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ORIGINAL ARTICLE

How methylglyoxal kills bacteria: An ultrastructural study

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ABSTRACT

Antibacterial activity of honey is due to the presence of methylglyoxal (MGO), H_2O_2, bee defensin as well as polyphenols. High MGO levels in manuka honey are the main source of antibacterial activity. Manuka honey has been reported to reduce the swarming and swimming motility of *Pseudomonas aeruginosa* due to de-flagellation. Due to the complexity of honey it is unknown if this effect is directly due to MGO. In this ultrastructural investigation the effects of MGO on the morphology of bacteria and specifically the structure of fimbriae and flagella were investigated. MGO effectively inhibited Gram positive (*Bacillus subtilis*; MIC 0.8 mM and *Staphylococcus aureus*; MIC 1.2 mM) and Gram negative (*P. aeruginosa*; MIC 1.0 mM and *Escherichia coli*; MIC 1.2 mM) bacteria growth. The ultrastructural effects of 0.5, 1.0 and 2 mM MGO on *B. substilis* and *E. coli* morphology was then evaluated. At 0.5 mM MGO, bacteria structure was unaltered. For both bacteria at 1 mM MGO fewer fimbriae were present and the flagella were less or absent. Identified structures appeared stunted and fragile. At 2 mM MGO fimbriae and flagella were absent while the bacteria were rounded with shrinkage and loss of membrane integrity. Antibacterial MGO causes alterations in the structure of bacterial fimbriae and flagella which would limit bacteria adherence and motility.

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Introduction

Honey is a supersaturated sugar solution with a high osmolarity that limits the growth of microorganisms, and its low pH (3.2–4.5) creates a hostile environment for most bacteria. In addition, molecules such MGO, H_2O_2, the peptide bee defensin, as well as flavonoids and phenolic acids such as catechin, apigenin, myricetin, caffeic acid, and ferrulic acid, also contribute to the antibacterial activity of honey [1–4]. MGO levels and bee defensin account for the specific antibacterial properties of therapeutic manuka and Revamil Source (RS) honey [5,6], respectively. Due to these unique antibacterial properties, these honeys are used for therapeutic treatment of many types of wound including skin grafts, abscesses, pressure ulcers, burns, and surgical wounds [7].

Manuka honey is derived from the monofloral *Leptospermum* tree which is indigenous to New Zealand. The unique manuka factor (UMF) is used to grade this honey and indicates the presence of dihydroxyacetone, leptosperin, and variable amounts of MGO [8]. Manuka honey with a UMF >10 is used for therapeutic purposes, has a MGO content of ≥263 mg/kg, and is sterilized using gamma radiation [7,9]. Manuka honey has been reported to have antibacterial activity against a wide range of bacteria including those resistant to other treatments [7,9]. MGO effectively kills *E. coli* and *S. aureus* [10] as well as methicillin- and oxacillin-resistant *S. aureus* [9]. Kilty et al. (2011) reported that MGO was also effective against biofilms of *P. aeruginosa*, *S. aureus*, and methicillin-resistant *S. aureus*, although effective concentrations were several fold greater than required for plantonic bacteria [11].

A recent study by Roberts et al. (2014) found that manuka honey reduced the swarming and swimming motility of *P. aeruginosa*, due to deflagellation. The expression of the major structural protein flagellin was reduced, as well as flagellin-associated genes, fliA, fliC, flhF, fleN, fleQ, and fleR. Deflagellation of bacteria by manuka honey limited bacterial mobility, reduced bacterial adhesion, and prevented biofilm formation [12].

Due to the complexity of honey it is unknown whether this deflagellation effect is directly due to MGO, the major antibacterial component of manuka honey. In this ultrastructural investigation, the effects of MGO on the morphology of *B. subtilis* and *E. coli* – and specifically the structure of fimbriae and flagella – were investigated.

Materials and methods

Bacterial strains

The following bacterial strains were used in this study: Gram-negative *E. coli* (ATCC 700928) and *P. aeruginosa* (ATCC 10145); and Gram-positive *B. subtilis* (ATCC

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13933) and *S. aureus* (U3300), donated by the University of Kwazulu Natal (UKZN). Bacteria were grown aerobically in Luria–Bertani (LB) broth at 37°C. To obtain bacteria in the mid-logarithmic phase, they were grown overnight, diluted 100 times in LB broth, and proliferated until attaining an OD_{600} of 0.5.

Antibacterial assay

The antibacterial activity of MGO was measured as described by Sherlock, et al. 2010 [6]. Mid- logarithmic-phase bacteria were diluted to an OD_{600} of 0.01 and were exposed to a serial dilution of 0.4–4.4 mM MGO (Sigma Aldrich, South Africa). Absorbance of the plate was measured immediately (T0) using a Multiscan Ascent V1.24 96-well micro-titre plate reader at 620 nm. The plate was then placed in an incubator for 24 h at 37°C, on a shaker set at 150 rpm. After this incubation period the absorbance was measured again (T24). The absorbance at T0 was subtracted from the absorbance at T24 in order to determine bacterial growth after exposure to MGO. The percent growth inhibition from T0 to T24 compared to the control was calculated. MIC is defined as the lowest MGO concentration that causes 100% inhibition of bacterial growth. MIC was calculated from the graph plotting percentage inhibition for different MGO concentrations.

Scanning electron microscopy

The effect of MGO on the ultrastructure of Gram-negative *E. coli* and Gram-positive *B. subtilis* was determined. The bacteria were exposed to low (0.5 mM), medium (1 mM), and high (2 mM) concentrations of MGO in the same way as for the antibacterial assay. For SEM, a 100 µl volume of the bacteria was transferred to the wells of a 24-well plate containing poly-L-lysine coated coverglass slides [13]. After 90 min incubation at 30°C, samples were fixed for 1 h using a solution of 2.5% formaldehyde and gluteraldehyde in 0.075 M sodium potassium phosphate (NaP) buffer (pH 7.4). The slides were then rinsed three times for 15 min, each time with the NaP buffer, before undergoing secondary fixation in 1% osmium tetraoxide for 30 min. The coverglass slides were then rinsed again three times (for 10 min) in NaP buffer. The samples were then dehydrated using increasing concentrations of ethanol (30, 50, 70, and 90%) with a final three rinsings in 100% ethanol. The coverglass slides were dried using critical point drying and were then mounted with carbon tape on aluminium stubs and coated with carbon before viewing with a Zeiss Ultra plus FEG SEM.

Table 1. MIC for MGO for gram-positive and -negative bacteria.

Gram-positive		Gram-negative	
S. aureus	1.2 mM	*P. aeruginosa*	1.2 mM
B. subtilis	0.8 mM	*E. coli*	1.0 mM

Results

MGO inhibited the growth of both Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. The MIC for Gram-positive bacteria was 1.2 and 0.8 mM for *S. aureus* and *B. subtilis*, respectively, while for Gram-negative bacteria *P. aeruginosa* and *E. coli* and the values were 1. and 1.0 mM, respectively (Table 1). An example of Gram-positive and -negative bacteria was selected and the effect of three MGO concentrations on the ultrastructure of *B. subtilis* and *E. coli* was evaluated.

Figure 1(A)–(D) are representative SEM micrographs of Gram-positive *B. subtilis* not (Figure 1A) and exposed to MGO (Figure 1B–D). In Figure 1(A) the typical features of a bacterium can be observed – namely the presence of

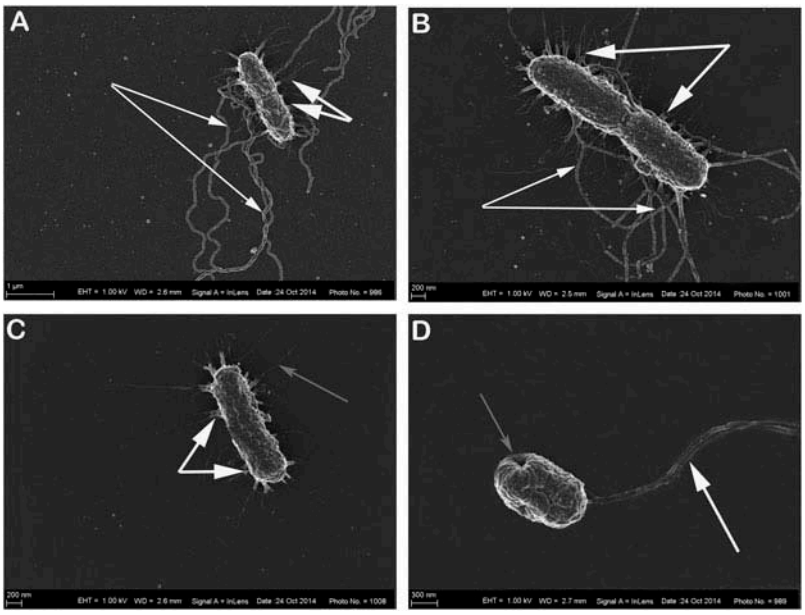

Figure 1. SEM micrographs of *B. subtilis* exposed to increasing concentrations of MGO. (A) Control; (B) 0.5 mM MGO; (C) 1.0 mM MGO; (D) 2.0 mM MGO. Thin white arrows indicate the flagella; thick white arrows indicate the fimbriae; the gray arrow in C shows a pilus; and the gray arrow in D indicates a hole in the cell.

numerous fimbriae (thick white arrows) and flagella (thin white arrows). The morphology of *B. subtilis* exposed to 0.5 mM MGO (<MIC) was similar to the control (Figure 1B). In the bacteria exposed to 1 mM MGO (>MIC), differences are observed when compared to the control – fewer fimbriae are present and the flagella were fewer or absent. Structures that could be identified appeared stunted and fragile. In bacteria exposed to the highest concentration of MGO (2 mM, <MIC), fimbriae were absent and only one flagellum was present in this specific example. Also, the bacteria were rounded with membrane damage, indicated by the gray arrow in Figure 1(D).

A similar effect was observed for Gram-negative *E. coli* exposed to increasing concentrations of MGO. There were no observable differences between the control and *E. coli* exposed to 0.5 mM MGO (<MIC). In these bacteria, flagella and fimbriae were present (Figure 2A and B). Increasing concentrations of MGO caused smoothing of the cell wall and loss of fimbriae, as can be seen in Figure 2(C) (exposed to 1 mM MGO, MIC for *E. coli*). At 2 mM MGO (>MIC), no fimbriae or flagella were present and bacterial shrinkage had occurred possibly due to the loss of intracellular content (Figure 2D).

Discussion

In bacteria, MGO is mainly synthesized from the glycolytic intermediate, dihydroxyacetone phosphate, catalyzed by MGO synthase. Glutathione (GSH) protects Gram-negative bacteria such as *E. coli* against the effect of MGO, via the spontaneous reaction of MGO with GSH to form a hemithiolacetyl which is converted to D-lactate by glyoxalase I and II, both enzymes of the glyoxalase pathway [14]. The KefB and KefC K^+ GSH efflux systems are integrated with the glyoxalase pathway. Depletion of GSH by the glyoxalase I and II enzymes results in these channels being open, leading to leakage of K^+. As K^+ efflux occurs there is a simultaneous influx of H^+, which results in a decrease in intracellular pH. This decrease in intracellular pH protects against MGO toxicity possibly by activating DNA repair systems or by reducing the protein reactivity of MGO [15].

However, excessive amounts of MGO such as found in manuka honey kill bacteria, and MGO has been found to have bacteriostatic and bactericidal effects across a broad spectrum of wound-associated bacteria [9]. Growth inhibition of bacteria occurs when MGO levels in the growth media reach 0.3 mM and viability decreases at levels above 0.6 mM. Inhibition and loss of activity is a function of cell density and the composition of the growth medium [15]. At a concentration of >1.2 mM, MGO inhibited the growth of both Gram-negative and -positive bacteria.

MGO has been reported to kill bacteria, with MIC for *E. coli* and *S. aureus* being around 1.1 mM MGO [10]. Studies have also shown that MGO is effective against methicillin- and oxacillin-resistant *S. aureus* [9]. Kilty et al. (2011) reported that MGO was also effective against *P. aeruginosa* and *S. aureus*, including MRSA biofilms. The effective concentration (EC) of MGO for plantonic MRSA was 1.1–4.16 mM, and for *P. aeruginosa* was 2.08–16.65 mM. For MRSA biofilms the EC was several fold higher than for plantonic bacteria – 6.94–50.0 and 24.98–101.30 mM, respectively [11]. The MGO content of therapeutic manuka honey, UMF > 10 is ≥263 mg/kg and 1.1 mM MGO is equivalent to a 72.27 mg/kg solution, which implies that manuka honey with UMF > 10 will cause lysis of the cell wall of the Gram-negative and -positive bacteria, as observed in this study for bacteria exposed to 2 mM MGO.

In the present study it was found that exposure to MGO concentrations at, or close to, MIC caused a loss of bacterial fimbriae and flagella. Fimbriae or attachment pili are bacterial appendages found in many Gram-positive and -negative bacteria. These structures are abundant, shorter, and thinner than

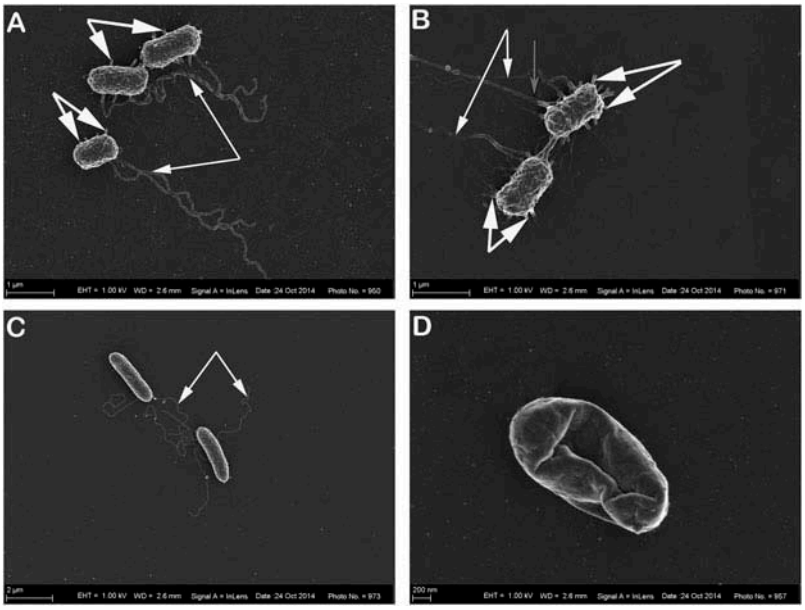

Figure 2. SEM micrographs of *E. coli* exposed to increasing concentrations of MGO. (A) Control; (B) 0.5 mM MGO; (C) 1.0 mM MGO; (D) 2.0 mM MGO. Thin white arrows indicate the flagella; thick white arrows indicate the fimbriae; gray arrow in B shows a pilus.

flagella and are several micrometers long and 3–10 nm in diameter. Fimbriae have an important attachment function and play a role in adherence between bacteria, between bacteria and host, as well as in attachment to innate surfaces [16]. Each pilus protrudes 1–2 μm into the external environment and is composed of a rod and tip segment which attaches to the host or surface. The biogenesis and adhesion properties of bacterial pili have been extensively reviewed by Lillington et al. (2014) [17].

In addition, flagella provide a propulsion system for bacteria and consequently they can swim in liquids and swarm over surfaces [18]. Flagella are simple proton-driven structures that are responsible for the rotary movement of bacteria. Flagella play an important role in the virulence of bacteria through chemotaxis, adhesion, invasion of the host's surfaces, and the release of virulence factors [16]. Inhibition of bacterial motility promotes the transition from planktonic bacteria to the formation of a biofilm. Guttenplan (2012) describe two flagella-associated events that occur with biofilm formation; the first and shorter event is the functional inhibition of flagellar rotation or modulation of their basal reversal frequency. Long-term inhibition involves the inhibition of gene transcription of flagella proteins and, as a consequence, synthesis of flagella-associated proteins is inhibited and the assembly of flagella ceases [18].

Fimbriae and flagella have been identified as important drug targets, and inhibition of genes associated with functioning and/or synthesis of structural proteins will result in decreased bacterial mobility and ability to adhere to innate and cellular surfaces, and this will reduce virulence and prevent biofilm formation. Macrolides, erythromycin, clarithromycin, and azithromycin at sub-MIC concentrations have been shown to inhibit the motility of *P. mirabilis* and *P. aeruginosa* [5]. Burt et al. (2007) determined that carvacol, a major ingredient of the essential oils of thyme and oregano, inhibited the synthesis of flagellin in *E. coli* O157:H7 [19]. Roberts (2014) reported that manuka honey caused the suppression of flagellum-associated genes of *P. aeruginosa* and subsequently reduction in the swimming and swarming capacity of these bacteria [12].

The cell wall of Gram-positive bacteria consists also of a plasma membrane, and in Gram-negative bacteria a plasma membrane, peptidoglycan layer, and outer membrane. These layers protect bacteria from the harsh extracellular environment. In contrast, the proteins of the fimbriae and flagella are in direct contact with the environment. Besides affecting gene expression and flagellum motility, MGO can bind directly to fimbriae and flagellar proteins such as FimA/PapA and flagellin, causing loss of structural integrity and subsequently function.

MGO is a highly electrophilic molecule and can also bind DNA and protein, thereby altering protein structure, function, and synthesis. MGO reacts with the nitrogenous base guanine [20], as well as the amino acids Arg, Lys, and Cys [20,21]. Reactions with these amino acids result in the formation of advanced glycation end products (AGE). The formation of AGE by MGO is well described for eukaryotic cells [10,22–24] but not for bacteria. Booth (2003) identified the principal events that occur following cellular exposure to MGO: rapid cytoplasmic formation of MGO-GSH adducts, simultaneous reaction with DNA guanine bases, and subsequent activation of DNA repair systems. In addition, MGO reacts with the thiol groups of proteins causing inhibition of enzyme activity [15]. Likewise in bacteria – as has been described for eukaryotic cells – MGO can disrupt GSH homeostasis as well as the structural integrity and function of bacterial DNA and proteins, such as the plasma membrane proteins, resulting in changes in permeability leading to cellular lysis.

Roberts et al. (2014) showed that manuka honey inhibited flagella-associated genes [12]. The present study clearly shows that MGO, the major antibacterial constituent of manuka honey, either directly damages or inhibits the formation of fimbriae and flagella. At concentrations >MIC, MGO causes bacteria lysis. How MGO affects fibrillin gene expression and protein structure is an important aspect that needs to be further investigated.

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