C, ciprofloxacin, 5 μg.

*Sharp edge of inhibition zone.

†Blur edge of inhibition zone.

‡Sporadic growth all the way in through the zone

§Repeated twice.

instrument (28). Some of the samples were also analysed in scan mode using a quadrupole GC–MS instrument. The 3-hydroxy fatty acids (3-OH FAs) monitored were 3-OH C 10:0–3-OH C 22:0.

Hydrogen peroxide

Hydrogen peroxide production from each LAB was analysed according to a previously described method (29). Shortly, LAB were initially cultured for 3 days in supplemented MRS. 3,3′,5,5′-Tetramethyl-benzidine (TMB) plates were prepared by adding solution A [25 mg of TMB (Sigma-Aldrich), dissolved in 6 ml methanol] and solution B [2 mg horseradish peroxidase, type 1, approximately 100 purpurogallin units/mg, dissolved in 2 ml of ddH_2O] to MRS agar. The TMB plates were then inoculated with each LAB and incubated anaerobically at 35°C for 48 hours before transfer to aerobic conditions at room temperature (RT). Blue colonies were observed after incubation at RT for 1, 24 or 90 hours.

Volatiles

All the tested LAB strains from *A. mellifera* were incubated anaerobically for 7 days in Pollen media (50 ml). Bacterial cultivations were performed separately in one anaerobic jar for each strain. The diffusive sampler was attached onto the inner side of the jar's lid. A diffusive sampler having uptake rates that fully agree with the theories behind diffusive sampling was used for sampling and analysis of formic and acetic acids (Ferm 2001, www.diffusivesampling.ivl.se). Analysis was made using ion chromatography with a gradient eluent generator (DIONEX ICS 2000). Diffusive sampling of other organic vapours was made with tube-typed sorbent tubes (PerkinElmer, Waltham, MA). For sampling of benzene, toluene, *n*-octane, ethylbenzene, *m*-, *p*-xylene, *o*-xylene and *n*-nonane, Carbopack B (Sigma-Aldrich) was used as an adsorbent. The pollutants were analysed via thermal desorption (ATD-400, PerkinElmer) and gas chromatography with a flame ionisation detector (GC-FID, Varian3800). More samplers could be analysed when Tenax TA was used as sorbent, and analysis was made with thermal desorption (Markes, Frankfurt, Germany) and GC–MS (Agilent Technologies). Experimentally determined uptake rates were used for the thermally desorbed hydrocarbons.

2-Heptanone

All the 13 LAB (from *A. mellifera*) were cultivated separately in 10 ml (15-ml tubes, Sarstedt) supplemented MRS and Pollen media (26) at 35°C for 3 days. A viable count was performed for all the LAB and their respective colony forming unit (CFU) values are shown in Table 4. Bacterial cultures were then cleaned by using 0·6 g of resin for 10 ml culture. Bacterial samples were centrifuged at 1200 g for 10 minutes; thereafter, 5 ml of each sample supernatant was transferred to a 10-ml glass test tube following extraction twice with 3 ml of dichloromethane (Sigma-Aldrich) containing deuterated *N*-octanol (D17) (Cambridge Isotopes Laboratories, Inc., Tewksbury, MA) as an internal standard. The bottom phase was transferred to a 1-ml GC test tube and analysed as described below.

A Varian model 3800 gas chromatograph equipped with a combiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and a silica capillary column (VF-5ms, 60 m × 0·25 mm ID, 1 µm film thickness, Agilent Technologies) coupled to a 1200 1 triple quadrupole MSMS detector (Varian, Inc., Walnut Creek, CA) was used. Helium was used as a carrier gas at a column flow rate of 1·0 ml/minute. The column temperature was programmed to rise from 50°C to 230°C at 7°C/minute, where it was held for 4 minute. The injector temperature was 200°C, the transfer line temperature 280°C, the ion source temperature 200°C, the electron energy 70 eV and the filament current 50 µA. One microlitre injections in the splitless mode were used.

Samples of the 13 LAB members ($n = 2$) cultivated in pollen medium were analysed in SCAN mode. Then, bacteria that were found to produce clearly detectable amounts of 2-heptanone were re-analysed. Quantification of 2-heptanone from these bacteria was performed using selected-ion monitoring (SIM). A standard curve was obtained by injecting 1·5–150 pg of 2-heptanone (Sigma-Aldrich) and 240 ng of deuterated *N*-octanol (internal standard). The detection limit of 2-heptanone was 1 ng/ml, and the extraction efficiency was 112%.

Biofilm formation

The LAB symbionts reside inside honeybees within the honey crop in biofilms (11). In order to assess if this biofilm can be formed outside the honey stomach, we investigated biofilm formation *in vitro* by a previously described method (30).Shortly, each LAB symbiont was grown in supplemented MRS and Pollen broths. LAB strains were allowed to reach early stationary phase (~10^8 CFU/ml), and 100 µl of the culture was inoculated into a polystyrene MicroWell plate (Nunc®, Sigma-Aldrich) in different dilutions and incubated at 35°C for 72 hours. The plates were then washed with sterile phosphate buffered saline (PBS; pH 7.2). The attached bacteria were stained by adding a crystal violet solution and rinsed with sterile water to remove excess stain. The plates were air-dried and the stain that bound to attached bacteria was released by adding an ethanol/acetone solution. The optical density (OD) was measured at 570 nm. Each LAB strain in MRS and Pollen broths was analysed in triplicates.

Protein and peptide analyses in honey

Samples were prepared and analysed as previously described (14) with some modifications. Different types of stored honey from the following nectars were used: heather, linden, raspberry, oil rapeseed and manuka (see above) were prepared in a 1:5 and 1:50 dilution (honey:water) and centrifuged at 3500 g for 25 minutes. Supernatant was taken from each tube and added to 30 K Amicon ultra centrifugal filters (Millipore, Merck, Darmstadt, Germany) and centrifuged for 10 minutes at 3500 g. Tris–HCl (0·2 M, pH 8·3) was added to the filter and samples were centrifuged as mentioned before. This step was repeated once again and 6 M urea (in 0·2 M Tris–HCl) was added to the filter and centrifuged as mentioned before (31,32). Samples were frozen at −20°C until further use.

Table 2 Bioactive substances produced by each of the 13 LAB symbionts from honeybees (*Apis mellifera*)*

Genus	Strain	Acetic acid	Formic acid	Lactic	H_2O_2	Benzene	Toluene	Octane	Ethylbenzene	Xylene	Nonane
Lactobacillus	Fhon2	>263	>17	680		0·0045	0·004	0·0	0·0022	0·39	0·0
Lactobacillus	Fhon13	>327	>28	600		0·0018	0·008	0·0	0·031	0·29	0·0068
Lactobacillus	Hma11	>306	>16	500	+	0·0005	0·036	0·027	0·0	0·23	0·0127
Lactobacillus	Hon2	>290	>16	770		0·001	0·045	0·049	0·0004	0·28	0·02
Lactobacillus	Bin4	161·8	9·3	600		0·074	0·0	0·0	0·017	0·01	0·0
Lactobacillus	Hma2	>271	>16	710	+	0·0003	0·057	0·049	0·0	0·25	0·0127
Lactobacillus	Bma5	>267	>16	900	+	0·0004	0·046	0·059	0·004	0·28	0·0163
Lactobacillus	Hma8	206·4	12·7	1060	+	0·0008	0·07	0·049	0·0005	0·24	0·02
Lactobacillus	Biut2	>258	>14	950	+	0·0006	0·036	0·039	0·0004	0·26	0·0159
Bifidobacterium	Bin2	>302	>20	260		0·0002	0·040	0·369	0·003	0·27	0·0147
Bifidobacterium	Bin7	>297	>25	420		0·009	0·045	0·579	0·004	0·25	0·02
Bifidobacterium	Hma3	>294	>20	220		0·0014	0·040	0·559	0·004	0·26	0·02
Bifidobacterium	Bma6	208·2	13·0	260		0·0005	0·0	0·419	0·003	0·01	0·0
Summation	All 13 LAB	>3451	>223	7930		0·094	0·427	2·198	0·0695	3·011	0·1594

*The table depicts organic acids (lactic-, acetic- and formic acids), hydrogen peroxide (H_2O_2) and volatiles (benzene, toluene, *n*-octane, ethylbenzene, xylene and *n*-nonane). The depicted amounts refer to microgram per sample and '+' refers to a positive reaction.

Tris–tricine SDS–PAGE and mass spectrometry

To identify any proteins found in honey samples, Mini-PROTEAN 10–20% Tris–Tricine precast gels (Bio-Rad, Hercules, CA) were used as per original protocol (33). Gel bands were prepared for mass spectrometry as outlined in the study by Shevchenko and coworkers (34), with some modifications (14).

Peptide mass fingerprinting

The resulting mass spectra files obtained from the mass spectrometry analysis were searched using MASCOT against a local database containing the predicted proteome of the 13 LAB (35). We used a cut-off ion score of 38 as a value for determining that the protein was identified. Individual ion scores greater than 38 indicated identity or extensive homology ($P < 0{\cdot}05$) of the protein. Protein sequence similarity searches were performed with software BLASTP in the software package BLAST 2·27+ against a non-redundant protein database at NCBI (36,37), Pfam (default database) (38) and InterProScan (default databases) (39,40).

Results

Antagonism assays

The overlay assays (Table 1) show that all the tested pathogens from clinical human wounds were inhibited by antimicrobial compounds diffusing from each of the 13 LAB originating from honeybees (*A. mellifera*) and when the 13 LAB were grown together. The results show that the different LAB strains produce different bioactive metabolites of varying inhibitory effects against the pathogens. We could observe the same inhibition results when *L. kunkeei* strains (Lahm1to13, Anhmro10 and Yubipro16) and combinations of LAB strains originating in other bees (*A. laboriosa*, *A. andreniformis* and *M. beechii*) were tested against the same pathogens. In all occasions, the effect from the collaborating LAB was greater than from the antibiotic discs.

Table 3 Free fatty acids, 3-OH Fas, (pmol/ml medium) in spent Pollen medium of cultivated bacteria

Samples	C 10:0	C 12:0	C 14:0	C 16:0
Blank (pollen)	–	–	–	–
Biut2	–	–	–	–
Hon2	–	–	–	–
Bma5	–	–	–	–
Bma6	–	–	–	–
Fhon2	34·5	36·9	–	132·4
Fhon13	307·9	252·4	26·7	51·9
Bin2	12·1	22·7	–	–
Bin4	–	–	–	–
Bin7	–	–	–	15·0
Hma2	–	–	–	–
Hma3	–	–	–	–
Hma8	–	–	–	–
Hma11	–	–	–	–

Bioactive products from the LAB

Our results demonstrate that every single member of the LAB microbiota of honeybees (*A. mellifera*) produces different bioactive metabolites (Tables 2, 3 and 4). In general (Table 2), organic acids were produced by all tested strains but in different amounts. Lactic-, formic- and acetic acids were produced by all 13 LAB. Five of the LAB strains, Hma11, Hma2, Bma5, Hma8 and Biut2, produced hydrogen peroxide. Different toxic volatiles were detected from every LAB. These included the following: benzene produced mainly by *L. mellifer* Bin4; toluene by 11 of the LAB strains; octane mainly by the bifidobacteria Bin7, Bin2, Hma3 and Bma6; ethylbenzene mostly by *L. apinorum* Fhon13; xylene by 11 LAB; and nonane mostly by lactobacilli Hon2 and Hma8 and *Bifidobacterium* Bin7 and Hma3.

3-Hydroxy fatty acids

Free fatty acids (3-OH FAs) were identified from 4 of the 13 LAB strains studied; these were C 10:0, C 12:0, C 14:0 and C 16:0 (Table 3). Only results from Pollen media are shown as the

Table 4 Results showing 2-heptanone production by one of the 13 LAB from honeybees (Fhon13)*

Samples	ng/sample	CFU
Quant1		
MRS blank	11·7	–
Fhon13 (1)	575·1	$3{\cdot}0\times10^{7}$
Fhon13 (2)	696·3	
Fhon13 (3)	611·7	
Pollen (Cleaned blank)	9·8	–
Fhon13 (1)	771·3	$3{\cdot}0\times10^{7}$
Fhon13 (2)	724·8	
Fhon13 (3)	875·8	
Pollen blank	140·6	–
Fhon13	888·2	$8{\cdot}0\times10^{8}$
Quant 2		
Pollen blank	44·1	–
Fhon13 (1)	926·5	$1{\cdot}5\times10^{8}$
Fhon13 (2)	863·6	
kohmto18 (1)	476·9	4×10^{8}
kohmto18 (2)	501·0	
kohmto18 (3)	495·4	
nuhmto23 (1)	565·1	$1{\cdot}5\times10^{9}$
nuhmto23 (2)	507·9	
nuhmto23 (3)	523·4	
cehmto2 (1)	1172·3	$2{\cdot}5\times10^{10}$
cehmto2 (2)	1349·3	
cehmto2 (3)	1418·2	

*Studied *L. apinorum* Fhon13 strains originating in other bee species were *Lactobacillus* kohmto18, *Lactobacillus* nuhmto23 and *Lactobacillus* cehmto2 in triplicate.

results from bacteria incubated in MRS contained 3-OH FAs in the blank. The relative amounts of the different 3-OH FAs varied between the different strains and were most abundant in *L. apinorum* Fhon13 and *L. kunkeei* Fhon2. *Bifidobacterium* Bin7 produced only C 16:0 and *Bifidobacterium* Bin2 produced two 3-OH FAs (C 10:0 and C 12:0), but in low amounts. In addition to the monitored 3-OH FAs compounds, both of the Fhon2 and Fhon13 strains contained a compound eluting just before 3-OH C 16:0. Its mass spectrum, as recorded by quadrupole GC–MS, showed a peak at *m/z* 341, strongly indicating that the compound represents 3-OH C16:1 (data not shown).

2-Heptanone

A clear peak representing 2-heptanone (2-HE) was found in the samples of *L. apinorum* Fhon13. Traces of 2-heptanone were also found in tested *L. melliventris* Hma8 and *L. kimbladii* Hma2 (data not shown). Different strains of *L. apinorum* Fhon13 originating in other honeybees were therefore tested further, and results are displayed in Table 4. Pollen medium, which was used for cultivation of the bacteria, was found to contain traces of 2-heptanone, which may explain the occurrence of the compound in small amounts in the analysed samples.

Fhon13 – SIM method

SIM analyses were made of *L. apinorum* Fhon13 and of the closely related strains isolated from *Apis koschevnikovi* (*Lactobacillus* Kohmto 18), *Apis nuluensis* (*Lactobacillus* Nuhmto 23) and *Apis cerana* (*Lactobacillus* Cehmto 2). The largest amount of 2-heptanone per CFU was found in the samples of *L. apinorum* Fhon13 cultivated in supplemented MRS medium. The amounts found in *L. apinorum* Fhon13 and Kohmto18 cultivated in pollen medium were similar but approximately 14 times smaller than those found in Fhon13 in MRS. Samples of *Lactobacillus* Nuhmto23 and Cehmto2 strains contained the smallest amount of 2-heptanone. Both media (supplemented MRS and Pollen) contain traces of 2-heptanone (Table 4). The pollen medium holds higher amounts of the analysed compound that may be explained by the fact that the same LAB strains are inoculated into the collected bee pollen in the production of bee bread (honeybee larval food) (41).

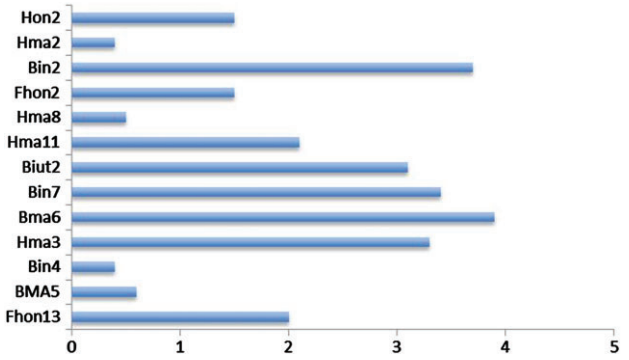

Figure 1 Biofilm formation in vitro of the lactic acid bacterial (LAB) strains derived from honeybees (*Apis mellifera*) varies between the species. Ability to adhere and form biofilm is shown (measured by OD).

Biofilm formation

Biofilm formation was detected in all the 13 LAB strains and it formed without induction or stress *in vitro* (Figure 1). The biofilm formation ability varied between the tested strains and showed that all the four *Bifidobacterium* strains (Bin2, Bin7, Bma6 and Hma3) and *L. kullabergensis* Biut2 were the ones that showed greatest ability to adhere and form biofilm *in vitro* independently of the growing medium used (data not shown).

LAB metabolites found in honey

We could detect nine LAB-produced proteins in stored honeys (Table 5). These originated in *Lactobacillus* Hma2, Hma8, Hon2 and *Bifidobacterium* Bin7, Hma3, Bma6. All the detected proteins had different putative functions and sizes between 33 and 60 kDa. In addition, free fatty acids were detected in varying amounts in freshly harvest honey and stored honeys (Table 6). It appears that these substances do not disappear with time, as they were detected in all sampled honeys. All honeys studied here were found to contain small amounts of most of the monitored 3-OH FAs, ranging between 0·1 and 2·5 pmol/mg (Table 6). 3-OH C 10:0 and 3-OH C 12:0 were the most common 3-OH FAs found in all stored honeys. Only linden honey and freshly harvest honey contained 3-OH C 16:0. None of the tested honeys contained any C 14:0. Overall, the concentrations were low (0.1–1.3 pmol/mg). 3-OH FAs C

Table 5 Proteins produced by lactic acid bacterial (LAB) symbionts found in different stored honey types.

Honey type	Dilution factor	Identified from	Gene number	Size (kDa)	Ion score	No. of peptide matches	Putative function	Closest species ID	Accession no.	Query alignment (%)	Max ID (%)	*E*-value	Signal peptide
Rape seed oil	1:5	Bin7	RIAT00292	41·1	41	1	CRISPR family associated protein	*Bifidobacterium asteroides*	YP_006865567.1	100	96	0	N
Linden	1:5	Bin7	RIAT00039	34·9	45	1	23S rRNA methyl-transferase	*Bifidobacterium asteroides*	YP_006865784.1	100	93	0	N
Linden	1:5	Hon2	RYBW01404	33·6	42	3	Ethanolamine ammonia-lyase light chain	*Lactobacillus rossiae*	WP_017262946.1	99	78	6·00E – 173	N
Raspberry	1:50	Hma3	RVKO00316	34	40	1	Unknown function	*Bifidobacterium catenulatum*	ZP_03324303.1	93	80	5·00E – 143	N
Raspberry	1:5	Bma6	RLWY00667	46·7	45	2	NaqC family transcriptional repressor	*Bifidobacterium longum*	YP_001955801.1	86	66	2·00E – 174	N
Raspberry	1:5	Hma2	ROUL00302	50	44	2	Glucose 6 phosphate isomerase	*Lactobacillus crispatus* ST1	YP_003601227.1	98	87	0·00E + 00	N
Manuka	1:5	Bin7	RIAT00292	41·4	53	1	CRISPR family associated protein	*Bifidobacterium asteroides*	YP_006865567.1	99	96	0·00E + 00	N
Heather	1:5 & 1:50	Hma8	RWLJ00689	42·6	41	1	Mannitol phosphate dehydrogenase	*Enterococcus raffinosus*	WP_010747016.1	99	58	4·00E – 154	N
Heather	1:50	Hon2	RYBW01023	59·9	40	1	Hypothetical protein	*Lactobacillus versmoldensis*	WP_010625130.1	68	29	43–16	Y

Table 6 3-OH FAs (pmol/mg) in fresh honey and stored honeys

Sample	C 10	C 12	C 14	C 16
Fresh honey	0·5	0·1	–	0·2
Two-week-old honey	0·3	0·1	–	–
One-month rapeseed	0·7	–	–	–
Rapeseed*	0·4	0·1	–	–
Linden*	0·9	0·3	–	0·9
Raspberry*	0·6	0·2	–	–
Honey dew*	0·7	0·2	–	–
Heather*	0·5	0·2	–	–
Manuka*	0·4	0·1	–	–

*Stored honeys.

18:0–C 22:0 were found in the sampled honeys, but as these are not of LAB origin these data are not shown.

Discussion

Microorganisms are well recognised to produce bioactive substances to defend themselves and their niche. LAB are known producers of antimicrobial compounds; however, it is known that the properties, qualities and substances produced by LAB are species- and strain-dependent. Furthermore, all LAB species neither exhibit the same antimicrobial qualities nor produce the same antibacterial substances (9,10). In this study, we demonstrate an overall inhibition of all the human wound pathogens analysed (Table 1). The inhibitory effect was greater than from the antibiotic discs regardless of the antibiotic resistance among these pathogens. In some cases, the zones of inhibition from antibiotic discs were very vague or absent. We can hypothesise that the LAB in this case are better than or just as effective as many of the widely used antibiotics in wound treatment today. Combined, the 13 LAB have another advantage over antibiotics as they have a broad spectrum against a wide variety of pathogens (Table 1), while as we know now, many antibiotics are active only against certain bacteria, for example, metranidazole and anaerobic bacteria. Antibiotics are now seldom used for chronic wound treatment because of increase in antibiotic resistance and their inability to penetrate the bacterial biofilm in the wound (42). It was evident that different LAB strains produce metabolites variably active against these wound pathogens as the inhibition zones from each member varied (Table 1). When looking at their individual antimicrobial effects, some are more potent than others against the tested pathogens. *L. mellifer* Bin4 inhibits all encountered pathogens, whereas *L. kunkeei* Fhon2 had the most potent activity

against the pathogens among all the LAB strains used in this study (Table 1).

Traditionally, honey is gathered from wild honeybee colonies by honey hunters when the wax combs contain a mixture of both ripe honey and almost ripe honey with a total water content between 22% and 30%. This method of harvesting fresh honey is still used in large parts of the world and was the only way for mankind to use honey before bee-keeping. We have previously studied fresh honeys and the amount of viable LAB microbiota in crops from all *Apis* species in the world and from some stingless bee species. We found honey with the highest concentration of viable LAB (10^8 per gram honey) in Nepalese honey of *A. laboriosa* and similar quantities in *A. mellifera* honey from Africa (11). Eventually, the LAB die after a couple of weeks in the harvested mature honey because of low water content. The water content of honey in EU is not allowed to exceed 20% with the exception of heather honey (22%). In such honey, sold by bee-keepers and stores, harvested only after the honey is totally ripe, with water content below 20%, zero LAB are viable. In addition, it is a well-known narrative in Europe that honey should not be heated as it will lose its antimicrobial properties. It is possible that this old knowledge is a remnant since from approximately 100 to 200 years ago when people still were hunting honey from wild honeybee colonies in Europe. Today honey is heated or sterilised before it can be used in a medical setting, killing off microorganisms including the LAB symbionts and destroying their bioactive products. Honey collected from wild colonies of honeybees has possibly reflected a myriad of beneficial effects of every specific LAB member in the honey crop.

We now know, however, that the microbiota is also rather consistent across *Apis* species (11,13). LAB diversity could be explained by variation in nutritional content of different nectars and pollen and also by the variation of microbes that they encounter in, for example, flowers. Transient floral microbes trigger the growth of resident LAB microbiota in honeybees and their production of putative antimicrobial proteins (14), a mechanism known for LAB strains in other niches (e.g. *Lactobacillus reuteri*) when producing reuterin (43,44).

In their natural environment, these LAB symbionts' production of active compounds is achieved when they are viable and encounter microbial threats. The LAB symbionts are shaped to defend their occupied niches, which are the honey stomach and honeybee products (honey and beebread) (7,11,25,41). These microbial threats are bacteria, yeast and moulds found in flowers and surrounding environment. Microbial genera and families that are commonly found are *Pseudomonas*, *Enterobacterioceae*, *Bacillus* and *Candida* (11). Interestingly, strains from these genera are also commonly isolated from chronic wounds and can cause major problems in choosing the correct treatment for the infection, as chronic wounds are usually polymicrobial in nature (45,46) . Our hypothesis is that the LAB need to produce bioactive metabolites to defend themselves in their niches and, therefore, their metabolites will be part of the ripe honey as they ferment nectar and counteract microorganisms introduced by foraging. We can also hypothesise that these LAB would have the same characteristics in defending themselves in a chronic wound environment when applied together with their natural food, honey. In this study, we detected some of these metabolites: 3-OH FAs in honey samples (Table 6) and extracellular proteins (Table 5). However, the longevity of produced compounds is to be associated to several factors such as storage time, light exposure, physical conditions of honey harvesting and so on. Another important consideration is that the LAB concentration varies within honeybees and their products (7,11). If certain LAB members within honeybees are in high numbers, they will produce certain active compounds originating in different LAB. When the entire microbiota work synergistically, a complex myriad of antimicrobial compounds is created, which remains in the honey stomach and end up in honeybee products. This could explain why that up to now the unknown factors contributing the antimicrobial properties of honey vary with honey type.

It has been shown that also honey from the stingless honeybees (*Meliponinae*) exhibit non-peroxide antibacterial activity against *S. aureus*, *Enterococcus faecalis*, *Escherichia coli* and *P. aeruginosa* (47). Yet nobody has been able to point out a consensus scientific reason for those activities. Here, we showed a clear antimicrobial activity that originates in bee's symbiotic LAB microbiota including isolates from stingless bees (*M. beechii*) (Table 1), which for the first time gives a reason to honey's well-recognised antimicrobial effect and historical use regardless of any specific nectar or flower. All bees possess these LAB microbiota, but the amount present changes depending on nectar source, bee health and exposure to other microbes (7,11). It appears to be a well-evolved defence mechanism of the bee in order to secure their health and food.

Our results demonstrate a potential explanation for why honey has been applied against threatening human and animal pathogens. One of the most frequent uses of honey by humans through history is wound management. A feasible explanation is that the honey used in folk medicine has been freshly harvest honey which would contain a large amount of viable and active LAB when applied onto wounds. Our recent results show that the LAB produce a large quantity of putative antimicrobial proteins and peptides (14). The LAB sense the presence of other threatening bacteria and start to produce substances to defend themselves. The most common bacterial genus found in flowers is *Pseudomonas*, which is one of most therapy-resistant pathogens in human chronic wounds. *P. aeruginosa* is a very significant chronic wound pathogen because of its biofilm formation, intrinsic multi-drug resistance and its proto cooperative action with other microbes in the wound environment (48). The ability of the LAB to inhibit or kill members of *Pseudomonas* spp. is very pronounced for *L. kunkeei* Fhon2. Other commonly found pathogens in wounds are from the family *Enterobacteriaceae* which, interestingly, also occur in nature therefore would most likely be in contact with honeybees and the LAB symbionts. This bacterial family contains a wide variety of significant wound species that are showing increased antibiotic resistance as well as being involved in biofilm formation (49).

Our previous results show that *L. kunkeei* Fhon2 is the dominant bacterial species found very frequently and in high numbers when sampling honeybee crops, honey, bee pollen and bee bread, and it is always present but in varying numbers regardless of honeybee species, geographic location of the bees or honey origin (7,11–13,41). *L. kunkeei* type strain was primary described as a wine spoiler because of its strong inhibition

properties against *Saccharomyces* yeast involved in wine production (50). This particular lactobacilli appears to be very important for honeybees as it is the very first LAB that establishes in the sterile honey stomach of an emerging bee callow (11). *L. kunkeei* Fhon2 was also shown to be the most potent LAB, potentially inhibiting both food spoiling microorganisms following nectar and pollen to the hive when bees are foraging and bee larval pathogens (11,25). As shown in this study, it is also the most potent one against all human wound pathogens tested. It produces a great variety of extracellular proteins on microbial stress (14) and three different 3-OH FAs (C 10:0, C 12:0 and C 16:0). However, *L. kunkeei* Fhon2 did not produce any bioactive volatiles (Table 2). At the moment, we do not know the exact function of all the produced proteins (14); however, analyses of these protein's known domains show a putative antimicrobial action in many. It is well known that LAB produce low-molecular-weight antifungal substances (51). *Lactobacillus plantarum* MiLAB 14 have been reported to produce 3-OH FAs with antifungal activity. Three of these 3-OH FAs were C10, C12 and C14, the same 3-OH FAs found to be produced by *L. kunkeei* Fhon2 and *L. apinorum* Fhon13 in this study. Sjögren and coworkers (52) found that these fatty acids were more active against yeast than moulds and suggested an antifungal activity connected to the detergent-like properties of the compounds that affect the cell membrane of target microorganisms. The only pathogenic yeast tested in this study was *Candida albicans* and strain Fhon2 showed an inhibition against this wound pathogen. However, there was sporadic growth throughout the inhibitory zone, suggesting different mechanisms of antimicrobial action are used (Table 1). The inhibitory mechanism appears not to be simply linked to 3-OH FAs as the other Lactobacilli (Fhon13) that also produced 3-OH FAs was not active against *C. albicans* by itself (Table 1). Thus, the function of bioactive compounds may need other compounds to work synergistically is shown in the present study.

We were able to find some proteins produced by LAB symbionts in different stored honey types (Table 5). These results show proteins of sizes above 30 kDa as the filters we used had a cut-off of 30 kDa. Larger proteins, these are more than 50 kDa, were not detected either as larger proteins could not diffuse through the electrophoretic gel that was used. Thus, our results show only a small amount of extracellular LAB proteins produced, which is in agreement with our tested nectar source hypothesis. The anti-inflammatory action of honey has been investigated by others (53,54). The mechanism by which honey reduces inflammation is not fully understood, but proteins may be an explanation. We have found one protein with a putative anti-inflammatory function produced by one of the LAB strains (14). However, this certain protein was neither found in any of the honeys studied here, nor any of the other more than 143 putative LAB proteins previously detected by us (14). Consequently, the fact that LAB-produced proteins vary in honeys make an application of the viable LAB much more attractive in future wound management to implement a standardised topical application with a constant amount of proteins with different functions.

Our overall results demonstrate that the LAB metabolites in combination work synergistically (Table 1) and form a myriad of bioactive substances. These bioactive substances (Table 2) are the key for any future application of these LAB in wounds and to elucidate their mechanism of action. Pathogens in wounds are very sensitive for an acidification of the wound environment. Production of organic acids decreases the pH and will form a hostile environment for wound pathogens. Formic acid is known to lower the pH of the wound environment, which lactic acid could also do, and these are produced by all of the LAB symbionts tested in this study. Acetic acid, which was produced by the 13 LAB strains, is known to inhibit the growth of *P. aeruginosa* in wounds (55). In addition, the volatiles produced were very interesting, as many of these compounds have known effects in wound management (Table 2). Benzene is a toxic volatile, but here produced mostly by *L. mellifer* Bin4, in minor amounts. But still, its action may be enough to influence the wound environment. Benzene extracts from fruits have shown an increased rate of wound closure and rate of epithelisation (56). The use of nonane as a solvent may indicate an antimicrobial effect caused by obstruction of the bacterial membranes. Hydrogen peroxide (H_2O_2) in small amounts is required for an optimal wound healing (57). As demonstrated in this study, five of the tested *Lactobacillus* strains (Hma11, Hma2, Bma5, Hma8 and Biut2) produced H_2O_2. 2-Heptanone is a known honeybee pheromone that we here, for the first time, show it is produced by one of the honeybee LAB symbionts, *L. apinorum* Fhon13, and all tested closely related strains isolated from other bees in the world (Table 4). It has recently been discovered that 2-heptonone acts as a local anaesthetic that paralyse *Varroa* mites and wax moth larvae by the honeybee bite (58). In a wound application, it may display the same function, which is promising as chronic wounds cause long-term pain in patients.

Furthermore, all the 13 LAB symbionts showed the ability to form biofilms *in vitro* (Figure 1) and colonisation by the 13 LAB in wounds may be secured by biofilm formation. We know that the investigated LAB symbionts are highly osmotolerant, stable and are viable much longer than other microorganisms, including other LAB, in a honey solution consisting of no more than 25% water content (26). The combination of osmotolerance with their studied antimicrobial and therapeutic characteristic and ability to form biofilms make these LAB symbionts very interesting for future wound applications. Therefore, these LAB symbionts with its myriad of bioactive products may be an optimal alternative in future wound management.

Present antibacterial dressings such as iodine or silver are associated with environmental and patient-related hazards as well as having a high cost for the patient and health sector. An ecological, environmental-friendly wound dressing with antimicrobial properties, as active honey dressing with viable LAB, which is also non-toxic and promotes healing, will be highly demanded in a near future.

Perspectives

The fact of finding new treatments in wound management is already one of the most important tasks in today's clinical and biochemical research. Although a new wave of research concerning honey has escalated during the last decades that may in part be explained by the increasing antibiotic resistance, it is assumed by many researchers that honey's mode of action is its osmolarity and release of hydrogen peroxide. However,

we have recently discovered a unique LAB microbiota in the honey-producing tract of the honeybee given, for the first time, an explanation to the before unknown factors contributing to honey's antimicrobial properties. In this study, we could confirm that LAB symbionts within honeybees are responsible for many of the antibacterial and therapeutic properties of honey. This is one of the most important steps forward in the understanding of the clinical effects of honey in wound management. The explanation model will take honey in combination with its viable and standardised amount of LAB into a much wider clinical use. This has implications not least in developing countries, where fresh honey is easily available, but also in western countries where antibiotic resistance is seriously increasing.

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