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Specific non-peroxide antibacterial effect of manuka honey on the *Staphylococcus aureus* proteome

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ABSTRACT

Manuka honey, derived from the New Zealand flowering plant *Leptospermum scoparium*, shows promise as a topical antibacterial agent and effective chronic wound dressing. The aim of this study was to determine the non-peroxide antibacterial effects of this honey on the proteome of the common wound pathogen *Staphylococcus aureus*. Proteomic analysis was performed on cells treated for a short time with manuka honey compared with the proteome of untreated cells as well as cells treated with a *Leptospermum* honey sample without antibacterial activity. Treatment with manuka honey resulted in a significant decrease in the bacterial cell growth rate as well as downregulation of ten and upregulation of two proteins. Nine of these proteins were also differentially expressed by cells treated with the inactive *Leptospermum* honey, but to a lesser degree, and the rate of bacterial growth was not affected. The differentially expressed proteins have roles in ribosomal function, protein synthesis, metabolic processes and transcription. Manuka honey uniquely caused downregulation of two proteins [dihydrolipoamide dehydrogenase (DLD) and elongation factor Tu (EF-Tu)] associated with two of these pathways as well as upregulation of one stress-related protein [cold shock protein C (CspC)]. The proteomic profile following treatment with manuka honey differed from the profiles of other antibacterial agents, indicating a unique mode of action and its potential value as a novel antimicrobial agent.

1. Introduction

The benefits of honey as a medicine have been reported since ancient Egyptian times [1]. However, use of honey as a wound treatment has become a developing area of research, with positive clinical results [2]. Honey has several properties that contribute to its wound-healing effects, including antibacterial, debriding and deodorising activity, as well as anti-inflammatory and tissue growth-promoting properties [3].

In addition to providing osmotic conditions that impede bacterial growth, most honeys produce hydrogen peroxide through the action of the bee-derived enzyme glucose oxidase upon dilution with water [4] and this can confer significant antimicrobial activity. However, in the presence of catalase, which breaks down hydrogen peroxide, some honeys, such as manuka honey derived from the New Zealand flowering plant *Leptospermum scoparium*, still retain potent antimicrobial activity [5]. This 'non-peroxide' activity is particularly advantageous in clinical situations as it is not destroyed by the catalase present in body fluids [6]. Jelly bush honey, also of the *Leptospermum* genus and native to Australia, can have similar high levels of non-peroxide antibacterial activity, although this varies in different honey samples (Fig. 1) [7]. Non-peroxide activity is unaffected by γ-irradiation [1], allowing *Leptospermum* honeys to be sterilised for medicinal use.

Recently, high concentrations of an antibacterial compound, methylglyoxal (MG), were found in manuka honeys [8,9]. MG is a reactive α-oxoaldehyde formed both enzymatically and non-enzymatically in mammalian and microbial cells as an intermediate in the glycolytic pathways [10,11]. MG is involved in the formation of advanced glycation end products implicated in growth arrest and cell death [10]. Clinical studies on the wound healing properties of manuka honey have found that it stimulates healing and promotes the re-growth of healthy tissue with no adverse effects [2], indicating that direct toxicity towards mammalian cells is either lacking or suppressed.

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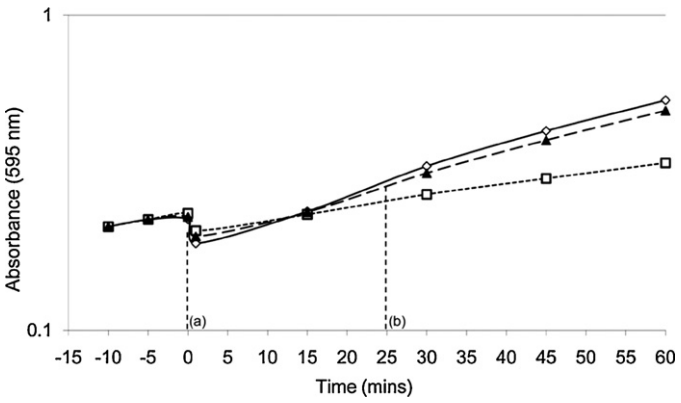

Fig. 1. Effect of 4% (w/v) jelly bush honey and manuka honey on the growth of *Staphylococcus aureus*. Briefly, 4% (w/v) manuka or jelly bush honeys were added to cultures of *S. aureus* at T_0 (a) and cells were harvested at 25 min for proteomic analysis (b). Error bars denote ± 1 standard deviation. The *y*-axis is in logarithmic scale. ◊, untreated control; ▲, 4% jelly bush honey treatment; □, 4% manuka honey treatment.

Honey inhibits a broad range of microorganisms and is equally as effective against antibiotic-resistant organisms as it is against those that are susceptible to antibiotics [12]. *Staphylococcus aureus* is a common pathogen found in chronic wounds and is sensitive to the antibacterial effects of manuka honey, with minimum inhibitory concentrations (MICs) of <5% regardless of the drug resistance profile [12,13]. Furthermore, resistance to honey could not be induced in *S. aureus* under in vitro conditions that rapidly induced resistance to conventional antibiotics [12]. This is especially pertinent in light of the current global antibiotic resistance crisis, as it suggests that honey has a unique mode of action and may be used where conventional antibiotics are no longer effective. However, the nature of this mode of action is not well understood. Elucidation of the mechanism(s) of action of manuka honey is required for its consideration as a viable option in effective chronic wound treatment. Here we perform a proteomic analysis of the specific effects of the non-peroxide activity of manuka honey on exponentially growing *S. aureus* cells. The results suggest that the non-peroxide component of manuka honey inhibits bacterial growth via a unique mode of action.

2. Materials and methods

2.1. Honey preparation

Manuka (*L. scoparium*) honey (Comvita WoundCare18+, batch number 1489733; Comvita New Zealand Ltd., Paengaroa, New Zealand) had non-peroxide activity equivalent to ≥18% (w/v) phenol against *S. aureus* [14]. The jelly bush (*Leptospermum polygalifolium*) honey (Capilano Honey Ltd., Brisbane, Australia) used in this study had no detectable antibacterial activity using the same assay and was used as an inactive honey control. Immediately before use, 50% (w/v) solutions of each honey in tryptone soya broth (TSB) (Oxoid Ltd., Basingstoke, UK), with catalase (Sigma, St Louis, MO) (5900 U/mL) to remove hydrogen peroxide, were prepared and sterilised by filtration through 0.2 μm pore filters (Millipore, Bedford, MA). An equivalent concentration of catalase was used in the no-honey control cultures.

2.2. Bacterial strain and growth conditions

Staphylococcus aureus RN4220 (derived from NCTC 8325-4) was used in this work since it provides a valuable comparison with the numerous genetic and proteomic studies employing this strain. Honey and/or catalase was added to exponentially growing cells of *S. aureus* [optical density at 595 nm (OD_{595}) = 0.2–0.3, which had already been grown to mid-exponential phase, then diluted back and allowed to reach mid-exponential phase a second time] in TSB at 37 °C at 200 rpm. Cells were collected after 25 min further incubation, centrifuged, washed twice in ice-cold TE buffer [10 mM Tris–HCl, 1 mM ethylene diamine tetra-acetic acid (EDTA) (pH 8)], re-suspended at 600 mg wet weight of cells/mL ice-cold TE containing a protease inhibitor cocktail (Roche, Sydney, Australia) and snap-frozen.

2.3. Protein extraction and quantification

Harvested cells (120 mg wet weight) were treated with lysostaphin (36 U) at 37 °C for 30 min followed by ultrasonication [8 × 20 s at 100% power with a DIGITAL Sonifier® S-450D (Branson, Danbury, CT)]. Protein was extracted under acidic conditions with 50 mM LiCl (pH 4) to dissociate proteins from non-protein macromolecules and simultaneously reduced and alkylated [15]. Protein yield was quantified, samples were diluted to 1.5 mg protein/mL with 7 M urea, 2 M thiourea and 1% C7BzO and stored at −20 °C.

Protein yield was determined using serial dilutions against a whole-cell *S. aureus* RN4220 reference protein extract that was briefly electrophoresed on a Criterion XT 1D gel (Bio-Rad, Hercules, CA) in MES [2-(*N*-morpholino)ethanesulfonic acid] buffer to form a single band for each sample. Gels were stained using Flamingo quantitative fluorescent stain (Bio-Rad) and were quantified using a Pharos FX Plus Molecular Imager and Quantity One® software (Bio-Rad).

2.4. Proteomic analysis

Three experimental conditions were analysed using three biological replicate experiments, each with triplicate gels (27 gels in total): (i) untreated (media plus catalase) control; (ii) 4% (w/v) jelly bush honey control (with catalase); and (iii) 4% (w/v) manuka honey (with catalase). The biological replicates of these treatment conditions were run in parallel using spectral counting quantitation with liquid chromatography mass spectrometry (LC–MS/MS).

2.4.1. Two-dimensional gel electrophoresis (2-DGE) image acquisition and analysis

Samples containing ca. 340 μg of protein were filtered (100 kDa cut-off), de-salted in Bio-Spin chromatography columns (Bio-Rad) and used to rehydrate pH 4–7, 11 cm immobilized pH gradient (IPG) strips (GE Healthcare Bio-Sciences, Uppsala, Sweden). IPG strips were focused as described in Herbert et al. [15] and were equilibrated for 25 min [6 M urea, 2% sodium dodecyl sulphate (SDS), 250 mM Tris–HCl (pH 6.8), 0.1% bromophenol blue]. Proteins were separated in the second dimension on Criterion XT 2D gels (Bio-Rad), with Precision Plus unstained marker (Bio-Rad), in MES buffer at 160 V. Gels were fixed and stained with Flamingo quantitative fluorescent stain (Bio-Rad) (2 h) and scanned at medium intensity with 100 μm pixel size resolution.

The nine gels for each treatment were analysed using ProteomWeaver™ (Bio-Rad). Paired spots on different gels were manually checked and re-matched as necessary. Spots that were significantly (Student's *t*-test, $P < 0.05$) upregulated or downregulated by a factor of ≥1.5 in at least two of the three biological replicates were excised. Gels containing these spots were overstained with Coomassie G-250 and spots were excised using an ExQuest™ spot cutter (Bio-Rad).

2.4.2. Protein identification using 2-DGE and liquid chromatography mass separation

Excised gel pieces were washed briefly with 100 mM NH_4HCO_3, then washed twice in a solution containing 50% acetonitrile, 50%