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ORIGINAL ARTICLE

A pilot study investigating lactic acid bacterial symbionts from the honeybee in inhibiting human chronic wound pathogens

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Abstract

Treatment and management of chronic wounds is a large burden on the health sector and causes substantial suffering for the patients. We believe that 13 lactic acid bacteria (LAB) symbionts isolated from the honey crop of the honeybee are important players in the antimicrobial action of honey, by producing antimicrobial substances and can be used in combination with heather honey as an effective treatment in wound management. A total of 22 patients with chronic ulcers were included; culture-dependent and molecular-based (MALDI-MS and 16S rRNA gene sequencing) techniques were used to identify bacteria from chronic wounds. These clinical isolates were used for *in vitro* antimicrobial testing with standardised viable LAB and sterilised heather honey mixture. Twenty of the patients' wounds were polymicrobial and 42 different species were isolated. Patient isolates that were tested *in vitro* were inhibited by the LAB and honey combination with inhibitory zones comparable with different antibiotics. LAB and heather honey in combination presents a new topical option in chronic wound management because of the healing properties of honey, antimicrobial metabolite production from the LAB and their bactericidal effect on common chronic wound pathogens. This new treatment may be a stepping stone towards an alternative solution to antibiotics.

Introduction

A chronic wound can be defined as a wound that does not follow normal wound healing trajectory or those that are persisting beyond 6 weeks. Treatment and management of chronic wounds puts a large economic burden on the health sector (1). In USA, direct costs for chronic ulcer management can be up to 25 billion dollars/year (2). Some of the reasons behind the difficulties in treating chronic wounds are microbial complexity with heavy bioburden within the wound, bacterial biofilm formation and the widespread emergence of antibiotic-resistant bacteria (3,4). This leads researchers to consider other previously unexplored ecological niches and traditional/ancient treatments, in particular, with focus on topical treatments.

Management and treatment of these wound types can be quite different and are usually treated according to their origin; for wounds with heavy bioburden, treatment is usually built on available antiseptic dressings such as iodine, silver, polyhexanide, or medical-grade honey (5). Increase in antibiotic resistance has made antibiotic treatment a less attractive alternative (1).

Key Messages

- in this study a mixture of 13 lactic acid bacterial (LAB) symbionts isolated from the honeybee are being investigated as a potential treatment for pathogen inhibition in chronic wounds, when combined with sterilized heather honey
- twenty-two patients with chronic wounds were sampled and microbial characterization was performed using both culture dependent and molecular methods
- identified isolates were used for *in vitro* antimicrobial tests using the LAB and honey mixture and forty-two different species were identified in total with the majority being *Staphylococcus* or *Enterobacteriaceae*
- all clinical isolates that were tested *in vitro* were inhibited by a combination of LAB and sterilized heather honey

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- this study also shows that LAB symbionts from the honeybee have effective antimicrobial activity against common chronic wound bacteria when used in a standardized combination with sterilized heather honey and could be used in the future as an alternative treatment for chronic wounds

Research has shown that chronic wounds are polymicrobial in nature and that pathogens will vary depending on the wound type with species from *Staphylococcus*, *Enterococcus*, *Enterobacter*, *Pseudomonas* and *Finegoldia* genera being the most commonly isolated from all types of wounds (6–8). It has also been reported that the prevalence of different bacterial species from wounds can be dependent on the detection methods used, with molecular-based methods such as full ribosome shotgun sequencing being superior to culture-dependent methods in nearly all cases (5,8). Owing to the variation in wound flora, biofilms and antibiotic resistance, finding an effective treatment can be difficult. Biofilm formation is essential for bacteria in chronic wounds to survive. A biofilm is a community of microorganisms attached to each other or a surface that is surrounded in a three-dimensional matrix of extracellular polymeric substances (EPS) such as extracellular DNA, polysaccharides and proteins (9). This way of surviving is used by bacteria to communicate, resist antibiotic treatment and evade the hosts' immune response. Recently, it has become obvious that bacteria survive in polymicrobial biofilm in a chronic wound scenario (10). The increasing resistance of biofilms against antibiotics suggests that alternative treatment practices must be used.

For hundreds of years, honey has been used as a folk medicine in treating bacterial infections and is still used in the treatment of ulcers and burns. There are some conflicting reports about efficacy of honey (11), nevertheless the prohealing and ecological properties it possesses make it an interesting alternative for wound treatment. The healing and antimicrobial activity of honey is partly attributed to its hygroscopy, high osmolality and low pH as well as its hydrogen peroxide (H_2O_2) content. The H_2O_2 activity has also been widely researched and is considered a large contributor to the antimicrobial efficacy of many widely used honey types. It has been discovered that H_2O_2 in combination with other unknown components of honey contribute to antimicrobial properties of honey (12). This discovery points towards other possibly unknown chemical substances in honey that could possibly contribute to its therapeutic and antimicrobial effects. The most widely used honeys in medicine are Manuka (*Leptospermum scoparium*) honey and Revamil® source (RS) honey that have varying degrees of antimicrobial activity towards different pathogens (13–15). Manuka honey has been shown to be effective in the healing of surgical wounds (16). Manuka honey contains the antimicrobial agent methylglyoxyl (MGO), which has been reported to originate from dihydroxyacetone of the Manuka flower (17) as well as a number of unidentified metabolites, proteins and peptides. RS honey has been reported to contain MGO in smaller concentrations as well as the antimicrobial peptide Bee-defensin 1 (13). However, the abovementioned bioactive substances found in honey, including MGO, can be of bacterial origin (18). In addition, other chemical factors affecting the antimicrobial and healing properties of different honey types and the unidentified compounds need to be characterised (19,20). One study has also shown that Scottish heather honey is just as effective as Manuka honey in inhibiting bacteria associated with equine wounds which suggests that other antimicrobial substances, other than MGO, are involved in the healing effect that is attributed to honey (21).

It has been discovered that a group of lactic acid bacteria (LAB) comprising nine *Lactobacillus* and four *Bifidobacterium* species inhabit the honey crop of the honeybee *Apis mellifera* during biofilm formation (22). In this group of LAB symbionts the majority are newly described species (23), are important for bee defence, and are consistent in all nine *Apis* species and in stingless bees in all continents, suggesting both honeybee and LAB have coevolved together (24,25). Interestingly, these LAB symbionts are involved in the production of honey and are present in fresh honey in large numbers (24,25). We have recently observed that these symbionts produce antimicrobial substances such as formic acid, hydrogen peroxide and free fatty acids as well as different extracellular proteins such as enzymes, bacteriocins and lysozymes (26,27). We also showed that proteins found in different honey types can be traced back to these LAB species including unknown proteins and enzymes (28). The LAB symbionts are very effective in the inhibition of the significant bee pathogens *Melisococcus plutonius* and *Paenibacillus larvae* that cause American and European Foulbrood Disease, and have antimicrobial activity against 55 bacterial species and five yeasts found in flowers (27,29). A recent exciting finding showed that these LAB symbionts separately and together have strong antimicrobial activity against a wide variety of human pathogens including antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) (28).

The LAB symbionts from *A. mellifera* that are involved in honey production and inoculate honey are important players in the antimicrobial action of honey by producing a myriad of antimicrobial metabolites and peptides, and therefore could be used in combination with heather honey as an ecological and effective alternative treatment in wound management.

The aims of this study were to examine the microbial composition of hard-to-heal chronic ulcers recruited from a wound healing centre in Sweden using both molecular- and culture-based techniques. The potential inhibitory effects were investigated for a combination of the 13 LAB symbionts reintroduced into Swedish heather honey on identified human wound bacteria *in vitro*.

Materials and methods

Patient information

The study period was between 2 October 2013 and 2 February 2014, that is, 4 months. All patients were treated at Blekinge Wound Healing Centre (BWHC), which is a primary care-based specialist centre for treatment of patients with chronic ulcers in the county of Blekinge (150 000 inhabitants).

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We registered the gender and age of the patient, ulcer aetiology, duration (in weeks) as well as ulcer pain, measured by VAS (the Visual Analogue Scale). Ulcer size was measured by a digital planimeter (Visitrak®; Smith & Nephew Medical Limited, Hull, UK).

We also noted if the patients had been treated with systemic antibiotics during the last 6 months before entering the study and if so, what kind of antibiotic treatment was given. All patients had an ulcer with heavy exudation. All patients received oral and written information about the study and gave informed consent before inclusion.

Bacterial culture

Samples were received as swabs in transport tubes containing Amies medium. A dilution series was made using sterile phosphate buffered saline (PBS; pH 7·2) and were inoculated into Blood, Haematin and Maconkey agar aerobically, and Fastidious anaerobe agar (FAA) anaerobically at 37°C for up to 48 hours. Colonies were counted and morphologically different colonies were then picked for identification.

Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS)

MALDI-TOF MS was performed on the isolates as previously described (30) with few modifications. Bacterial isolates were cultured as described above. Bacteria were applied in duplicate as a thin film to a 96-spot steel plate (Bruker Daltonics, Solna, Sweden) and allowed to visibly dry at room temperature. The direct-transfer formic acid method was used for all samples (31). Each bacterial target was covered first with formic acid (Sigma-Aldrich, Stockholm, Sweden) and left to dry, and then covered with 1 μl of α-cyano 4-hydroxycinnamic acid (HCCA) matrix solution [50% acetonitrile, 47·5% water, 2·5% triflouroacetic acid (Sigma-Aldrich, Sweden)] and air-dried. The plate was then loaded into an ultraflextreme MALDI-TOF/TOF MS instrument and experiments were performed in linear positive mode in a mass range of 2–20 kDa. Mass spectra were analysed using the FlexControl and MALDI Biotyper 3·1 software and reference databases (MBT-BDAL-5627). The Biotyper software provides additional information on the isolate identification through a comment, informing the user of the limitations of the technique [e.g. species that are difficult to discriminate (*Enterobacter cloacae* group), species included in the same bacterial complex or group (*Klebsiella oxytoca* group), species for which additional tests are needed]. Identification scores above 2 and between 1·7 and 2 were considered to be reliable at the species and genus levels, respectively. Identification scores below 1·7 were considered unacceptable and were interpreted as no identification. Samples that were not identified by MALDI-TOF MS were prepared for 16S rRNA gene polymerase chain reaction (PCR) amplification and sequencing.

16S rRNA gene genotypic characterisation

Characterisation of unidentified bacteria using 16S rRNA gene sequencing was carried out according to previous work (22) with some modifications that are described here briefly. Colonies of each unidentified bacteria were recultured for 24–48 hours depending on their growth conditions. DNA was extracted before PCR amplification of 16S rRNA genes. Amplification of isolates was performed using universal primers ENV1 and ENV2 (TAG, Copenhagen, Denmark). PCR products were sent for Value Read sequencing at Eurofins MWG operon (Ebersberg, Germany) and identified sequences were then searched against GenBank [National Centre for Biotechnology Information (NCBI), Rockville Pike, MD] using the advanced BLAST similarity search option (available at http://www.ncbi.nlm.nih.gov).

Preparation of LAB and honey mixture

The LAB and heather honey mixture used for the antimicrobial assays consists of 13 viable species of LAB (*Lactobacillus kunkeei* Fhon2, *Lactobacillus apinorum* Fhon13, *Lactobacillus mellis* Hon2, *Lactobacillus mellifer* Bin4, *Lactobacillus kullabergensis* Biut2, *Lactobacillus kimbladii* Hma2, *Lactobacillus helsingborgensis* Bma5, *Lactobacillus melliventris* Hma8, *Lactobacillus apis* Hma11, *Bifidobacterium coryneforme* Bma6, *Bifidobacterium asteroides* Bin2, *Bifidobacterium* sp Bin7 and *Bifidobacterium* sp Hma3 (total cell count of all 13 LAB;10^9 cfu/g honey)] (22,23) and their bioactive substances in a matrix of Swedish-sterilised heather (*Calluna vulgaris*) honey (gel form). Sterilisation of the honey was performed at 102°C for 30 minutes (29). To obtain the spray form, the gel was mixed with sterile water (1 g/5 ml). These mixtures were then prepared and incubated for 24 hours at 35°C to allow the LAB to become active (total cell count after incubation: 10^{11} cfu/ml) and produce bioactive metabolites (26). These activated mixtures were evaluated for antimicrobial activity.

Dual culture overlay assay

Antimicrobial activity was measured by using dual culture overlay assay as previously described (27,32) with few modifications.

Antimicrobial action was measured by adding LAB and honey mixture in gel or spray form (10^{8-10} cfu/disc depending on activation time) (incubated as mentioned in a previous method to allow for production of bioactive metabolites) and their respective controls (heather honey without LAB symbionts) into a filter disc (10 μl) and placed on de Man, Rogosa & Sharpe (MRS) agar plates (supplemented with 0·1% L-cysteine and 2% fructose) followed by overnight incubation at 35°C. Culture collection isolates from American Type Culture Collection (ATCC) of the most common wound pathogens were first tested as positive quality controls (data not shown). The identified wound pathogen cultures containing their respective media were mixed with 10 ml soft agar (0·8%) at a temperature of 42°C. Prior to mixing, pathogenic cultures were adjusted to 10^8 cells/ml (OD of 0·5–0·6 at 540 nm). Each mixture of soft agar was poured as a layer on top of MRS plates with the overnight cultivated LAB. The plates were incubated at 37°C for 24 hours. All the tests with the 13 LAB symbionts were performed in triplicate for each pathogen tested. Zone sizes were measured from the centre of the disc to zone edge, and doubled for diameter.

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Antibiotic susceptibility assay

Antibiotic susceptibility test was performed on wound isolates using the EUCAST (European Committee on Antimicrobial Susceptibility testing) disc diffusion method as previously described (http://www.eucast.org) with some modifications. Briefly, wound isolates were grown overnight on their respective medium and adjusted to 0·5 MacFarland concentration (0·08–0·1 at 600 nm). Isolates were placed on Muller Hinton (MH) agar and antibiotic discs were placed on top. Plates were incubated overnight at 37°C. Zone sizes were measured from the centre of the disc to zone edge, and doubled for diameter.

LAB survival assay

LAB survival in wound exudate was studied using well diffusion assay and dual culture overlay assay, as previously described, with some modifications (32). The overnight culture of the LAB and honey mixture were mixed with 10 ml soft agar (0·8%) containing MRS (supplemented with 0·1% L-cysteine and 2% fructose), and holding at a temperature of 42°C. Prior to mixing, the culture was adjusted to 10^8 cells/ml (OD of 0·5–0·6 at 540 nm). Each mixture of soft agar was poured as a layer on top of Tryptone Soya Broth (TSB) plates where the exudate was added (10 μl spots). The plates were incubated at 37°C anaerobically for 48 hours.

Well diffusion assay was performed as previously described with some modifications (33). LAB and honey mixture was incubated overnight and added to 20 ml soft MRS agar (supplemented with 0·1% L-cysteine and 2% fructose) after being adjusted to 10^8 cells/ml. The mixture was then poured into an empty petri dish and dried. Small wells were cut (17 mm) into the agar and the wound exudate was added (40 μl) and dried. Plates were then incubated for 48 hours anaerobically at 37°C. Ampicillin (10 μg) antimicrobial discs were used as a positive control in both methods.

Results

The study population included 22 patients from whom 29 samples were taken, that is, there were more than one sample in some patients. There were 9 men (41%) and 13 women (59%). The mean age was 77 years (median 82 years, ranging from 27 to 99 years). The mean ulcer duration was 47 weeks (median 36 weeks, ranging from 3 to 208 weeks) and mean ulcer size was 6·5 cm^2 (median 4·85 cm^2, ranging from 0·3 to 111·6 cm^2). Fourteen patients (64%) experienced ulcer pain with a VAS mean of 6·3, ranging from 2 to 10.

The ulcer diagnosis was venous ulcers in ten cases (45%), arterial ulcers in three cases, foot ulcers in diabetic patients in three cases, pressure ulcers in two cases, one patient with a venous-arterial ulcer, one with osteomyelitis, one with a traumatic ulcer and another patient with pemphigus. One patient died during the study period.

Six patients had been treated with antibiotics because of ulcer infection before entering the study. In three cases, the patients were treated with flucloxacillin (patient number 6, 12 and 22). Two patients (patient number 11 and 14) were treated with clindamycin and another patient (patient number 15) with phenoxymethylpenicillin. Two other patients were treated with ciprofloxacin because of a urinary tract infection (patients 3 and 20). No patients were taking antibiotics during the course of this study.

We identified 20 different bacterial genera with 42 species from 22 patients by culture-dependent and molecular-based techniques (MALDI-TOF and 16S RNA gene sequencing). The most commonly isolated bacterial species are depicted in Figure 1. Nearly all patients (91%) had a polymicrobial colonisation in their wound with more than two isolates being identified in each case (data not shown). Twenty-one patients were positive for *Staphylococcus* species with *S. aureus* being the most commonly isolated species (64% of patients) from this genus. *Enterococcus faecalis* was the second highest identified isolate in 64% of all patients. *Escherichia coli* (32%), *E. cloacae* complex (27%), *Proteus mirabilis* (27%), *Pseudomonas aeruginosa* (23%), *Finegoldia magna* (23%), *Staphylococcus pettenkoferi* (23%), *Streptococcus* species (14%), *Morganella morganii* (14%), and *Bacteroides fragilis* (14%) were the next most frequently isolated pathogens (Figure 1). Eleven gram negative and ten gram positive genera were recognised from all wounds with the majority of species being facultative anaerobes (Table 1). Five genera of obligate anaerobes were found. Only two species of yeast were identified, both belonging to the genus *Candida* (Table 1).

Three patients had a second sample taken after 4–8 weeks to assess if the wound stayed colonised by the same bacteria (data not shown). At least three bacterial species identified in the first samples were present in the recurring sample that was taken a few weeks later.

All wound isolates tested against LAB and honey combination were inhibited successfully (Table 2). The zones of inhibition varied between the different genera and the *Enterobacteriacae* family and *Pseudomonas* showed to be most susceptible to the LAB honey combination (Table 2, Figure 2). All zones of inhibition with LAB and honey combination were comparable to antibiotic susceptibility tests (Table 2). All zones for LAB antimicrobial activity were clear sharp zones except for some species such as *K. oxytoca* that had a clear zone with a hazy outline (Figure 2). It was observed that the LAB symbionts used in this study had the ability to survive in chronic wound exudate received from three of the patients' wounds for at least 24 hours (data not shown).

Discussion

As previously demonstrated by others, multiple species reside together in chronic wounds and the most commonly detected bacterial species belonged to the *Staphylococcus*, *Enterobacter* and *Enterococcus* genera. The results of this study are in accordance with previous studies (6,34,35) in that *S. aureus* or other *Staphylococcus* species were identified in the majority of patients (Table 1, Figure 1). We noticed that all wounds were moderately different from each other in their overall microbial wound composition and the identified bacteria reflected previous studies in most cases (6), except for the percentage of patients with *S. aureus* infecting their wounds, as this was lower than data from other studies

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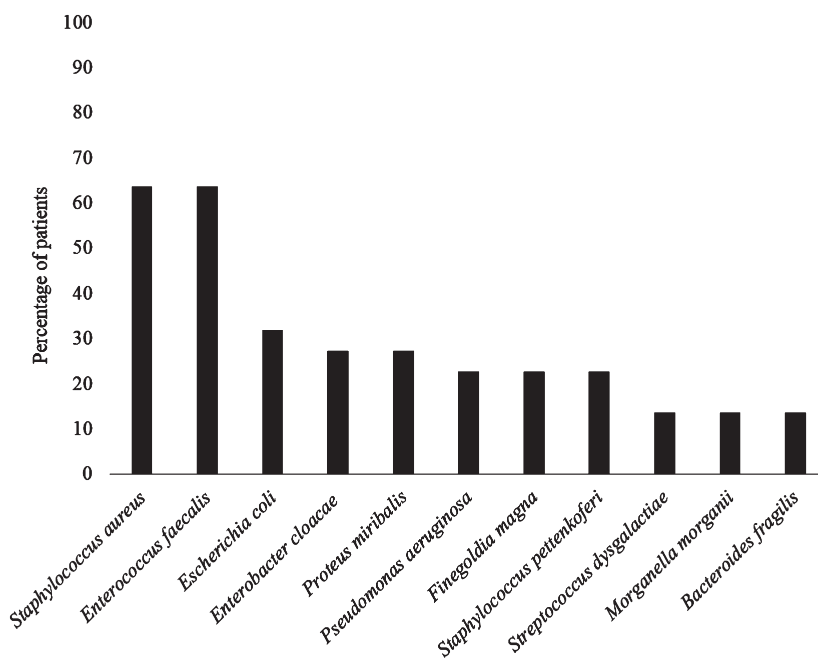

Figure 1 Percentage of most frequently identified bacterial species from hard-to-heal ulcers.

S. aureus colonisation in wounds is known to impair wound healing on a significant level and with multiple-antibiotic-resistant strains becoming more widespread, a need for new treatments for *Staphylococcus* infection is becoming increasingly important. We isolated many morphologically different *S. aureus* colonies on agar from patients which suggest strain variation; however, this is common in chronic wounds and infections caused by biofilm formation (36). *S. pettenkoferi*, a coagulase-negative Staphylococci (CNS), is another significant *Staphylococcus* species that is problematic in chronic infections such as osteomyelitis in diabetic foot ulcers (37). In this study, *S. pettenkoferi* was isolated in 23% of the patients, which suggests that this is a significant species in the colonisation of certain chronic ulcers. All patients who harbour *S. pettenkoferi* had a venous ulcer and in one case osteomyelitis was present, suggesting a trend not previously observed in other studies as it has been associated with infection only recently in the last decade (38) (data not shown).

Interestingly, only a small percentage (13%) of the patients, were colonised by *Streptococcus dysgalactiae* (Table 1, Figure 1). This species has been implicated in many studies as a significant coloniser of chronic wounds; yet in this study it was not the case. However, in another study from the same centre, the percentage of patients colonised with this *Streptococcus* was almost the same (8).

Different *Enterococcus* species were also detected from 14 of the patients tested in this study (Table 1, Figure 1). *Enterococcus* species are one of the most common pathogens isolated from chronic wounds and are known to be involved in biofilm formation (6). The case with *P. aeruginosa* is similar, as 23% of patients were colonised with this bacterial species (Figure 1). *P. aeruginosa* is of key importance in wound infections because of its high resistance against antibiotics, role in biofilm formation and utilization of other bacterial species to survive in the host (36). Anaerobic bacteria such as *F. magna* and *B. fragilis* were readily isolated from the patient samples. *F. magna* (formerly known as *Peptostreptococcus magnus*) is a skin and mucous membrane commensal. However, it has recently been shown to be an opportunistic pathogen associated with other infections and diseases including chronic wounds (39). Many species from the *Enterobacteriacae* family were identified in the patient samples, such as *E. coli*, and species *K. oxytoca* group, *P. mirabilis*, *M. morganii*, *E. cloacae* complex and *Citrobacter freundii* (Table 1). These bacterial genera and species are significant in wound infections (6).

It was also observed in patients who were retested that the majority of bacteria identified from the first sample were still present when tested again at a later sampling occasion (data not shown). This can indicate which bacterial species are most likely involved in the delayed healing of the wounds. A Danish study has previously shown similar results suggesting the polymicrobial environment and resident pathogens in chronic wounds (40). They showed that *S. aureus*, *E. faecalis*, *Proteus* species and *P. aeruginosa* were among the most common bacteria identified, which is similar to the results of this study (Table 1). Another study has shown that the majority of chronic wound infections are polymicrobial (80% of wounds colonised by more than five species) with most colonisation by aerobic bacteria or a mixture of both aerobes and anaerobes (41). This is in accordance with the results of this study where 89% of patients were colonised by more than three different bacterial species. It is now possible to assume that one bacterial species on its own does not cause the non-healing of chronic wounds but that a mixture of microorganisms work together in synergy to cause this wound pathology (42,43). Consequently, a treatment that is active against several pathogens causing a chronic wound is needed.

In this study, we have shown that a mixture of the 13 LAB symbionts in a matrix of heather honey has antimicrobial activity against all isolated bacterial strains associated with

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Table 1 Identification of pathogens at a genus and species level by MALDI-MS and 16S rRNA gene sequencing*

Genus identified	Isolates from patients ($n = 22$)	Species identified	Gram	Aerotolerance
Staphylococcus	21	*S. aureus*	+	FA
		S. pettenkoferi		
		S. epidermidis		
		S. warnerii		
		S. haemolyticus		
		S. lugdunensis		
		S. caprae		
		S. capitis		
		S. saprophyticus		
		S. pseudointermedius		
		S. cohni		
Enterococcus	14	*E. faecalis*	−	A, FA
		E. avium		
Proteus	8	*P. mirabilis*	−	FA
		P. vulgaris		
		P. penneri		
Escherichia	7	*E. coli*	−	FA
Enterobacter	6	*E. cloacae* complex	−	FA
		E. hormaechei		
		E. kobei		
Streptococcus	6	*S. dysgalactiae*	+	FA
		S. agalactiae		
		S. gordonii		
Finegoldia	5	*F. magna*	+	OA
Pseudomonas	5	*P. aeruginosa*	−	A
Corynebacterium	3	*C. striatum*	+	A, FA
		C. coylae		
Bacteroides	3	*B. fragilis*	−	OA
Klebsiella	3	*K. oxytoca* group	−	FA
		K. pneumoniae		
Morganella	3	*M. morganii*	−	A
Clostridium	2	*C. perfringens*	+	OA
		C. halophilum		
Candida	2	*C. albicans*	Yeast	FA
		C. parapsilosis		
Peptonophilus	2	*P. harei*	+	OA
Actinomyces	2	*A. neuii*	+	OA
		A. turicencisis		
Dermobacter	2	*D. hominis*	+	A
Arthrobacter	1	*A. cuminsii*	+	A
Citrobacter	1	*C. freundii*	−	FA
Brevibacterium	1	*B. celere*	+	A
Acinobacter	1	*A. pitii*	−	A
Oligella	1	*O. urethralis*	−	A

A, aerobic; FA, facultative anaerobe; OA, obligate anaerobe.
*The table displays the amount of wounds colonised by such genus, and a breakdown of the identified pathogens to the species level, Gram stain and aerotolerance.

chronic wounds (Table 2). The ability of these LAB symbionts to survive and possibly be involved in production of honey makes honey the perfect candidate as an effective carrier medium for the LAB in the treatment of chronic ulcers. As mentioned earlier, honey has the potential to treat bacterial infections because of its hygroscopic properties and low pH, which helps inhibit bacterial growth, improves epithelialisation and reduces inflammation as well as deodorising malodorous wounds (44). Manuka honey is the most widely known and used in medical applications, yet many reports show that other honey types have similar healing effects (21,45). This is the reasoning behind the choice of using Swedish wild heather honey in this case as it was observed to be just as effective in bacteria inhibition as Manuka or other medical-grade honey types and is also widely produced throughout Europe (21).

We also discovered that the LAB symbionts caused the largest inhibition against *P. aeruginosa* (Figure 2, Table 1). These LAB symbionts could have more activity against the genus *Pseudomonas* as it is a widely found genus in nature and in flowers. Therefore, it is reasonable to assume that the

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Table 2 *In vitro* antimicrobial activity of LAB and honey treatment*

Identified microbe	LAB and honey gel	LAB and honey spray	Honey control	Antibiotic susceptibility			
S. aureus	23	22	0	F 24	V 16	Ox 34	
E. faecalis	17	18	0	Amp 28			V 28
E. coli	32	22	0	Amp 16	V 0	A 22	
E. cloacae complex	27	25	0	G 22			
P. mirabilis	23	19	0	Amp 24			
P. aeruginosa	34	26	0	G 24			A 26
F. magna	21	20	0	P 42	Met 46	A 44	C 18
S. pettenkoferi	23	21	0	F 36	V 16	Ox 32	
S. dysgalactiae	24	20	0	P 16	G 30	F 42	A 40
M. morganii	27	24	0	Cip 26	Amp R	F R	
B. fragilis	26	20	0	Met 46			C R
K. oxytoca group	24	19	0	G 20			A 20

*Table showing the results of the zones of inhibition (diameter in millimetres) from the most common wound bacterial isolates against LAB and honey treatment. Antibiotic susceptibility test performed; F, fusidic acid (10 μg/ml); V, vancomycin (5 μg/ml); Ox, oxacillin (5 μg/ml); Amp, ampicillin (30 μg/ml); G, gentamicin (30 μg/ml); Met, metronidazole (10 μg/ml); P, penicillin (10 μg/ml); A, Amykacin (30 μg/ml); Cip, ciprofloxacin (5 μg/ml); C, clindamycin (2 μg/ml). All assays have been performed in duplicate.

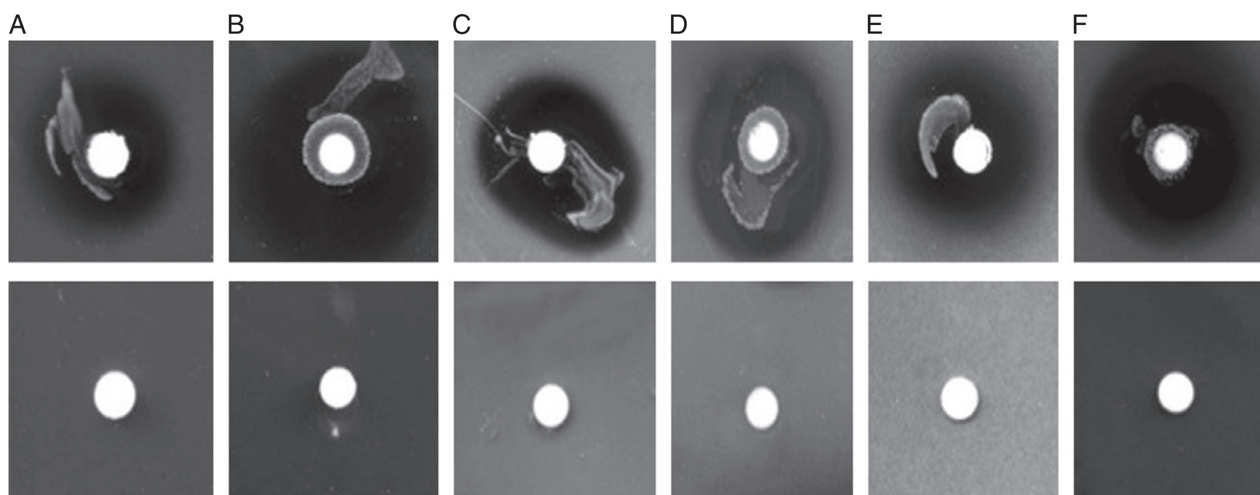

Figure 2 Dual culture overlay zones of inhibition for most commonly isolated bacterial species from patient wound samples. Top line shows lactic acid bacteria (LAB) and honey gel or spray and bottom line shows control (honey without LAB) (A = *S. aureus*, B = *E. coli*, C = *E. cloacae* complex, D = *P. aeruginosa*, E = *S. pettenkoferi*, F = *K. oxytoca* group).

LAB symbionts would have had more exposure to these species because of the high concentration of these bacteria in nectar transported in the honeybee stomach. We have previously shown that the LAB symbionts inhibit microorganisms present in flowers and nectars (27). They appear to have evolved a defence strategy against incoming microorganisms surrounding them in order to defend their niche and host.

In previous research, we have detected different bioactive metabolites including free fatty acids, 2-heptanone and other toxic volatiles, as well as organic acids such as formic acid, and hydrogen peroxide that can lower the pH of their surrounding environment and work in inhibiting a wide variety of pathogens (28). Most of these bioactive substances have an antimicrobial effect and partly cause the inhibitory effects depicted in the

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present experiments. Another possible source is the LAB's production of active peptides and proteins. We recently published that these LAB symbionts produce different extracellular proteins during microbial stress, such as lysozymes, enzymes and putative bacteriocins, which could also account for some of the inhibition results in this study (26). Therefore when combined, it is reasonable to assume that these LAB are extremely powerful in producing an inhibitory effect. It is a well-established fact that most LAB species produce antimicrobial peptides known as bacteriocins and other antimicrobial metabolites that have been shown to have antimicrobial activity against a broad range of pathogens (46). One study has observed that *Lactobacillus plantarum* supernatant has antipathogenic effects against *P. aeruginosa* (47).

Extracellular metabolites act differently on different pathogens as some can be bacteriostatic or bactericidal as well as have different mechanisms of action such as interfering with cell membrane permeability, DNA synthesis or by changing the growth environment, for example by decreasing the pH. In most cases for this study, in the antimicrobial assays, the zones of inhibition were clear with an outer ring of hazy growth (Figure 2; *K. oxytoca* group has obvious pattern). This could possibly reflect that there is more than one metabolite or protein having a different mechanism of action on inhibiting the isolates; however more experiments need to be performed to confirm this.

Research has shown that other species of LAB can have positive immunemodulatory effects in the wound healing process, for example, *Lactobacillus reuteri*. It was demonstrated to accelerate collagen deposition at the site of the wound, which is essential for the healing process (48). However, to our knowledge no research has used any viable Lactobacilli or Bifidobacteria directly on the wound surface, only with indirect techniques such as ingestion, or *in vitro* studies. No study had ever used a mixture of 13 LAB species with their metabolites that work synergistically in order to combat microorganisms as a broad barrier. Thus, if animal and human studies are in accordance with the *in vitro* results observed in this work, the 13 LAB symbionts in the honey matrix could possibly be applied as a novel alternative and environmental friendly treatment in future wound management.

Concluding remarks

Chronic wounds are a significant burden on society because of their high economic impact and decrease in the quality of life of patients. Novel research that investigates effective treatments is therefore extremely important especially because of biofilm formation in the wound, pathogen variation and of course the continuing emergence of antibiotic-resistant bacteria. This is the first time in history that since their discovery, both the novel honeybee LAB and honey have been standardised together in a wound management application in a manner similar to fold medicine, where fresh honey was applied as medicine for a millennia. We believe that the LAB, their metabolites and heather honey in combination present a new topical option in the treatment and management of chronic wounds. This is attributed to the healing properties of honey, the presence of antimicrobial LAB metabolites and the LAB's ability to combat pathogens most commonly involved in chronic wounds. This new standardised mixture may be a stepping stone towards an alternative solution to antibiotics.

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