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ARTICLE

The unusual antibacterial activity of medical-grade *Leptospermum* honey: antibacterial spectrum, resistance and transcriptome analysis

S. E. Blair · N. N. Cokcetin · E. J. Harry · D. A. Carter

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Abstract There is an urgent need for new, effective agents in topical wound care, and selected honeys show potential in this regard. Using a medical-grade honey, eight species of problematic wound pathogens, including those with high levels of innate or acquired antibiotic resistance, were killed by 4.0–14.8% honey, which is a concentration that can be maintained in the wound environment. Resistance to honey could not be induced under conditions that rapidly induced resistance to antibiotics. *Escherichia coli* macroarrays were used to determine the response of bacterial cells to a sub-lethal dose of honey. The pattern of gene expression differed to that reported for other antimicrobial agents, indicating that honey acts in a unique and multifactorial way; 78 (2%) genes were upregulated and 46 (1%) genes were downregulated more than two-fold upon exposure to the medical-grade honey. Most of the upregulated genes clustered into distinct functional regulatory groups, with many involved in stress responses, and the majority of downregulated genes encoded for products involved in protein synthesis. Taken together, these data indicate that honey is an effective topical antimicrobial agent that could help reduce some of the current pressures that are promoting antibiotic resistance.

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S. E. Blair · N. N. Cokcetin · D. A. Carter (✉)
School of Molecular and Microbial Biosciences,
University of Sydney,
Sydney, NSW, Australia
e-mail: d.carter@mmb.usyd.edu.au

S. E. Blair · E. J. Harry
Institute for the Biotechnology of Infectious Diseases,
University of Technology,
Sydney, NSW, Australia

Introduction

Bacterial pathogens have developed resistance to every antibiotic introduced into clinical practice [1]. Today, most bacteria that cause infections in hospitals are resistant to at least one antibiotic [2], and some are resistant to all commonly employed antibacterial drugs [3]. There is an urgent need for new antibiotics with novel modes of action, but few are currently under development [4]. Many large pharmaceutical companies have abandoned antimicrobial research as the cost of bringing new drugs to market is not being recovered, in part because micro-organisms so rapidly develop resistance to new products [1].

Staphylococcus aureus is one of the most commonly acquired pathogens in both the community and the hospital settings, and it is particularly problematic in skin and wound infections [5]. The emergence of methicillin-resistant *S. aureus* (MRSA), and, more recently, vancomycin-resistant strains [6], has seriously compromised treatment options. Multi-drug resistance is, likewise, a major problem among the Enterobacteriaceae, which are the most frequent pathogens isolated by clinical microbiological laboratories [7]. Members of the Enterobacteriaceae that commonly cause wound infections include *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella* and *Serratia*. Many hospitals are now also seeing the emergence of intrinsically resistant pathogens that were previously uncommon. For example, *Acinetobacter* spp. cause a multitude of infections in skin and soft tissue, with a high associated mortality and a particularly broad spectrum of

resistance phenotypes [8]. In one hospital study, 89% of *Acinetobacter* sp. isolates were resistant to at least three classes of antibiotics, and 15% were resistant to the nine antibiotics tested [9]. Multi-drug resistance limits treatment options and results in the use of more expensive or more toxic drugs, with corresponding increases in patient morbidity and mortality [10–12].

Prior to the introduction of modern antibiotics, dressing wounds with honey was a common practice, and it has been known for almost a century that honey has antibacterial properties. A number of recent studies have shown that honey is an effective infection control agent in specific situations, particularly in wound care [13]. The current issues surrounding antibiotic resistance, and a growing body of evidence supporting the use of honey as a dressing for a wide range of wounds, has increased interest in its use in the clinic [14–20]. Honey provides a moist healing environment and can prevent (if used early enough) or rapidly clear existing wound infections. Several other properties make it an attractive wound dressing: honey debrides wounds and removes malodour, it reduces oedema and exudate, prevents or minimises hypertrophic scarring and hastens healing [13]. Certain honeys are now available in the form of various sterile products licensed for use in wound care in Australia, Canada, the European Union, Hong Kong, New Zealand and the USA.

Various antimicrobial factors present in honey have been identified. Honey is high in sugar (~80%) and is relatively acidic (typical pH ranges from 3.2 to 4.5), making it unsuitable for most microbial growth [21]. Most honeys, when diluted with water, produce hydrogen peroxide, which is toxic to microbes [22]. This is due to glucose oxidase, a bee-derived enzyme that is introduced during honey production. In addition, a few honeys possess an extra, florally derived antibacterial activity [23]. This activity is largely restricted to certain *Leptospermum* honeys from New Zealand and Australia (colloquially known as manuka and jelly bush honey, respectively). *Leptospermum* honeys are particularly well suited to therapeutic use as they are very stable during storage and in the presence of body fluids, and they show no toxicity towards mammalian cells [13]. Two laboratories have recently reported that the activity of *Leptospermum* honeys correlates with the presence of methylglyoxal (MG), an alpha-oxoaldehyde that reacts with macromolecules such as DNA, RNA and proteins [24, 25]. Toxicity issues with MG have been raised [26]; however, the extensive consumption of manuka honey and its widespread use as a wound dressing indicate that other components in the honey may suppress mammalian toxicity.

Transcription analysis using small-format microarrays or larger-format macroarrays allows the action of antimicrobials on the entire target organism to be investigated and quantified. Analysis of the transcriptome response to a range of antibiotics has revealed that they often have much more complex mechanisms of action and a greater range of targets than was previously appreciated [27, 28]. Antibiotics used at or below their minimum inhibitory concentrations (MICs) induce or repress the transcription of dozens to hundreds of genes, with different classes of antibiotics producing characteristic signatures of differential expression. Often, these relate to the general mechanism of action of the antibiotic through the inhibition of a primary target, but they can also encompass action on secondary targets and other downstream effects [27–29]. Monitoring gene signatures with microarrays provides valuable information about the mode of action of inhibitors, and potentially elucidates novel targets for new antimicrobial drug discovery [27, 29–33].

Although there are a number of studies showing that honey is effective against common pathogens, it is not commonly used in modern infection control. More information on the spectrum and mode of activity of honey is required for it to gain widespread acceptance as a therapeutic agent in conventional medicine. The broad objective of this work was, therefore, to determine how bacterial cells respond, at the molecular level, to the presence of medical-grade *Leptospermum* honey with activity beyond that which can be accounted for by hydrogen peroxide production or osmolarity.

Methods

Honeys

The following honeys were used in this study: (1) active *Leptospermum* honeys (Medihoney® Antibacterial Honey Barrier, Medihoney Pty Ltd., Brisbane, Australia, a proprietary blend of active *Leptospermum* honey and honey with high levels of hydrogen peroxide-dependent activity, or Comvita Manuka Woundcare 18+, Comvita New Zealand Ltd., Paengaroa, New Zealand, a pure *Leptospermum scoparium* honey); (2) control *Leptospermum* sp. honey (Capilano Honey Ltd., Brisbane, Australia), derived from *Leptospermum* sp. but without the active floral factor present in Medihoney or Comvita honey (as the addition of catalase removed all detectable activity from this honey); (3) Lucerne Blueweed honey (LuBl) (supplied by Dr. Doug Somerville, Senior Apiary Officer, NSW Department of Primary Industries, Australia), hydrogen peroxide-dependent activity only; (4) artificial honey, included as an osmotic control and composed of 7.5 g sucrose, 37.5 g maltose, 167.5 g glucose and 202.5 g fructose in 85 ml of sterile deionised water. All of the natural honeys had a level of activity equivalent to approximately 18% phenol, which

was previously determined using the standard test against *S. aureus* [23].

Bacterial strains and growth media

Clinical isolates of a variety of bacterial pathogens were obtained from the Royal Prince Alfred Hospital (RPA), Sydney, Australia, and are listed in Table 1. All cultures were supplied and maintained on nutrient agar (NA) (Oxoid, Basingstoke, UK). The RPA Microbiology Department supplied resistance profiles. Reference strains of *S. aureus* (ATCC 9144) and *Pseudomonas aeruginosa* (ATCC 27853) that are commonly used in susceptibility testing were obtained from Oxoid and maintained on Mueller-Hinton (MH) media (Oxoid). *Escherichia coli* (K-12) was maintained on Luria-Bertani (LB) broth and agar media.

Assessment of MICs and MBCs

Agar incorporation method

The MIC of active *Leptospermum* honey blend (Medihoney) was determined against all isolates listed in Table 1 using previously published solid-media methods [34]. Briefly, Medihoney agar plates were prepared in 1% increments by incorporating various concentrations of honey into NA (Oxoid). Artificial honey plates were prepared in the same manner, but in 5% (w/v) increments. Honey stock solutions and honey plates were freshly prepared for each experiment. Clinical isolates were grown overnight in nutrient broth (Oxoid) at 37°C and were inoculated onto the dried honey plates in 1 μl volumes using an antibiotic sensitivity replicator (HI Clements Pty Ltd., Sydney, Australia). Plates were incubated at 37°C overnight, and the MIC was determined as the lowest concentration of honey that completely inhibited visible growth of the test organism. At least two replicate plates were done for each bacterial strain on a given day and the entire process was repeated on at least two separate days.

Macrodilution method

The NCCLS tube dilution method with liquid media [35] was used in the studies aimed at generating antibiotic-resistant and honey-resistant phenotypes. Cultures of *P. aeruginosa* and *S. aureus* were prepared by picking five colonies from an MH agar plate and suspending them in 5 ml of MH broth. The suspensions were incubated at 35°C for 2–6 h with shaking. Following incubation, transmittance at 530 nm was adjusted to 0.8–0.88 with sterile MH broth, and cultures were then further diluted to 1:100 in MH broth. One ml of the bacterial suspensions was added to each bottle (containing the appropriate honey or antibiotic concentration) to give a final working concentration of 5×10^5 cfu/ml. Honey solutions were prepared in MH broth in 1% w/v increments. Serial dilutions of each of the antibiotics tetracycline (broad-spectrum; MP Biochemicals, St. Louis, MO, USA), ciprofloxacin (narrow-spectrum for *P. aeruginosa*; MP Biochemicals, St. Louis, MO, USA) and oxacillin (narrow-spectrum for *S. aureus*; Sigma-Aldrich, Irvine, Scotland) were prepared in sterile MH broth from the stock solutions (1,280 μg/ml in sterile deionised water). MICs were defined as the lowest concentration of honey or antibiotic that resulted in no visible growth of the organism.

Table 1 Summary of minimum inhibitory concentrations (MICs) for honey against antibiotic-resistant clinical isolates

Organism (*n*)	MIC (w/v % honey)[1]		
	Medihoney		Artificial[3]
	Average±1SD	Range	
MRSA (13)[2]	4.2±0.4	4.0–5.0	>25
R *Staphylococcus aureus* (5)	4.1±0.2	4.0–4.5	>25
S *Staphylococcus aureus* (2)	4.4±0.2	4.3–4.5	>25
All *Staphylococcus aureus* (20)	4.2±0.3	4.0–5.0	>25
Acinetobacter calcoaceticus (4)	8.1±1.5	6.0–9.3	20–25
Citrobacter freundii (2)	9.1±3.0	7.0–11.3	20–25
Enterobacter cloacae (18)	11.8±1.4	8.8–14.3	20–25
Enterobacter aerogenes (1)	13.8±0.5	–	20–25
Enterobacter agglomerans (1)	7.0±0.0	–	>25
All *Enterobacter* (20)	11.7±1.8	8.8–14.3	>20
Escherichia coli (9)	7.5±0.8	6.3–8.5	20–25
Klebsiella pneumoniae (7)	13.0±2.4	8.0–14.8	20–25
Morganella morganii (1)	7.8±0.5	–	20–25
Serratia marcescens (1)	14.8±0.5	–	20–25

[1] MICs were determined using the agar dilution method

[2] MRSA = methicillin-resistant *Staphylococcus aureus*; R = isolates resistant to antibiotics other than methicillin; S = isolates sensitive to all antibiotics tested

[3] 25% was the highest concentration of artificial honey prepared

The viability of bacteria in any tubes with no turbidity was determined by spreading 100 μl onto an NA plate (Oxoid) and incubating at 37°C for 24 h. The lowest honey or antibiotic concentration that resulted in no survival of viable bacteria was recorded as the minimum bactericidal concentration (MBC). At least two replicate plates were done for each bacterial strain on a given day and the entire process was repeated on at least two separate days. As specified in the NCCLS reference methods, all susceptibility testing with oxacillin was done with the addition of 2% NaCl to the growth media.

Statistical analysis of the MIC results was performed with SPSS 15.0 statistical software, using the Mann-Whitney *U*-test.

Selection for resistance to honey and antibiotics

S. aureus strain ATCC 9144 and *P. aeruginosa* strain ATCC 27853 were prepared as above and exposed to active *Leptospermum* honey (Comvita), LuBl honey or antibiotics (tetracycline, ciprofloxacin and oxacillin), starting at 0.5 MIC. Cultures were incubated for 48 h at 37°C, after which 1 ml of the culture was removed and added to a new honey or antibiotic solution, which had a 5% increase in concentration of the inhibitor. Cultures were again incubated for 48 h at 37°C, and the process was repeated, incrementing the appropriate inhibitor by 5% each time. The entire process was repeated for each strain/inhibitor combination. Any strain developing a resistant phenotype to any of the inhibitors was retested to determine its MIC and MBC for all of the honeys and antibiotics.

Macroarray analysis

Macroarray analysis was performed with the Panorama™ *E. coli* Gene Array system (Sigma-Genosys), in which amplified DNA from the 4,290 open reading frames of *E. coli* K-12 (MG1655) is spotted in duplicate onto replicate nylon filters.

Two duplicate 20-ml solutions of 6% (w/v) of active *Leptospermum* honey (Medihoney) or 6% (w/v) control inactive *Leptospermum* honey were prepared in LB broth. The concentration of honey chosen was below the MIC for *E. coli* K12 (10%) to allow inhibitory effects to be determined without killing the cells, as dying cells would confound the analysis (see Supplementary Figure S1). Catalase was added to each honey solution (57.2 mg/20 ml) (Sigma-Aldrich) to remove any hydrogen peroxide-dependent antibacterial activity, and the honey solutions were filter sterilised. An overnight culture of *E. coli* K-12 (MG1655) in LB broth was diluted (1:100) in fresh medium and incubated on a shaker at 37°C to mid-exponential phase (OD_{600} 0.3). Aliquots of this culture (500 μl) were used to inoculate the honey solutions, which were incubated on a shaker for 3 h at 37°C.

Total RNA was extracted from two 6-ml aliquots of the honey-treated cells using the protocol supplied by the manufacturer of the Panorama™ *E. coli* Gene Array, with the following minor modifications: (1) cells were washed three times in ice-cold resuspension buffer that contained 0.5 M EDTA and then resuspended in 500 μl of DEPC-treated water containing 0.5 M EDTA prior to hot, acidic phenol RNA extraction; (2) 0.5 μl of RNase Inhibitor (Roche) was added during RNA precipitation and during DNA-free™ (Ambion) treatment. The two samples of a given treatment (active or control honey) were then pooled for the macroarray analysis.

Labelled cDNA was generated according to the manufacturer's instructions, with the addition of 0.5 μl of RNase Inhibitor to the reaction mix and 20 μCi[α-^{33}P] dCTP. All of the gene probes on the macroarrays were spotted in duplicate. After hybridisation and washing, the Panorama™ *E. coli* Gene Arrays were exposed to a phosphor screen (Molecular Dynamics) overnight. The resulting scanned images were analysed by Sigma Genosys USA, using ArrayVision™ (Imaging Research Inc.) software, and the fold difference in gene expression between the two samples was calculated.

Results

Active *Leptospermum* honey (Medihoney) is potently active against antibiotic-resistant clinical pathogens

MICs for the commercially available active *Leptospermum* honey, Medihoney, were determined for a range of important clinical pathogens using the agar dilution method. All of the bacterial pathogens tested were sensitive to Medihoney at concentrations well below any inhibition exerted by the osmotic effect of the artificial honey (Table 1; Supplementary Table S1). Medihoney was particularly potent against *S. aureus*, with an average MIC of 4.2% and a range of 4–5% (w/v). In contrast, 25% artificial honey did not inhibit growth of any of the *S. aureus* isolates.

Resistance profiles determined by the Royal Prince Alfred Microbiology Department (Sydney, Australia) found that only two strains of *S. aureus* (S14 and S19) were sensitive to all of the antibiotics tested. All other isolates were resistant to at least one antibiotic, many to at least seven antibiotics, and one to 13 different antibiotics (Supplementary Table S1). There was no significant difference in the Medihoney MICs for the drug-sensitive or drug-resistant *S. aureus* isolates, including MRSA (P= 0.6). Similarly, the Gram-negative clinical isolates, which

included isolates resistant to six or more different antibiotics, were all significantly more sensitive to Medihoney than to artificial honey, with MICs ranging from 6.3 to 14.8% Medihoney (P=0.006). Even isolates of the intrinsically resistant species *Acinetobacter calcoaceticus* were susceptible to low levels of Medihoney (average 8.1% honey; range 6.0–9.3%). There was no discernable correlation between the sensitivity of the organisms to honey and their drug resistance profiles.

Resistance to honey is not acquired following continuous exposure to sub-lethal concentrations

Bacteria rapidly acquire resistance to antimicrobial agents, and, theoretically, the increased use of honey could likewise promote honey resistance and could induce cross-resistance to other antimicrobials. We therefore investigated the ability of bacterial cells to become resistant to increasing sub-lethal concentrations of honey. MICs and MBCs of the honeys and control drugs were assessed before and after continuous exposure to the inhibitors. Exposure to sub-lethal concentrations of the antibiotics tetracycline, oxacillin and ciprofloxacilin rapidly induced a resistance phenotype in the antibiotic-susceptible strains of *S. aureus* (ATCC 9144) and *P. aeruginosa* (ATCC 27853) (Table 2). In contrast, constant exposure of these organisms to increasing sub-lethal concentrations of active *Leptospermum* honey (Comvita) and LuBl (hydrogen peroxide-type) honey could not raise the level of resistance past the initial honey MICs, and it was not possible to generate a honey-resistant strain under these conditions (Table 2). Resistance to one antibiotic resulted in increased cross-resistance to the second antibiotic for both *S. aureus* and *P. aeruginosa*. For example, the tetracycline-exposed strain of *S. aureus* developed a 64-fold increase in resistance to tetracycline and a 32-fold increase in cross-resistance to oxacillin (Table 2). When challenged with honey, however, the strains with antibiotic-resistant phenotypes remained unchanged in their levels of honey susceptibility (Table 2). The MICs and MBCs for the honeys were similar (Table 2). Bactericidal inhibitors have MBC values that are close to the MIC; for bacteriostatic agents, the MIC is much lower than the MBC. These results, therefore, indicate that honey is bactericidal toward these pathogens.

The global cellular response of *E. coli* to active *Leptospermum* honey

The effect of a sub-lethal concentration of active *Leptospermum* honey (Medihoney) on *E. coli* gene expression was compared to the same concentration of an inactive *Leptospermum* honey. *E. coli* growth was retarded but not completely inhibited by 6% active *Leptospermum* honey vs. 6% inactive honey (see supplementary Figure S1), and this concentration was, therefore, selected for the macroarray analysis.

Figure 1 shows a section of the Panorama™ *E. coli* Gene Array following exposure to labelled RNA generated from cells exposed to active *Leptospermum* honey (Medihoney) or control (inactive) *Leptospermum* honey. All of the gene

Table 2 Induction of resistance to honey and antibiotics

	Initial values		Final values for 'TE resistant' strain		[1]Final values for 'OX/CP resistant' strain	
	MIC[a]	MBC[a]	MIC	MBC	MIC	MBC
S. aureus						
LuBl honey (% w/v)	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0
Active *Leptospermum* honey (% w/v)	5.0±0.0	7.0±0.0	4.8±0.5	6.8±0.5	5.0±0.0	7.0±0.0
Tetracycline (μg)	1.0±0.0	>16*	64±0.0	>64*	[3]32±0.0	>32*
Oxacillin (μg)	0.5±0.0	>16*	[2]16.0±0.0	>32*	32±0.0	>32*
P. aeruginosa						
LuBl honey (% w/v)	13.0±0.0	16.0±0.0	13.0±0.0	15.3±0.5	13.0±0.0	16.0±0.0
Leptospermum honey (% w/v)	14.0±0.0	16.0±0.0	13.5±0.6	16.0±0.0	14.0±0.0	16.0±0.0
Tetracycline (μg)	16.0±0.0	>64*	512±0.0	>1028*	[3]128±0.0	>512*
Ciprofloxacin (μg)	0.5±0.0	4±0.0	[2]16.0±0.0	>32*	32±0.0	>32*

TE = tetracycline; OX = oxacillin; CP = ciprofloxacin

[a,b] MICs and MBCs were determined using the NCCLS broth dilution methods

*Highest concentration tested

[1] OX = oxacillin-induced resistance in *S. aureus*; CP = ciprofloxacin-induced resistance in *P. aeruginosa*

[2] Cross-resistance of the strains with induced tetracycline (TE) resistance to OX (*S. aureus*) or CP (*P. aeruginosa*)

[3] Cross-resistance of the strains with induced OX resistance (*S. aureus*) or CP resistance (*P. aeruginosa*) to TE

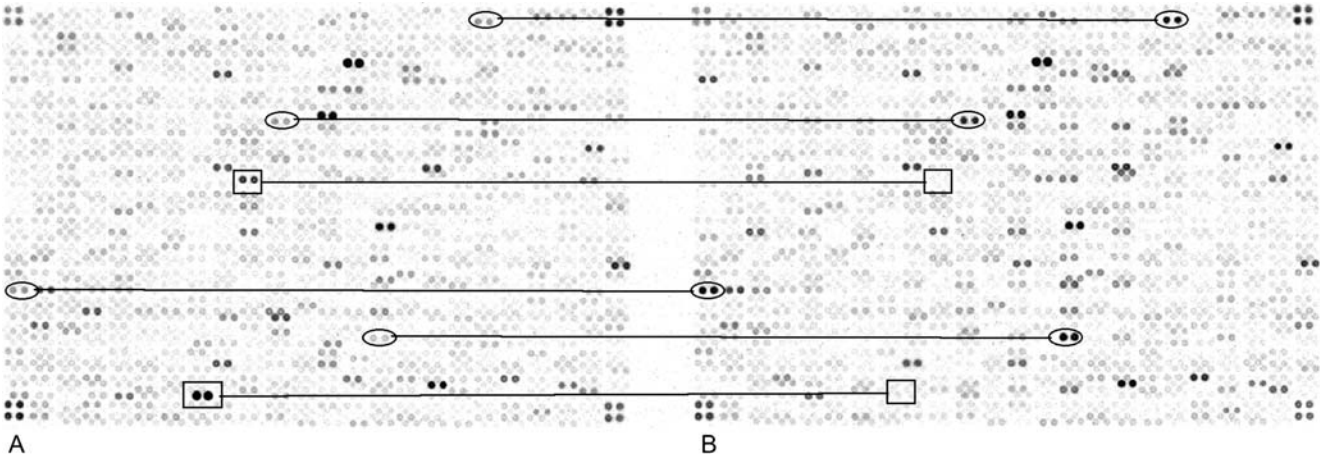

Fig. 1a, b Detail of *E. coli* macroarray following exposure to: (**a**) active *Leptospermum* honey or (**b**) control honey. Two identical arrays were probed with P^{33}-labelled cDNA produced from total RNA extracted from cells exposed to active or control honey. This figure shows a section of one of the macroarrays, with some examples of genes that were differentially expressed. All genes are spotted in duplicate, and there was a high level of consistency between duplicate spots. The four spots in each corner are of control genomic DNA. ▭ Genes significantly upregulated by exposure to active *Leptospermum* honey. ⬭ Genes significantly downregulated by exposure to active *Leptospermum* honey

probes were spotted in duplicate on the arrays and the expression levels were consistent between the duplicate spots. The expression levels of most of the 4,290 *E. coli* genes were similar in cells exposed to active *Leptospermum* or control inactive honey. However, 78 (1.82%) genes were upregulated and 46 (1.07%) genes were downregulated more than two-fold upon exposure to Medihoney.

Most of the genes upregulated by Medihoney clustered into distinct functional regulatory groups, with many involved in stress responses (Fig. 2). These included genes regulated by histone-like nucleoid-structuring (H-NS) protein, which is well conserved in bacteria and is considered to be a universal repressor of gene expression [36]. Numerous RpoS-controlled genes were likewise upregulated by Medihoney. RpoS plays an important role in cell adaptation by controlling the expression of a large set of genes under numerous stress conditions and as cells enter stationary phase, and it also modulates gene expression during exponential growth [37–39]. A third group of upregulated stress-response genes were part of the EvgAS regulon, a two-component system used by *E. coli* in adaptive responses to acid resistance, osmotic resistance and drug resistance [40, 41]. Several chaperones were upregulated, as were genes involved in dealing with oxidative damage. Around 16% of upregulated genes were of unknown function. The majority of genes downregulated

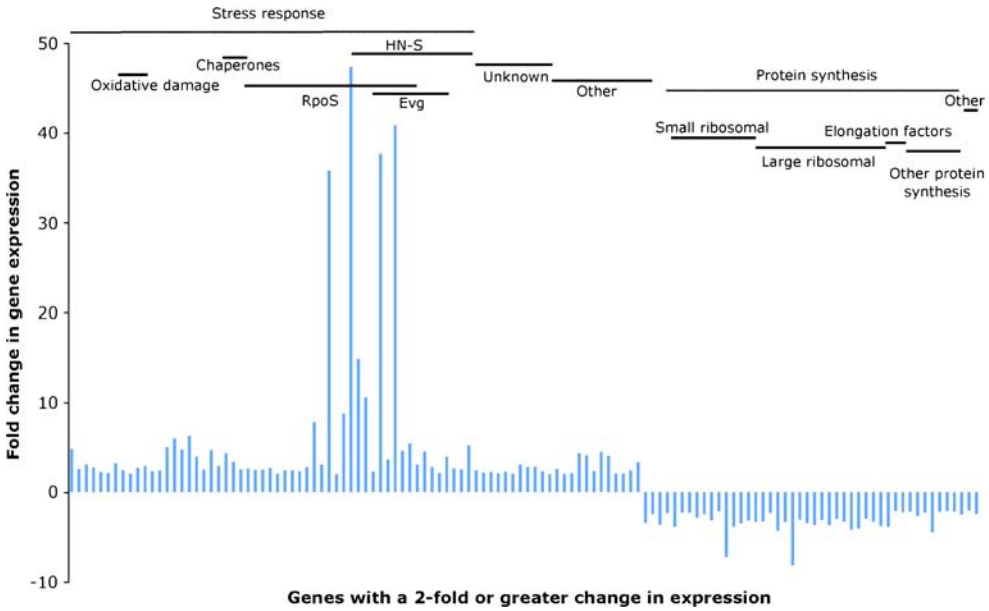

Fig. 2 Functional grouping of genes differentially expressed following exposure to active *Leptospermum* honey. The expression levels of most of the 4,290 *E. coli* genes were similar in cells exposed to active *Leptospermum* or control inactive honey. However, 78 (1.82%) genes were upregulated and 46 (1.07%) genes were downregulated more than two-fold upon exposure to Medihoney

by Medihoney encode products involved in protein synthesis (Fig. 2), including various ribosomal proteins and elongation factors.

Discussion

Honey has an unusually broad spectrum of antimicrobial activity

Honey has significant antibacterial properties that have been well documented [13, 42, 43]; however, few studies to date have used a readily available, registered, medical-grade honey, making it difficult to apply these findings in clinical practice. Here, we have shown that the medical-grade *Leptospermum* honey, Medihoney, has antibacterial activity against a wide range of Gram-negative and Gram-positive wound pathogens, regardless of their antibiotic-resistance profile. This includes clinical isolates that were resistant to up to 13 different antibiotics. The MICs and MBCs for honey were similar for *S. aureus* and *P. aeruginosa*, indicating that, at least for these pathogens, honey is a bactericidal agent. Other studies have found similar results when using *Leptospermum* honey from New Zealand [34, 44–46].

Honey resists resistance

The emergence of extensive resistance to antibiotics has arguably occurred due to their overuse and misuse, and it is widely recognised that sub-lethal concentrations of antibiotics favour the development of a resistant phenotype [47]. Recent studies attribute the antibacterial activity of *Leptospermum* honey to methylglyoxal (MG) [24, 25], and as this is a small, simple molecule, it might be expected to induce resistance in similar ways to other antibacterial agents, e.g. by reduced uptake, increased efflux, direct degradation or increased detoxification. MG detoxification pathways have been identified in bacterial cells, as these must deal with some level of endogenous MG production [48]. However, although isolates of *S. aureus* and *P. aeruginosa* exposed to increasing, sub-inhibitory levels of tetracycline and oxacillin (*S. aureus*) or ciprofloxacin (*P. aeruginosa*) rapidly became resistant to these inhibitors, they were not able to develop any form of resistance to active *Leptospermum* honey or to honey with antibacterial activity due to hydrogen peroxide production.

A combination of antimicrobial agents is known to slow or prevent resistance. While the low pH, high sugar content and hydrogen peroxide production in honey all play a part in it antibacterial activity, these were either reduced or removed in our experiments. Honey is a complex substance with over 200 components [49], and it is possible that it contains one or more compounds that block the ability of a bacterial cell to develop honey resistance. Furthermore, although bacterial cells that were resistant to one antibiotic displayed considerable cross-resistance to a second antibiotic, no cross-resistance to honey was seen. Cross-resistance often occurs via a generic structural modification, such as decreased cell wall permeability, decreased drug accumulation or increased drug efflux, and the cross-protection conferred by biocides against other antimicrobials is of increasing concern [50, 51]. Importantly, these results demonstrate that an increased use of honey is unlikely to result in honey resistance, and that its use will not compromise the efficacy of any other antimicrobials.

Active *Leptospermum* honey induces a unique global cellular response

Active *Leptospermum* honey induced a complex and unique transcriptional response in *E. coli*. A two-fold or greater change was seen in the expression of approximately 3% of all *E. coli* genes when exposed to Medihoney, with 63% of these upregulated and 37% downregulated. This number of differentially expressed genes falls within the range reported for several other studies investigating transcriptional changes to antibacterials [52–57]. Most of the downregulated genes (~89%) have a role in protein synthesis. This response is frequently seen upon exposure to toxic agents, and it is likely that it occurs as a matter of economy while an organism is exposed to stress [52, 57, 58]. Many of the upregulated genes are involved in stress responses, including genes influenced by RpoS, EvgAS and H-NS, genes encoding a number of chaperones, and some affected by oxidative damage. Overall, when compared to the published data on the effects of various growth inhibitors, including antibiotics, on the gene expression of *E. coli*, active *Leptospermum* honey produced a unique expression signature, suggesting that it works by a different mode of action to the other inhibitors [27].

RpoS plays an important role in cell adaptation by controlling the expression of a large set of genes when cells enter stationary phase or are exposed to various stress conditions [38]. Recently, RpoS has been found to affect gene modulation during exponential growth in *E. coli*, where it is involved in iron scavenging [39]. RpoS also protects exponentially growing *E. coli* cells against osmotic shock [59], toxins, including MG [60], and acid stress [61].

Iron sequestration is crucial for pathogenic micro-organisms [62]. During exponential phase, the expression of iron acquisition functions comprises a key component of the RpoS regulon in *E. coli* [39]. It is interesting that, although numerous RpoS-controlled genes were upregulated by active *Leptospermum* honey, we did not see an increase in iron acquisition gene expression. Active

Leptospermum honey has been shown to be effective against non-bacterial pathogens, such as the yeast *Candida* [63], and dermatophytes, which are filamentous fungi [64]. It is possible that one or more components in the honey interfere with the ability of microbes to acquire useful iron. As iron is essential for many metabolic processes, this might explain, in part, the broad-spectrum activity and the lack of adaptive resistance to the effects of honey

Ferguson et al. found the induction of RpoS to be important in protecting cells against MG, but a transcriptome analysis of the effects that MG alone on microbial gene expression has never been carried out. Although MG is toxic to microbial cells, they also produce it, which is thought to relieve cells from stress caused by elevated levels of sugar phosphates [65, 66]. To counter MG production, cells employ MG detoxification systems, and three different systems have been found in the enteric bacteria [65]. The most important of these is the glutathione-dependent GlxI/GlxII (glyoxylase II) pathway, encoded by the genes *gloA* and *gloB* in *E. coli* [67]. *E. coli* also possesses an aldo-keto reductase with low affinity for MG [68] and a novel glyoxylase (GlxIII) pathway, which catalyses the glutathione-independent conversion of MG into D-lactate [69]. Finally, *E. coli* possesses KefB and KefC K^+ efflux systems that are linked to the Glx system and aid in protection from MG [48, 70]. In the present study, we did not see a change in the expression of any genes involved in the MG detoxification through the Glx system or in MG metabolism via aldo-keto reductases, nor were the KefB/C efflux systems differentially expressed. *E. coli* growth is inhibited when MG reaches 0.3 mM, and loss of viability occurs above 0.6 mM [48]. Based on a comparison with previously published data [24], we estimate the MG level in the 6% honey used to be a maximum of 0.29 mM. As this is just below the limit where *E. coli* growth is inhibited, it is possible that transcription of the detoxification/metabolism genes is not induced. Alternatively, transcription of these genes may be repressed by honey, allowing it to inhibit *E. coli* growth at what should be sub-inhibitory levels.

The potential of honey in infection control

The data presented here argue for a greater use of medicinal-grade honey in wound care, particularly where antibiotic resistance is an issue. Our study suggests that it is unlikely that resistance to honey will develop, even with increased use. Honey has many ideal properties of a wound dressing, including the maintenance of a moist wound environment (which is essential for timely healing), non-toxicity, anti-inflammatory actions, debriding activity, reduction in scarring and the stimulation of re-epithelisation (reviewed in [13]). The gene expression signature of *E. coli* cells exposed to active *Leptospermum* honey indicates that it has a mode of action that is distinct from conventional antibiotics. Further investigations into the mode of action of the non-peroxide antibacterial activity of this honey are warranted, as these may lead to new classes of antimicrobials, which are desperately needed.

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