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.

Key words: methylglyoxal, antibacterial, flagella, fimbriae.

Introduction

Honey is a supersaturated sugar solution with a high osmolarity that limits the growth of microorganisms and the low pH (3.2 - 4.5) of honey 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 accounts 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 wounds including skin grafts, abscesses, pressure ulcers, burns and surgical wounds [7].

Manuka honey is 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 and has a MGO content of ≥ 263 mg/kg and is sterilised using gamma radiation [7, 9]. Manuka honey has been reported to have antibacterial activity against a wide range of bacteria including bacteria 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* as well as 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 de-flagellation. The expression of the major structural protein flagellin was reduced as well as flagellin-associated genes, fliA, fliC, flhF, fleN, fleQ and fleR. De-flagellation of bacteria by manuka honey would limit bacteria mobility, reduce bacterial adhesion and prevent biofilm formation [12].

Due to the complexity of honey it is unknown if this de-flagellation 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. substilis* 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), *P. aeruginosa* (ATCC 10145) and Gram positive: *B. subtilis* (ATCC 13933), and *S. aureus* (U3300), donated by the University of Kwazulu Natal (UKZN), were used in this study. Bacteria were grown aerobically in Luria-Bertani (LB) broth at 37°C. To obtain bacteria in the mid-logarithmic phase, bacteria were grown overnight, diluted 100 times in LB broth and proliferated until an OD_{600} of 0.5 was reached.

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 % growth inhibition from T0 to T24, compared to the control was calculated. The 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 a 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 cover glass 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 3 times for 15 min each time with the NaP buffer before undergoing secondary fixation in 1 % osmium tetraoxide for 30 min. The cover glass slides were then rinsed again 3 times for 10 min each in NaP buffer. The samples were then dehydrated using increasing concentrations of ethanol (30%, 50%, 70% and 90%) with a final rinse of 3 times in 100% ethanol. The cover glass 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.

Results

MGO inhibited the growth of 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 mM and 0.8 mM and for *S. aureus,* and *B. subtilis* respectively, while for Gram-negative bacteria *P. aeruginosa* and *E. coli* and the MIC was 1.2 mM 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. substilis* and *E. coli was* evaluated.

Table 1: MIC for MGO for Gram-positive and negative bacteria

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

Gram-positive

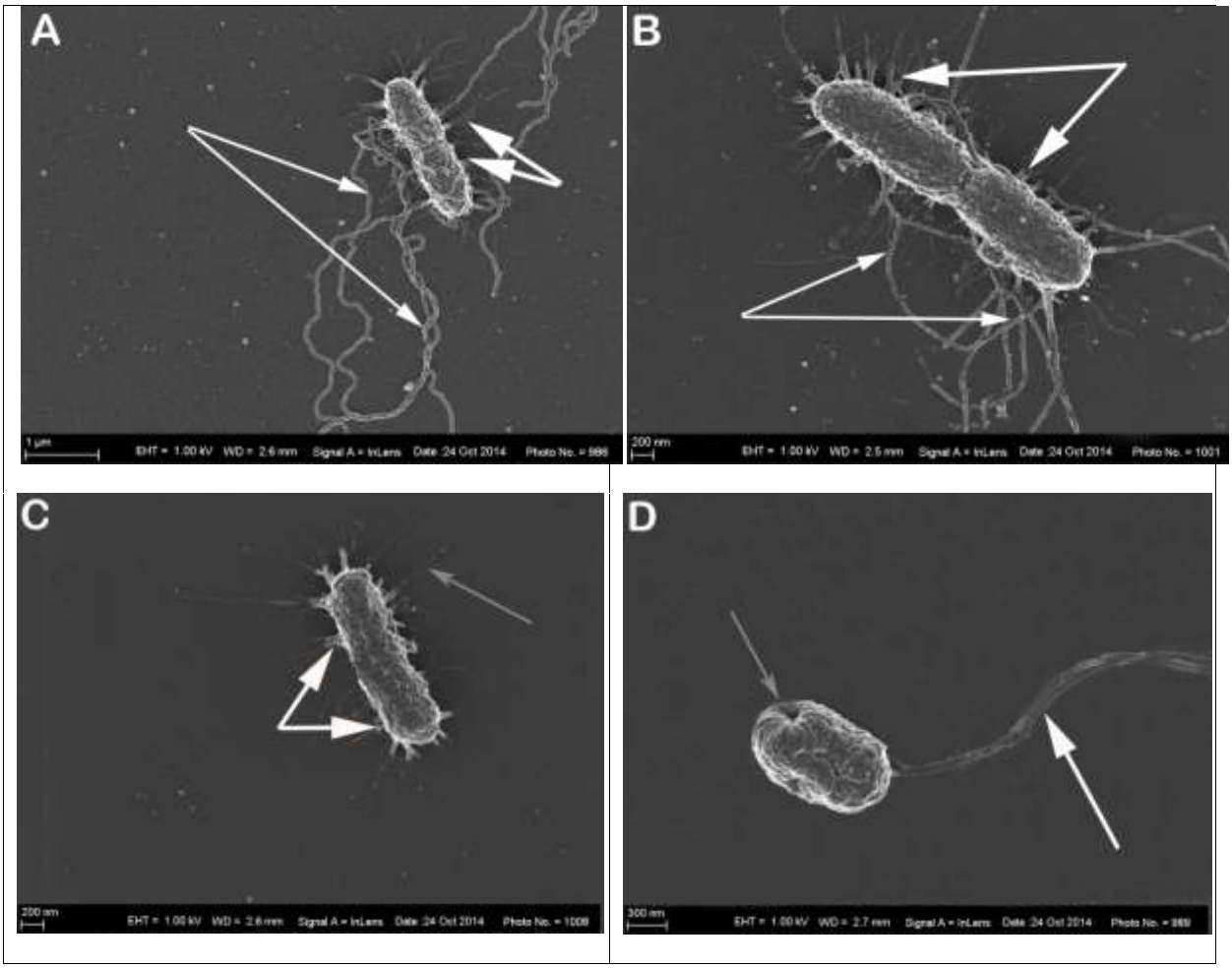

Figure 1: SEM micrographs of *B. subtilis* exposed to increasing concentrations of MGO. (**A**) Control; (**B**) 0.5 mM MGO; (**C**) 1.0 mM MGO; (**D**) 2.0 mM MGO. Thin white arrows indicate the flagella; thick white arrows indicate the fimbriae, the grey arrow in C shows a pilus and the grey arrow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

Figures 1 A - D are representative SEM micrographs of Gram positive *B. substilis* not (Figure 1 A) and exposed to MGO (Figure 1 B - D). In Figure 1 A the typical features of a bacterium can be observed namely the presence of numerous fimbriae (thick white arrows) and flagella (thin white arrows). The morphology of *B. substilis* exposed to 0.5 mM MGO (< MIC) was similar to the control (Figure 1 B). In the bacteria exposed to 1 mM MGO (> MIC) some differences are observed when compared to the control, fewer fimbriae are present and the flagella were less or absent. Structures that could be identified appeared stunted and fragile. In the bacteria exposed to the highest concentration of MGO (2 mM, >> MIC), fimbriae were absent and only one flagellum is present in this specific example. Also, the bacteria were rounded with membrane damage, indicated by the grey 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 2 A and B). Increasing concentrations of MGO caused smoothing of the cell wall, loss in 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 shrinkage of the bacteria had occurred possibly due to the loss of intracellular content (Figure 2D).

Gram-negative

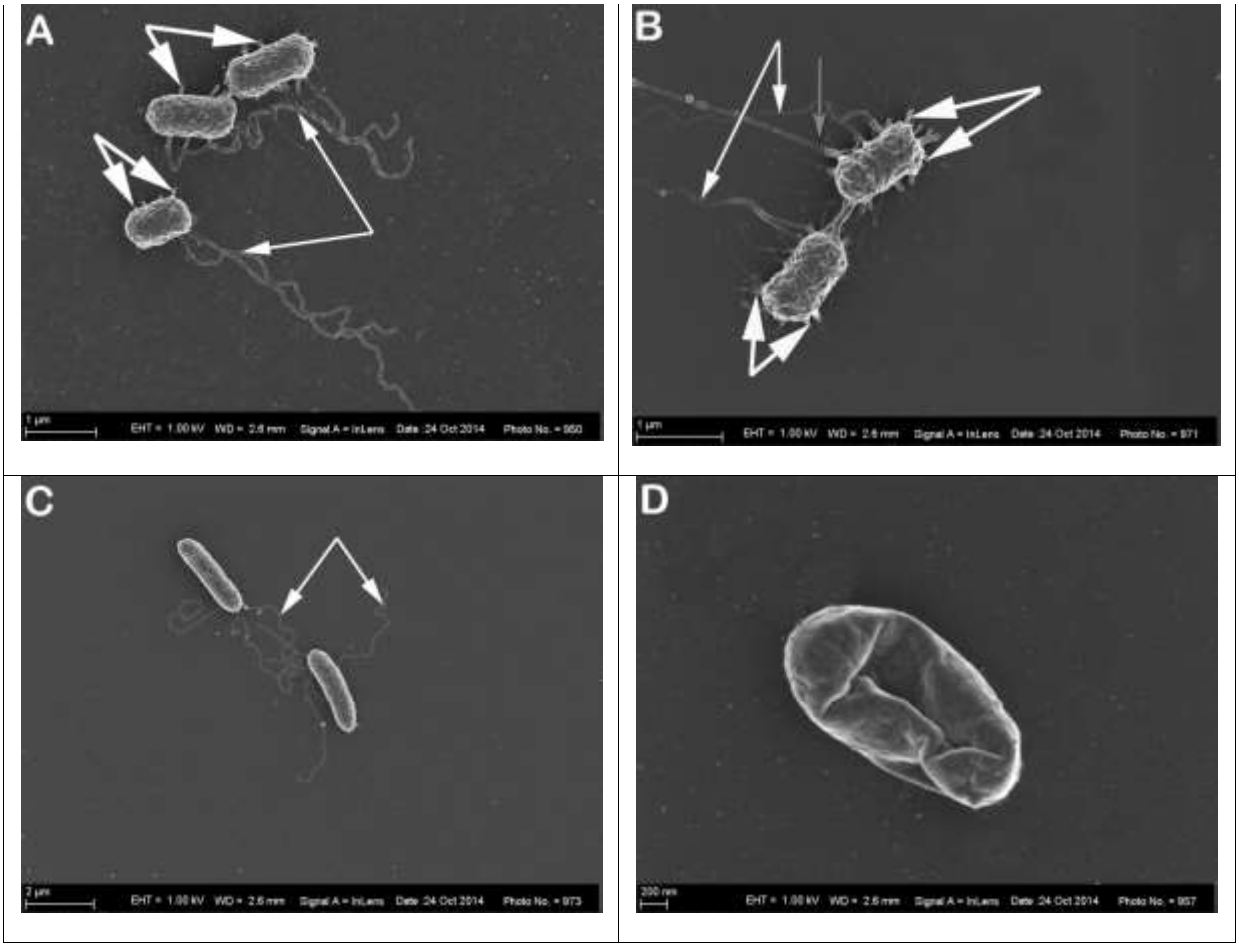

Figure 2: SEM micrographs of *E. coli* exposed to increasing concentrations of MGO. (**A**) Control; (**B**) 0.5 mM MGO; (**C**) 1.0 mM MGO; (**D**) 2.0 mM MGO. Thin white arrows indicate the flagella; thick white arrows indicate the fimbriae; grey arrow in B shows a pilus.

Discussion

In bacteria MGO is mainly synthesized from the glycolytic intermediate, dihydroxyacetone phosphate, catalysed 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 the 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 the 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 by possibly 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 a bacteriostatic and bactericidal effects across a broad spectrum of wound bacteria [9]. Growth inhibition of bacteria occurs when MGO levels in the growth media reaches 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 media [15]. At a concentration > 1.2 mM, MGO inhibited the growth of both Gram-negative and positive bacteria.

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

In the present study it was found that exposure to MGO concentrations at the MIC or close to the 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 flagella and in size are several µm 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 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 bacteria 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 flagella rotation or modulation of the basal reversal frequency of the flagella. 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].

The fimbriae and the flagella have been identified as important drug targets as inhibition of genes associated with functioning and/or synthesis of structural proteins will result in decreased bacterial mobility, 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* bacteria [5]. Burt *et al.* (2007) determined that carvacol a major ingredient of 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 the reduction in the swimming and swarming capacity of these bacteria [12].

The cell wall of Gram positive bacteria consists of plasma membrane and cell wall and in Gram negative bacteria, a plasma membrane, peptidoglycan layer and an outer membrane. These layers protect bacteria from a 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 flagella 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 amino acids, Arg, Lys and Cys [20, 21]. Reactions with these amino acids results 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 that the principle events that occurs following cellular exposure to MGO, is the rapid cytoplasmic formation of MGO-GSH adducts, the 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 the GSH homeostasis as well as the structural integrity

and function of bacterial DNA and protein 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.

Conflict of Interest

The authors report no conflict of interest.

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