

Manuka honey inhibits cell division in methicillin-resistant *Staphylococcus aureus*

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Objectives: The aim of this study was to investigate the effect of manuka honey, artificial honey and an antibacterial component (methylglyoxal) on cell division in methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods: Viability of epidemic MRSA-15 NCTC 13142 incubated with manuka honey, artificial honey and methylglyoxal was determined, and structural effects monitored by electron microscopy. Activity of murein hydrolase (a peptidoglycan-degrading enzyme implicated in cell separation, encoded by *atl*) was estimated by cell wall hydrolysis and zymography; expression of *atl* was quantified by real-time PCR.

Results: Growth of MRSA was inhibited by 5%, 10% and 20% (w/v) manuka honey and 10% (w/v) artificial honey containing methylglyoxal, but not 10% (w/v) artificial honey. Statistically significantly increased numbers of cells containing septa and increased cell diameter ($P < 0.001$ and $P < 0.001$, respectively) were found in MRSA exposed to 5%, 10% or 20% (w/v) manuka honey, but not 10% (w/v) artificial honey with and without methylglyoxal. Intracellular activity of murein hydrolase was elevated in MRSA grown in 10% (w/v) artificial honey and at undetectable levels in MRSA treated with 10% (w/v) manuka honey. Increased *atl* expression was found in MRSA treated with 10% (w/v) manuka honey and 10% artificial honey containing methylglyoxal.

Conclusions: Enlarged cells containing septa were observed in MRSA exposed to inhibitory concentrations of manuka honey, suggesting that cell division was interrupted. These changes were not caused by either the sugars or methylglyoxal in honey and indicate the presence of additional antibacterial components in manuka honey.

Keywords: cytokinesis, cell cycle, murein hydrolase, MRSA, manuka honey

Introduction

Staphylococcus aureus is a major cause of wound infection throughout the world and the prevalence of multidrug-resistant strains demands innovative interventions. Honey is an ancient wound remedy that has been reintroduced into modern medicine for the topical treatment of wounds, and licensed products containing medical-grade honey are available in Australasia, Europe and North America.

Honey has a complex chemistry, and its broad-spectrum antimicrobial activity varies with floral source, climate and harvesting conditions. Although the precise mode of action of honey is unknown, components that contribute to its antimicrobial activity include a high sugar content, low water content, low pH and the formation of hydrogen peroxide on dilution.¹ Antibacterial phenolic components have been identified in honey² and an antimicrobial peptide has been discovered in a Dutch medical-grade honey that was produced from an undisclosed

floral source cultivated in glasshouses.³ Methylglyoxal (MGO) was identified as the dominant active antibacterial component of manuka honey (MH),^{4,5} but its effect on bacterial structure and function is unknown.

MH is derived from nectar that has been collected by honey bees (*Apis mellifera*) foraging on a shrub known as manuka (*Leptospermum scoparium*) that is indigenous to New Zealand. It is used in modern wound-care formulations and has been shown to eradicate methicillin-resistant *S. aureus* (MRSA) from wounds.^{6–9} *In vitro* clinical isolates of methicillin-susceptible and methicillin-resistant staphylococci were shown to be equally susceptible to MH with MICs reported as <3% (v/v) [equivalent to 41000 mg/L or 4.1% (w/v)].^{10,11} Exposure of *S. aureus* to 10% (w/v) MH for 4 h was found to interrupt the cell cycle during cytokinesis when cells containing septa accumulated.¹² Cell division in staphylococci is normally preceded by the formation of a septum at the cell equator, closely followed by cleavage to achieve the separation of the two daughter

cells. Accumulation of cells containing septa would result if either defective septa had formed or if septum cleavage was deficient. Murein hydrolases, otherwise known as autolysins, are a diverse family of enzymes that hydrolyse structural components in the cell wall, such as peptidoglycan. A deficiency in murein hydrolases has been reported to lead to the failure of cells to separate.^{13–16} These enzymes are encoded by the *atl* gene¹⁷ and increased sensitivity to MH in *S. aureus atl* mutants has implicated this target site in failure to complete cell division.¹²

The aim of this study was to investigate the effect of MH on the cell cycle of MRSA. It was important to establish the kinetics of inhibition *in vitro* in order to determine the contributions made by sugars and MGO, and to select appropriate times and honey concentrations for structural studies. To investigate whether interrupted cell division was due to defective cell separation, the effect of MH on the activity of murein hydrolases was determined by enzyme assay and zymography, and the expression of *atl* was determined by real-time PCR.

Materials and methods

General

Throughout this study, epidemic MRSA-15 (EMRSA-15) NCTC 13142 was utilized.

A non-sterile MH (M109) was provided by Professor Molan of the University of Waikato, New Zealand for the structural studies, and sterile MH (Activon®) was provided by Advancis Medical (Nottingham, UK) for other experiments. Both MH samples had antibacterial activity equivalent to 18% (w/v) phenol, which was determined against *S. aureus* NCTC 6571 by an agar well diffusion assay.¹⁸ In order to demonstrate whether any observed effects were due to sugar content, 100 g of an artificial honey (AH) was prepared by dissolving 1.5 g of sucrose in 17 mL of sterile deionized water and adding 7.5 g of maltose, 40.5 g of fructose and 33.5 g of D-(+)-glucose with vigorous mixing and gentle warming to dissolve.^{11,19}

Cultivation conditions

EMRSA-15 NCTC 13142 was grown in nutrient broth (NB) (Oxoid, Cambridge, UK) containing a range of concentrations of MH [0%, 2.5%, 5%, 10% and 20% (w/v)] and in NB with either 10% (w/v) AH or 10% (w/v) AH containing 614 mg/kg MGO. The concentration of MGO employed was appropriate for a 10% (w/v) solution of the samples of MH used here.^{4,5} All cultures were incubated at 37°C with shaking at 120 cycles per min, and samples were removed at known time intervals for time–kill analyses, electron microscopy, murein hydrolase assays and zymography.

Time–kill studies

The viability of MRSA, cultivated as described above, was determined at time 0, 30, 60, 90, 120, 240 and 1440 min by serial decimal dilution in one-quarter strength Ringer's solution and Miles Misra surface drop counts using tryptone soya agar plates (Oxoid) incubated at 37°C for 24 h.

Transmission electron microscopy (TEM)

Samples (4.5 mL) of MRSA broth cultures prepared as described above were collected at time 0, 30, 60, 90, 120, 240 and 1440 min, and

centrifuged for 1 min at 12000 g in a microfuge (MSE Micro Centaur, Sanyo). Each pellet was resuspended in 750 µL of 3% phosphate-buffered glutaraldehyde for 1 h at room temperature, reharvested and washed twice in PBS before fixing in 1% phosphate-buffered osmium tetroxide.²⁰ After dehydration in a graded series of ethanol alcohol and two changes in propylene oxide, cells were embedded in Araldite resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined in a 1210 JEOL transmission electron microscope.^{20,21} Up to 12 micrographs for each timepoint and each honey concentration were prepared at ×20000 magnification, and cells were observed for evidence of septal components and percentages calculated. Images were also collected at ×32000 magnification in order to assess fine structural changes. Using ImageJ software (NIH), cell diameters were measured using an average of 677 cells for each timepoint and each honey concentration.

Preparation of extracts for enzyme studies

EMRSA-15 NCTC 13142 was cultivated in NB with and without sterile 10% (w/v) MH and in NB containing 10% (w/v) AH at 37°C with shaking at 120 cycles per min. Samples (200 mL) were collected at time 0, 60, 120, 240, 720, 1080 and 1440 min, and harvested by centrifugation (Sorvall RC-5B; Du Pont Instruments) for 20 min at 6500 g and 4°C. Supernatants and cell pellets were collected. To investigate the activity of extracellular murein hydrolase, supernatants were concentrated ~100-fold by using Centricon PL-10 concentrators (Millipore, Bedford, UK) and centrifuging (Sorvall RC-5B; Du Pont Instruments) for 115 min at 3000 g and 4°C; pellets were stored at –20°C until assayed. To investigate the activity of intracellular murein hydrolase, each cell pellet was resuspended in 20 mL of sterile PBS (Oxoid) and a cell-free extract was prepared by passage through a French press (American Instruments) at 3000 pounds per square inch. The resulting suspension was centrifuged (Sorvall RC-5B; Du Pont Instruments) for 30 min at 4500 g and 4°C to remove whole cells or large agglutinated proteins. The resultant supernatant (intracellular extract) was collected and stored at –20°C. The protein concentration of all extracts was estimated using the Bradford method (Bio-Rad, Hemel Hempstead, UK).^{22–24}

Murein hydrolase enzyme assays

Murein hydrolase activity was estimated by a cell wall hydrolysis assay.²⁵ Briefly, 200 µg of protein from either intracellular (cell-free extract) or extracellular extracts (supernatant) was added to a cell wall suspension containing 1.0 mg/mL autoclaved and lyophilized *Micrococcus lysodeikticus* cells (Sigma, Gillingham, Dorset, UK) in 100 mM Tris-HCl (pH 8.0) and incubated at 37°C with shaking (250 cycles per min). The optical density at 580 nm was monitored at 30 min intervals using a spectrophotometer (Cecil, Cambridge, UK) and the hydrolysis of cell walls was demonstrated by decreasing optical density.

Murein hydrolase profiles by zymography

Extracellular and intracellular extracts (containing 15 µg of protein) were electrophoresed in SDS–polyacrylamide gel (15%) containing 1% (w/v) *M. lysodeikticus* cells (Sigma) using a Criterion cassette (Bio-Rad). Color-burst™ size markers were included (Bio-Rad). Following electrophoresis, gels were incubated in renaturation buffer (25 mM Tris-HCl, pH 8.0) containing Triton X-100 overnight at 37°C, stained with 0.01% (w/v) methylene blue in 0.1% (w/v) KOH and destained with deionized water.²⁶ Gels were photographed using an AutoChemi system (Ultra-Violet Products Ltd, Cambridge, UK) and murein hydrolase activity was detected as unstained bands in the gel.

Extraction of RNA and real-time PCR

To determine the effect of MH and MGO on the expression of the *atl* gene that encodes murein hydrolases, MRSA was grown in NB with and without 10% (w/v) MH and in NB with 10% (w/v) AH containing MGO at 37°C for 4 h. RNA was isolated using the Promega SV Total RNA Isolation Kit and cDNA was prepared using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit, according to the manufacturers' instructions. Real-time PCR was performed on all samples, using Fast SYBR Green (Applied Biosystems), according to the manufacturer's instructions, on a CFX96 Real-Time PCR System (Bio-Rad). Primers for *atl* and *yqjI* (which is a housekeeping gene that codes for acetyl coenzyme A) were designed using NCBI Primer-BLAST to be 20–24 bases long, have a GC content of >50% and a melting temperature of ~60°C, and to amplify PCR products of 137 and 243 bp for *yqjI* and *atl*, respectively (Table 1). All reactions were carried out in triplicate and expression of *Atl* was analysed by reference to the expression of the housekeeping gene.

Statistical analysis

Descriptive statistics and ANOVA significance tests were performed with Minitab version 15.

Results

Time–kill studies

The growth curves of EMRSA-15 NCTC 13142 in NB, NB containing 10% (w/v) AH and NB containing 2.5% (w/v) MH were similar

Table 1. Primers used in this study

Target gene	Direction	Sequence (5'–3')
<i>YqjI</i>	forward	GACGTGCCAGCCTATGATTT
<i>YqjI</i>	reverse	ATTCGTGCTGGATTTTGTC
<i>Atl</i>	forward	AATCAAGGTGGCACACAACA
<i>Atl</i>	reverse	CTCGATGCTCATGATTGACG

(Figure 1), with generation times of 30 min. Cultures of MRSA incubated in NB containing 5%, 10% and 20% (w/v) MH, and in NB containing 10% (w/v) AH and MGO showed loss of viability (Figure 1). The extent of inhibition with AH containing MGO was less than that with 5%, 10% or 20% (w/v) MH.

TEM

For each of the seven cultivation conditions tested here, the percentage of cells possessing evidence of partial and complete septa was determined and plotted against time (Figure 2). On average, 41% of untreated MRSA cells were found to contain septal components over the observation period. Similarly, cells treated with 2.5% (w/v) MH, 10% (w/v) AH and 10% (w/v) AH containing MGO were found to exhibit septal components in 41%, 36% and 43% of cells, respectively, which was not statistically significantly different from untreated cultures ($P > 0.05$ using one-way ANOVA). However, MRSA cultures exposed to inhibitory concentrations of MH [5%, 10% and 20% (w/v)] revealed a significantly higher proportion of cells containing partial and complete septa compared with untreated cells (64%, 71% and 67%, respectively; $P < 0.001$ using one-way ANOVA). These increases were established by 30 min and persisted over 24 h. The effect did not appear to be dependent on honey concentration between 5% and 20% (w/v) MH.

As a large number of electron micrographs were collected during this study, only images of samples collected at 120 min are presented to illustrate the structural changes observed in MRSA (Figure 3). It was evident that uninhibited MRSA (Figure 3a, b and c) appeared to be smaller than MRSA exposed to inhibitory concentrations of MH (Figure 3d, e and f). Diameters of >500 cells for every timepoint for each culture condition were measured and the means calculated. Data after 4 h of incubation are presented here (Table 2). Using two-way ANOVA, it was determined that MRSA cells treated with 5%, 10% or 20% (w/v) MH from 30 min onwards had significantly larger cell diameters than cells in untreated or uninhibited cultures [2.5% (w/v) MH] ($P < 0.001$).

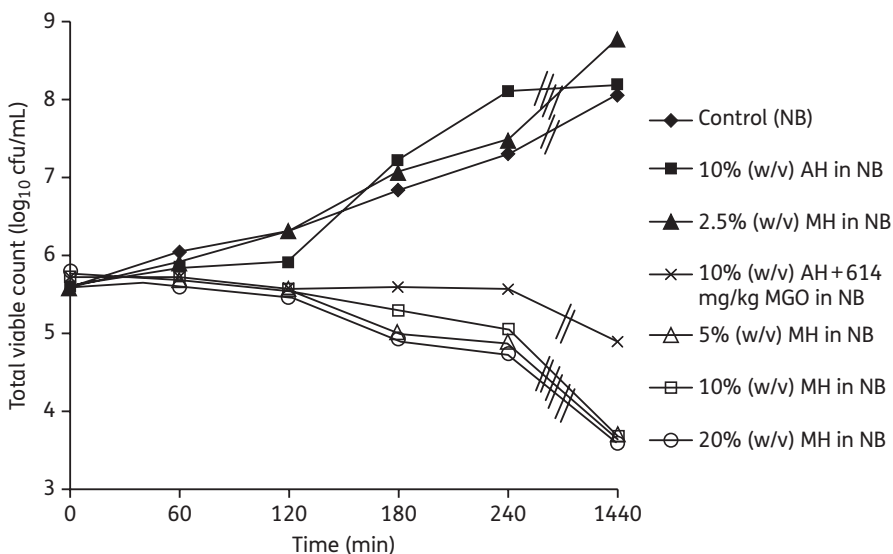


Figure 1. Time–kill curves of EMRSA-15 NCTC 13142.

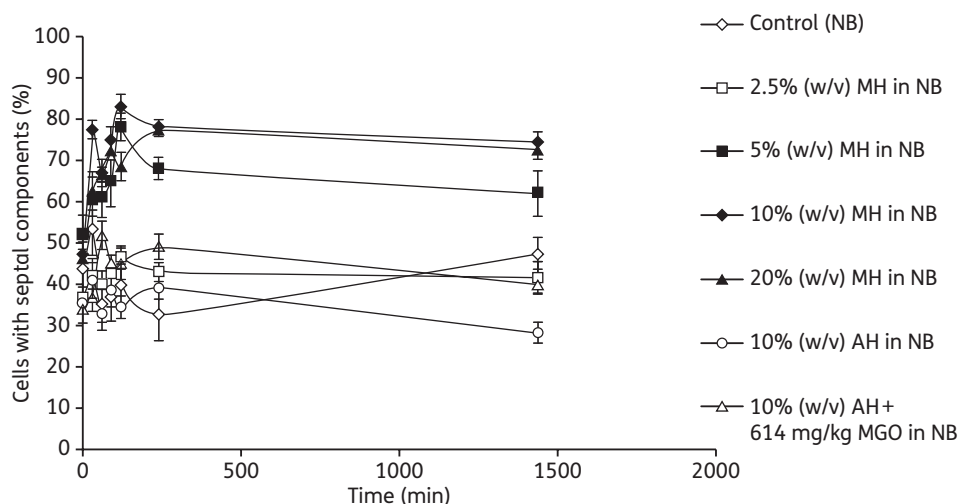


Figure 2. Effect of honey with time on the percentage of MRSA cells containing septal components. The percentage of cells containing septal components was determined from transmission electron micrographs of EMRSA-15 NCTC 13142 at $\times 20000$ magnification. At least 12 images were used for each sample collected.

MRSA treated with AH or AH containing MGO or 2.5% (w/v) MH were not significantly different in size from untreated cells at any timepoint.

Determination of murein hydrolase activity

Murein hydrolase activity was estimated in the intracellular and extracellular extracts prepared from MRSA cultivated in NB with and without 10% (w/v) MH and in NB containing 10% AH at seven different timepoints. Using the cell wall hydrolysis assay, the activity of murein hydrolase was at undetectable levels in all of the extracellular extracts prepared during this study and marked differences in the activity of murein hydrolases were detected only in intracellular extracts prepared at 24 h (Figure 4). Compared with untreated MRSA, MRSA exposed to AH exhibited increased levels of enzyme activity (by enhanced hydrolysis of turbid cell wall suspension), but murein hydrolase activity was not detected in MRSA exposed to MH (Figure 4).

These observations were confirmed by zymography (Figure 5), where digestion of the cell walls was markedly reduced in intracellular extracts of 24 h MH-treated cells compared with untreated and AH-treated cells, but cell wall hydrolytic activity was not at detectable levels in extracellular extracts.

Real-time PCR

Compared with untreated MRSA, the exposure of MRSA to 10% (w/v) MH and 10% (w/v) AH containing MGO resulted in increased levels of expression of *atl* by a factor of 5.5 and 5, respectively.

Discussion

Time-kill studies

Although the rate of inhibition of MRSA caused by MH was relatively slow, with <3 log reductions achieved in 24 h (Figure 1), the time-kill curves presented here demonstrated a loss of viability by MH at concentrations $\geq 5\%$ (w/v) and concurred with

susceptibilities reported for staphylococci.^{11,12} These data indicate that MH would have to be diluted by a factor of ≥ 20 to reach subinhibitory levels. There is a need to know whether honey would reach subinhibitory concentrations under the circumstances that may arise during clinical use. Undiluted medical-grade honey destined for the topical treatment of wounds is usually packaged into tubes or impregnated onto wound dressing materials and sterilized by γ irradiation. When applied to wound surfaces, the sugars contained in honey normally promote the release of wound exudate, which inevitably leads to dilution *in vivo*. It is unlikely that MH would be diluted 20 times in practice.

Undoubtedly, the sugars in undiluted honey contribute to its antibacterial activity,¹ but it was evident that 10% (w/v) AH did not inhibit MRSA whereas 10% (w/v) MH did (Figure 1). An inference that the four major constituent sugars present in honey were not entirely responsible for the inhibition of MRSA on dilution was justified. MGO was added to AH and its efficacy was compared with that of MH. The extent of inhibition of MRSA exposed to 10% (w/v) AH containing MGO was less than that of MRSA exposed to 10% (w/v) MH (Figure 1). Although MGO has been claimed to be the prime active antibacterial component of MH,^{4,5} our data suggest that it does not entirely account for the inhibitory activity against MRSA. A recent study in which the MGO contained in honey was neutralized showed that residual inhibitory activity was detected against *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, but not against *S. aureus*.²⁷ Additionally, a laboratory study with MH and honey infused with MGO indicated that MGO was only partially responsible for antibiofilm activity against *S. aureus*.²⁸ Hence, the antibacterial components of MH appear not to have been completely characterized at present.

Structural studies

The benefit of using electron microscopy to elucidate the cytological effects of antimicrobial agents on *S. aureus* as a means to identify possible intracellular targets has been illustrated in

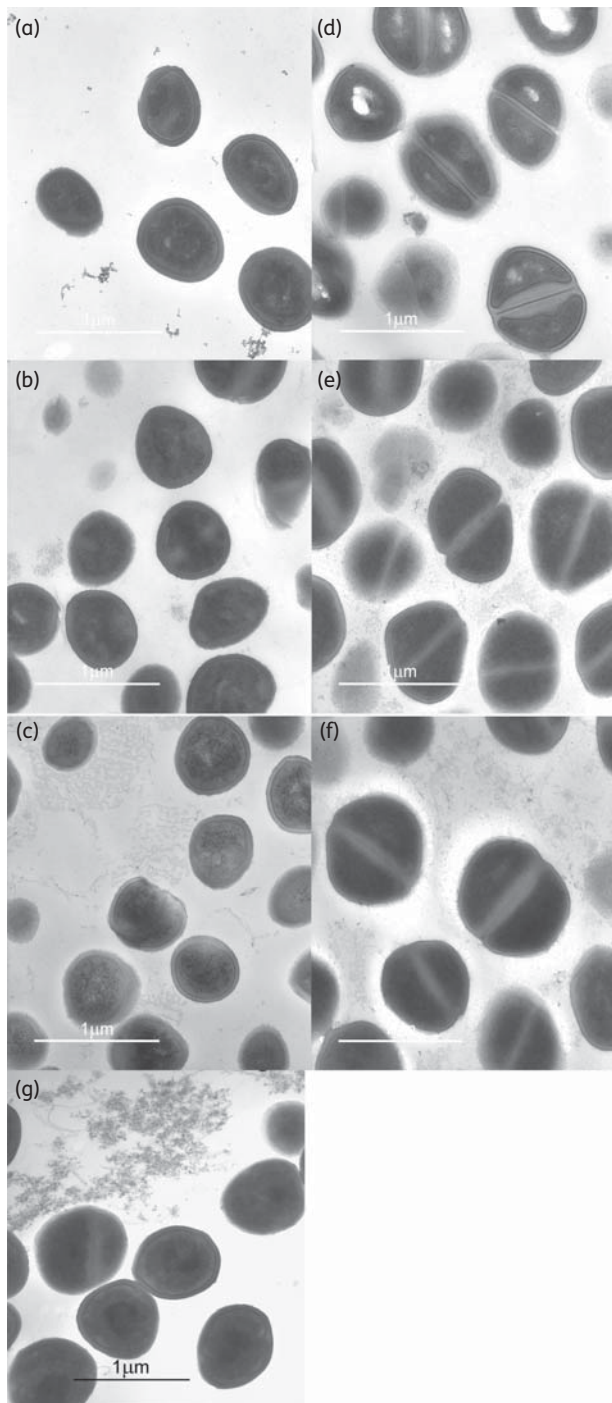


Figure 3. Effect of honey on the structure of MRSA cells. Transmission electron micrographs show MRSA at $\times 32000$ magnification following incubation for 120 min in (a) NB, (b) NB containing 10% (w/v) AH, (c) NB containing 2.5% (w/v) MH, (d) NB containing 5% (w/v) MH, (e) NB containing 10% (w/v) MH, (f) NB containing 20% (w/v) MH, and (g) NB containing 10% (w/v) AH with 614 mg/kg MGO. The percentage of cells with septa was significantly increased following treatment with concentrations of MH $>2.5\%$ (w/v) ($P < 0.001$) and cells were also significantly larger than control cells ($P < 0.001$).

Table 2. Effect of honey on cell size in MRSA

Treatment conditions	Cell diameter (μm)	P
NB (untreated cells)	0.68 ± 0.08	—
NB + 2.5% (w/v) MH	0.67 ± 0.07	>0.05
NB + 10% (w/v) AH	0.62 ± 0.09	>0.05
NB + 10% (w/v) AH + MGO	0.65 ± 0.08	>0.05
NB + 5% (w/v) MH	0.81 ± 0.09	<0.001
NB + 10% (w/v) MH	0.88 ± 0.04	<0.001
NB + 20% (w/v) MH	0.80 ± 0.08	<0.001

Cell diameters (μm) of *S. aureus* were estimated after 4 h of incubation.

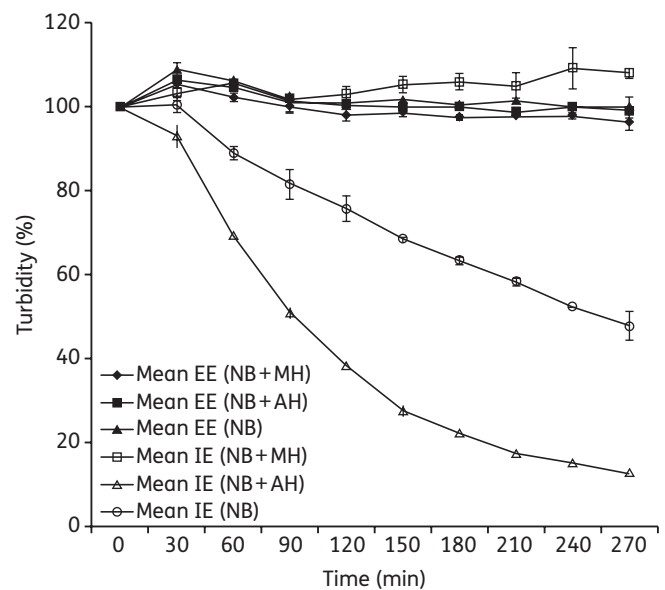


Figure 4. Effect of MH on murein hydrolase activity. EMRSA-15 NCTC 13142 was cultivated in NB for 24 h with and without 10% (w/v) honey, and extracellular extracts (EE) and intracellular extracts (IE) prepared. The activity of murein hydrolase was assayed in triplicate and the mean relative activity is presented as the percentage turbidity \pm standard deviation.

previous studies.^{29–32} Importantly, the accumulation of cells containing whole or incomplete septa has shown that MH interrupted the cell cycle of *S. aureus*.¹² In this study, MRSA was incubated with a range of honey concentrations for 24 h, rather than 10% (w/v) MH for 4 h.¹² Despite the gradual loss of viability of MRSA exposed to inhibitory concentrations of MH (Figure 1), increased numbers of cells containing septal components were evident within 30 min (Figure 2), suggesting that effects on the cell cycle were rapid. The inability of MRSA to complete cell division will necessarily limit its ability to establish wound infection and is of clinical significance.

The accumulation of cells with significantly larger diameters following incubation with inhibitory concentrations of MH (Figure 3 and Table 2) indicates that cells had reached a late stage in the cell cycle, where cell division rather than metabolic

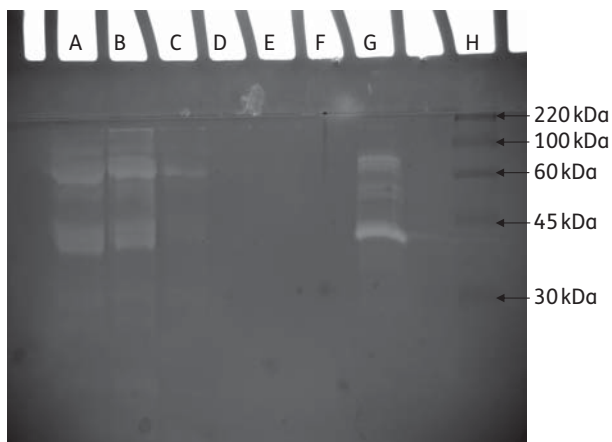


Figure 5. Zymograph showing inhibition of murein hydrolase production in intracellular extracts treated with honey for 24 h. Intracellular extracts from untreated cells (lane A), cells exposed to 10% (w/v) AH (lane B) and cells exposed to 10% (w/v) MH (lane C), and extracellular extracts from untreated cells (lane D), cells exposed to 10% (w/v) AH (lane E) and cells exposed to 10% (w/v) MH (lane F). Lysostaphin was in lane G and Colorburst™ electrophoresis size standards (Bio-Rad) in lane H.

activity associated with increased cell volume was impaired. The absence of lysed cells and cellular debris in electron micrographs of MRSA exposed to $\geq 5\%$ (w/v) MH suggests that the target site of MH is not associated with cellular events that lead to autolysis. MGO did not induce an increased formation of septal components (Figure 2) or cell enlargement (Figure 3), which further indicates the presence of unidentified inhibitory components in MH. A search for additional antibacterial agents in MH might be influenced by the observation of cell cycle perturbations in MRSA that were induced by a component of green tea.³² So, characterizing the polyphenols present in MH and evaluating their effects on cell division in staphylococci may be a profitable line of future enquiry.

Murein hydrolase activity

Inhibition of *atl*-negative mutants by MH suggested that murein hydrolases were implicated in the failure of *S. aureus* to divide.¹² The loss of activity of intracellular murein hydrolase in MRSA incubated with NB containing 10% (w/v) MH for 24 h was demonstrated in cell wall hydrolysis assays (Figure 4) and by zymography (Figure 5). Conversely, real-time PCR demonstrated that MH enhanced the expression of *atl* after 4 h compared with untreated MRSA. This apparent contradiction may be explained if MH affected the functionality of murein hydrolase by interfering with the post-translational modification of the enzyme. A similar deduction was reported in a strain of *S. aureus* exhibiting intermediate resistance to vancomycin, where zymographic analysis and microarray had revealed an increased expression of autolytic enzymes, even though a decreased autolysis was manifest in the phenotype.³³

This study provides an insight into the effects of MH in the inhibition of MRSA. The data demonstrate that the events of the cell cycle are affected and that cell separation is interrupted, although the precise mechanism is not explained. These effects

cannot be solely attributed to either the main constituent sugars of honey or MGO, an important bioactive component. It is feasible that MGO acts synergistically with other components in honey. Honey has a complex chemistry and it is probable that a multitude of effects will be induced by more than one of its constituent components. Investigation of the inhibition of *E. coli* by MH using transcriptome analysis demonstrated that it induced multiple effects, with 2% of genes being up-regulated and 1% down-regulated.³⁴ Further investigation of gene expression may help to elucidate the effects of MH on MRSA, which are not yet explained.

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