The intracellular effects of manuka honey on *Staphylococcus aureus*

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Abstract

Purpose: To investigate the effect of manuka honey on *Staphylococcus aureus* in order to identify the intracellular target site.

Methods: Mode of inhibition of manuka honey against *Staphylococcus aureus* NCTC 10017 was investigated by determining MIC, MBC and the effect of time on viability. Structural changes were observed by scanning and transmission electron microscopy of cells suspended for 4 hours at 37ºC in 0.05 mM Tris buffer containing 10% (w/v) manuka honey and compared to cells in buffer alone or buffer containing 10 % (w/v) artificial honey (to assess osmotic damage).

Results: A bactericidal mode of inhibition for manuka honey on *Staphylococcus aureus* was established. Marked structural changes in honey-treated cells were seen only with transmission electron microscopy, where a statistically significant increase in the number of whole cells with completed septa compared to untreated cells were observed ($p<0.05$).

Conclusion: Structural changes found with transmission electron microscopy suggest that honey-treated cells had failed to progress normally through the cell cycle and accumulated with fully formed septa at the point of cell division without separating. Sugars were not implicated in this effect. The staphylococcal target site of manuka honey involves the cell division machinery.

Keywords: Manuka honey, bacterial cell cycle, cell division, wound infection, *Staphylococcus aureus*.

Introduction

Honey is a sweet, sticky substance that is produced by bees following the collection of either nectar or plant secretions induced by insect damage. It has been highly valued by generations for thousands of years and its virtues are

documented in ancient religious, secular and medical texts. Therapeutic claims range from the clearance of wound infection to the enhancement of healing in chronic wounds. Traditional remedies containing honey were used in the topical treatment of wounds by diverse ancient civilizations and are still used in remote communities. New formulations of honey have been introduced into modern medicine by the development of licensed wound care products and those using manuka honey are available in Australia, New Zealand, Hong Kong, the European Union, Canada and USA. Yet some practitioners are reluctant to accept these products because their mechanism of action is not known.

Honey is such a complex and variable natural product [1] that the search for specific inhibitors has been extensive. The antimicrobial activity of honey is derived from multiple factors, with contributions from high sugar content, low water content, low acidity, hydrogen peroxide and phytochemicals [2]. Using a syrup containing the sugars typically found in honey, the inhibitory effects of diluted natural honey solutions have been demonstrated to be independent of sugar content [3,4]. Honey exhibits broad spectrum antimicrobial activity that extends to more than 80 species [2,5]. Inhibition of pathogens capable of causing wound infection has been demonstrated [3,4,6,7,8], with both antibiotic-resistant and antibiotic-sensitive strains exhibiting susceptibility to honey [3,4,9]. An increasing number of clinical reports demonstrate the clearance of infection by topical application of manuka honey, including the eradication of methicillin-resistant *Staphylococcus aureus* (MRSA) from colonised and infected wounds [10,11,12,13].

Manuka honey is derived from nectar collected by honeybees (*Apis mellifera*) foraging on the manuka tree (*Leptospermum scoparium*) in New Zealand. Unlike many honeys, the activity of this honey at low dilutions is not limited to the production of hydrogen peroxide by glucose oxidase, but linked to plant derived components. One of these is methylglyoxal [14,15] which has been shown to originate from the high levels of dihyroxyacetone present in the nectar of manuka flowers [16]. Although the inhibition of Gram positive bacteria by manuka honey has been reported, cellular target sites and mechanisms of action have not yet been established, and the effects of honey on the structure of bacteria have not been studied. Often it is assumed that the effects of honey are attributable to osmotic effects. Using *Staphylococcus aureus,* a common cause of wound infection, this study was designed to investigate the effects of manuka honey on bacterial structure that were independent of sugars.

Methods and materials

General

Staphylococcus aureus NCTC 10017 was the test organism used throughout this study. Additionally two strains of mutant *Staph. aureus* together with their respective parental strains were used to determine intracellular target sites. One parental strain was SH 1000 (reference number 682) and its respective *SigB* stress mutant (in SH 1000, TET 5, reference number 1028); the other parental strain was 8325-4 (reference number 57) with its autolysin *atl* mutant (in 8325-4, ERY 5, reference number 187). These cultures were created in Professor Simon Foster's laboratory in Sheffield University, UK and kindly provided to us for this study.

A sample of manuka honey (M109) that was a gift from Prof. Molan at the University of Waikato in New Zealand was used. Antibacterial potency of the sample was related to phenol using a standardised bioassay developed in New Zealand [17] and non-peroxide activity was found to be equivalent to 18 % (w/v) phenol. The median antibacterial activity for manuka honey (MH) is 15.5 % (w/v) [17].

A syrup of the sugars predominantly found in honey (artificial honey or AH) was used to determine whether cytological changes were caused by osmotic effects [4]. 100 g AH was prepared as follows: 1.5 g sucrose, 7.5 g maltose, 40.5 g D-fructose and 33.5 g D-glucose were dissolved in 17.5 mL deionised water.

Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations

MIC was determined in 96-well, flat bottomed microtitre plates (Nunc, Roskilde, Denmark) using 100 µL double strength nutrient broth (NB; Oxoid, Basingstoke, UK) and 100 µL MH diluted in deionised water to achieve concentrations varying by 1 % (w/v) intervals. Wells were inoculated with 1 µL overnight broth cultures, and total viable counts (TVCs) performed by Miles Misra surface drop counts, to check retrospectively that each well had received approximately 1 x 10^6 cells. Briefly to estimate TVCs, samples were serially diluted in ¼ strength Ringers solution, plated onto nutrient agar (NA; Oxoid, Basingstoke UK) plates and incubated at 37^0C for 24 hours. After inoculation, microtitre plates were incubated at 37^0C for 24 hours and turbidity was measured at 400 nm in a plate reader (Anthos Labtec Instruments). Positive (NB with inoculum) and negative (NB with manuka honey at the highest respective concentration, without inoculum) controls were included. The lowest concentration of honey in the wells without growth was recorded as MIC. MBC was determined by streaking

20 µL from wells without growth onto NA plates, incubating at 37^0C for 24 hours and recording the lowest concentration without viable bacteria.

In order to identify possible target sites for MH, MICs of two mutant strains of *Staph. aureus* were compared to their parental strains.

Time-kill curve

The inhibitory concentration of MH that was chosen for subsequent experiments was 10%(w/v) because it was approximately three times the MIC. The effect of MH on viability of cells was monitored by inoculating 40 µL of an overnight culture of *Staph. aureus* NCTC 10017 into 20 mL NB with and without 10 % (w/v) MH and incubated at 37^0C in a shaking water bath (120 cycles min^{-1}). Samples were removed at known intervals and TVCs determined as above.

Reversibility of inhibitory effects

Cultures of *Staph. aureus* NCTC 10017 with and without 10 %(w/v) MH in NB were set up as in time-kill studies and incubated at 37^0C in a shaking water bath. At time 0 and at hourly intervals, 100 µL samples were removed from each of the flasks, transferred to 10 mL NB, incubated overnight at 37^0C and viability deduced by the presence of turbidity.

Electron microscopy

Staph. aureus NCTC 10017 was cultivated in 100 mL isosensitest broth (ISB; Oxoid, Basingstoke, UK) at 37^0C in a shaking water bath for either 3 hours or overnight to obtain cells in either the exponential or the stationary phase of growth, respectively. Cultures were harvested by centrifugation at 3000 g for 30 minutes (MSE Harrier 15/80 centrifuge, Sanyo) at room temperature and suspended in 0.05 mM Tris buffer (pH 7.2) with and without 10 %(w/v) MH. To evaluate whether structural changes were attributable to the osmotic effect of

honey, cultures were also suspended in buffer containing 10 %(w/v) artificial honey solution [4]. Cell suspensions at time 0 and after 4 hours incubation at 37^0C were fixed and processed for scanning (SEM) and transmission electron microscopy (TEM) by the methods of Lemar, Turner & Lloyd [18], except that pellets for (TEM) were embedded in Araldite resin, not Spurr.

Analysis of SEM images

Using the scanning electron microscope (5200LV Jeol, Herts, UK) electron micrographs of untreated and honey treated cells were prepared. In total 8 samples were processed and at least 6 images of each sample were collected. To investigate cell surface changes, at least 600 different cells were observed and counted in typically 8-12 scanning micrographs taken at 10,000 times magnification for untreated, AH- and MH treated cells. Data was analysed for statistically significant structural differences by Mann-Whitney test using Mini-Tab (version 15).

Analysis of TEM images

Thin sections of samples were observed by transmission electron microscopy (1210 Jeol, Herts, UK). Twelve images of each of 10 samples (5 were exponential phase cells and 5 were stationary phase) were collected for each test organism at magnifications between 10,000 and 40,000. Cells were scrutinised for structural changes, and the presence of completely formed cross walls (septa) was counted in more than 1000 cells in each of MH-treated and untreated cultures. Data was analysed by Mann-Whitney test with Mini-Tab (version 14).

Results

Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations

Although inhibition of staphylococci by manuka honey has been reported [4,8], the culture used in this study had not previously been tested. Also, because honey is a natural product whose characteristics may vary, it was important to determine MIC And MBC values at the start of this study to establish appropriate concentrations to use in time-kill studies and electron microscopy. Furthermore, altered sensitivity to manuka honey in mutants was used to provide insight into possible intracellular target sites.

The close proximity of MIC and MBCs values (Table 1) indicated a bactericidal mode of action for manuka honey with *Staph. aureus* NCTC 10017 and increased sensitivity of autolysin (*atl)* mutant compared to its parental strain ($p<0.001$) suggested that the target site was associated with bacterial cell division.

Time-kill studies

Loss of viability was observed when bacteria were incubated in 10% (w/v) MH in NB with time compared to untreated cells (Figure 1). Extrapolation of viable bacterial population sizes in the presence of MH, estimated that the mean time to achieve a 2 log reduction was 427 minutes.

Reversibility of inhibitory effects

Removing samples from cultures incubated in 10 %(w/v) MH in NB to NB alone, demonstrated that viable cells were not recovered after 8 hours. This suggested that the inhibitory effect of MH was irreversible.

Scanning electron microscopy (SEM)

Cultures in both the exponential and the stationary phases of growth were utilised in structural studies because stationary phase cells are often less susceptible to antimicrobial agents than exponential cells. However similar

changes were seen in both types of cell and therefore only electron micrographs of exponential cells are presented here.

SEM images demonstrated that the appearance of *Staph. aureus* exposed to MH and AH was indistinguishable to that of untreated cells and it was deduced that a bactericidal concentration of MH did not induce marked cellular lysis. Cells with regular, smooth cell surfaces were observed in SEMs of 99% of the untreated cells that were incubated in buffer for 4 hours (Figure 2a), as well as in cells incubated in buffer containing 10 %(w/v) AH (Figure 2b). In the samples treated with manuka honey for 4 hours few surface changes were found and 90% of the cells retained a smooth surface; there was limited evidence of cell lysis (Figure 2c).

Transmission electron microscopy (TEM)

As with SEM, in TEM images honey was found to cause minimal alteration to the cell surface of *Staph. aureus* and there was limited evidence of cellular debris and lysis. Compared to untreated cells (Figure 3a) and cells exposed to 10%(w/v) artificial honey (Figure 3b), cells treated with 10%(w/v) manuka honey (Figure 3c) showed a difference in that entire cells with fully formed cross walls were a bit more common. The percentage of cells with septa in MH cells increased by 10 % in both exponential and stationary phase cells (Table 2) compared to untreated cells and was statistically significant ($p=0.014$ and 0.03 respectively). The similar appearance of untreated and AH-treated cells ($p>0.05$) indicates that the increased number of cells with septa following treatment with MH was not likely to be caused by the sugars in honey.

Discussion

Infection always interrupts the wound healing process and undiluted honey, or honey mixed with grease and plant extracts, was traditionally applied to the surface of wounds to treat infections [19]. However, when honey is used topically in wounds, its osmotic potential causes increased release of fluid which dilutes honey. It is therefore important to determine the lowest concentration at which the antimicrobial activity of honey is demonstrated, in order to judge whether samples of honey will be effective in clinical use. The sample of manuka honey utilised in this study gave mean MIC and MBC values of 2.9 and 4.5%(w/v) respectively, which indicated that sufficient activity to inhibit the test bacteria in laboratory conditions would be present if MH were diluted approximately 33 and 20 times respectively. Since this extent of dilution is unlikely to occur in practice, clinicians may be confident that activity will be retained; of the licensed wound care products that contain manuka honey, concentration usually exceeds 80% by weight. It was notable that *Staph. aureus* NCTC 10017 was susceptible to relatively low concentrations of manuka honey, despite being an osmotolerant bacterium. The ratio of MBC to MIC was less than 4 and indicates a bactericidal mode of inhibition [20]. Reductions in viable cells seen in the time-kill experiments (Figure 1) confirmed a bactericidal mode of action with a time to achieve 2 log reductions estimated as 427 minutes. Failure to recover viable bacteria after 8 hours in the reversibility experiment confirmed that the inhibition was irreversible. Clinical isolates may be less susceptible to antimicrobial agents than reference strains, yet results with 5 clinical isolates (not shown here) demonstrated a mean estimated time of 770 minutes to achieve 3 log reductions for MRSA.

The value of electron microscopy in studying cytological effects of antiseptics in order to elucidate intracellular target sites has been demonstrated for chlorhexidine [21], iodine [22] and mupirocin [23]. Here the cytological changes observed with TEM indicate that manuka honey elicited a specific effect in *Staph. aureus* that led to a statistically significant increase in numbers of cells with fully formed septa compared to untreated cells. No structural differences between the septa of treated and untreated cells were detected; this suggests that honey-treated cells were able to complete septum formation, but were unable to separate after the cross walls had formed. It is possible that septa were formed prematurely in the cell cycle and that cell division was interrupted because mandatory cellular events had not been completed. Alternatively, cell division might have been prevented by a defect in the process that facilitates cell separation. Bacterial cell division is a complex process that has not yet been fully characterized. Initially structural division proteins such as FtsZ assemble a ring-like structure at the cell equator and septum formation is achieved by the deposition of flanking layers of peptidoglycan. Separation of daughter cells is then mediated by autolysins that cleave the median line of the septum, with or without cell wall constriction according to species [24].

In *Escherichia coli* treated with manuka honey, transcriptome analysis has recently indicated multiple effects on protein expression [25]. Increased susceptibility of the autolysin mutant to manuka honey suggests that one of the effects of manuka honey may be on cell wall components in *Staph. aureus*. The intracellular effects of 10%(w/v) manuka honey observed here on *Staph. aureus* are unlikely to be due to the sugars present, as the MIC of artificial

honey has been shown to be greater than 30%(v/v) [4]. Furthermore, the cells exposed to 10%(w/v) artificial honey and observed in SEM and TEM did not manifest the increased number of cross walls seen in manuka treated cells. This cell cycle effect of manuka honey in staphylococci has not previously been reported and we are investigating this mechanism further at present.

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363

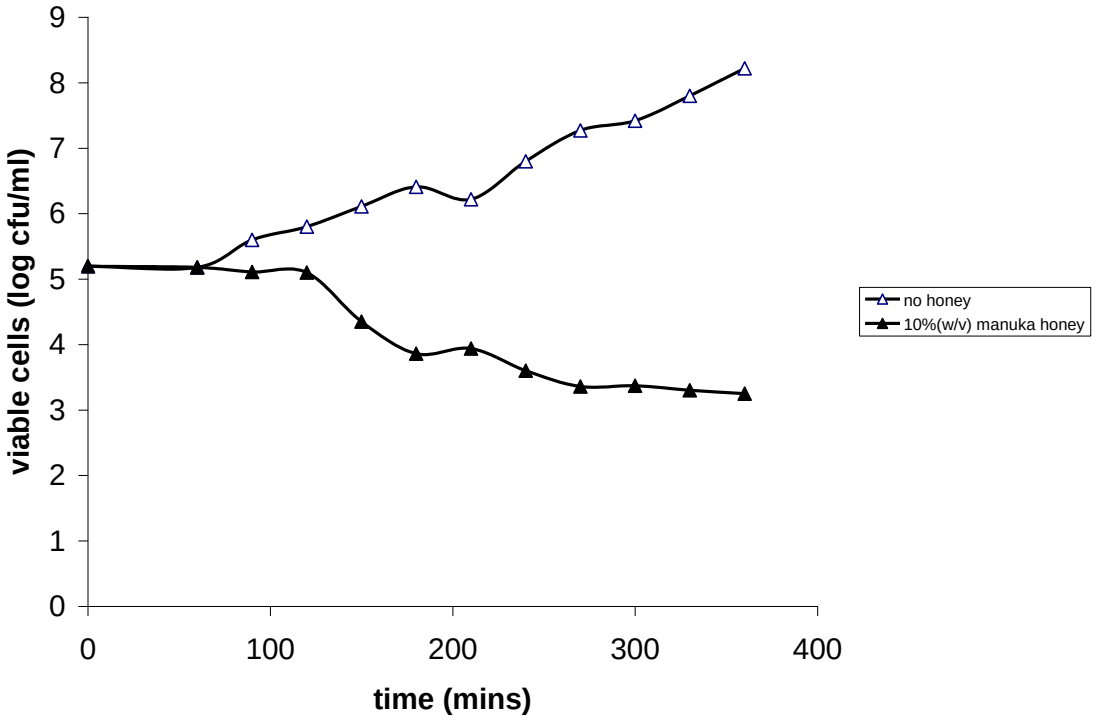
364 Table 1: Sensitivity of *Staphylococcus aureus* cultures to manuka honey

	Staph. aureus NCTN 10017	*Staph. aureus* parental *atl*	*Staph. aureus* *atl* mutant	*Staph. aureus* parental *Sig B*	*Staph. aureus* *Sig B* mutant
Mean MIC (% w/v), range (n)	2.9, 2.5-3.4 (9)	1.45, 1- 1.8 (10)	0.5, 0.2-0.8 (10)	1.83, 1.2-2.2 (10)	1.6, 1.2- 2 (10)
Mean MBC (%w/v), range (n)	4.5, 3.2-5 (7)	NT	NT	NT	NT
p (between parental strain and mutant)	-	<0.001		>0.05	

365 NT: not tested; (n): number of determinations.

366 Figure 1: The effect of manuka honey on the viability of *Staph. aureus* NCTC

367 10017



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369 Table 2: Percentage of *Staph. aureus* cells with complete septa in TEM images

	Exponential phase cells with septa (%)	Statistical significance between treated and untreated cells	Stationary phase cells with septa (%)	Statistical significance between treated and untreated cells
No honey	16 (n =1025)	-	10 (n=1178)	-
10 % (w/v) artificial honey	20 (n=1235)	NS	NT	NT
10 % (w/v) manuka honey	25 (n =1135)	p = 0.014	16 (n=1617)	p = 0.03

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371 n = total number of cells counted; NT = not tested; NS = not significant

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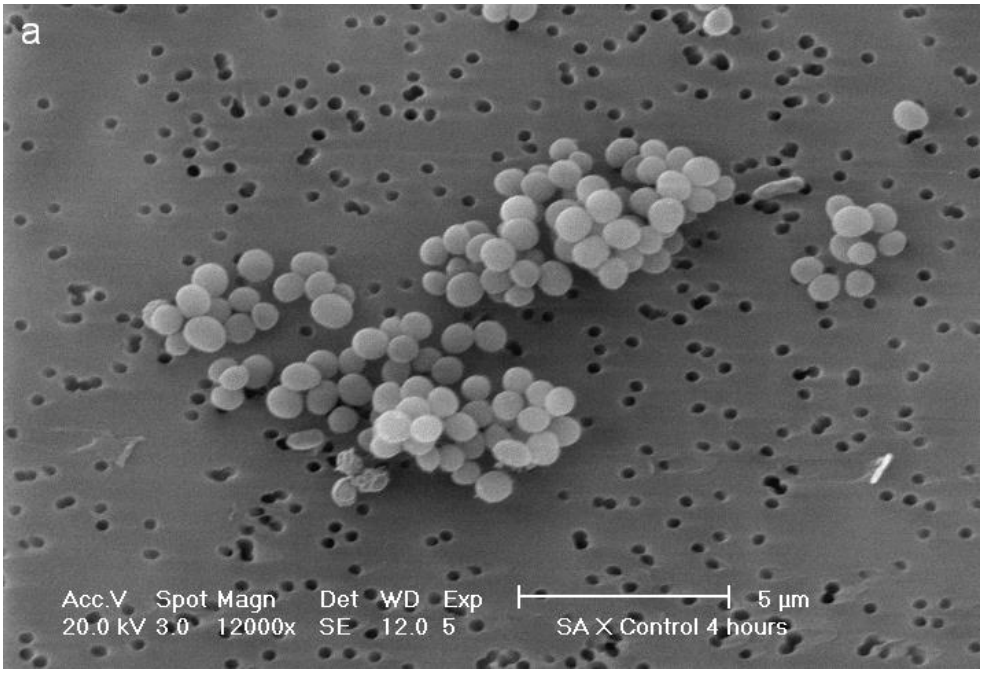
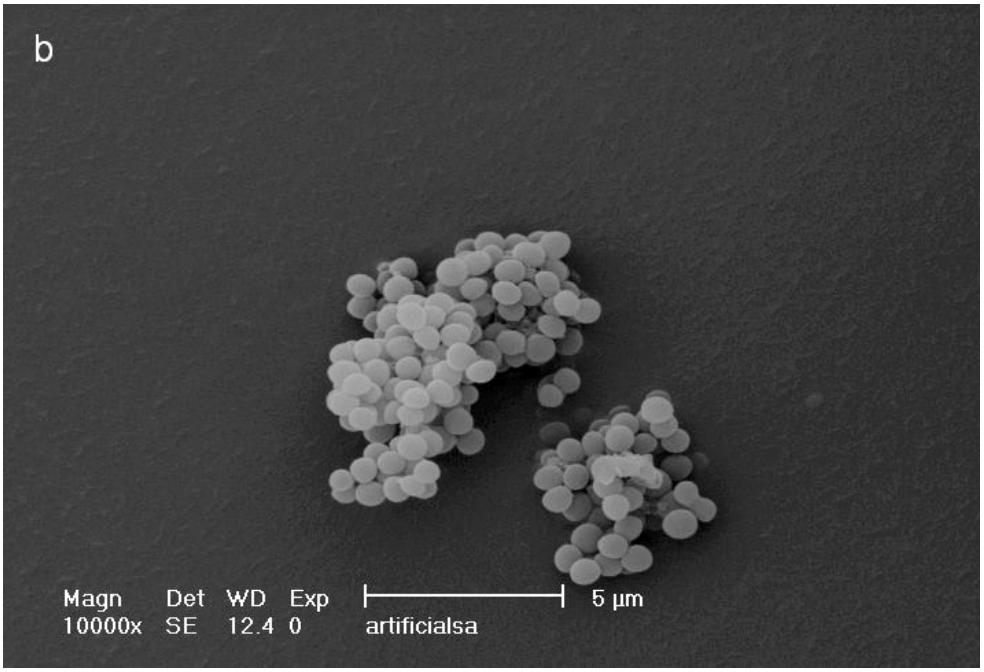
Figure 2: The effect of manuka honey on the appearance of exponential phase *Staphylococcus aureus* as seen by scanning electron microscopy

(a) Untreated exponential cells of *Staph. aureus* incubated in 0.05 mM Tris buffer pH 7.2 for 4 hours at 37^0C observed by SEM using 12,000 times magnification

(b) Exponential cells of *Staph. aureus* incubated in 0.05 mM Tris buffer pH 7.2 containing 10 % (w/v) artificial honey for 4 hours at 37^0C observed by SEM using 10,000 times magnification

(c) Exponential cells of *Staph. aureus* incubated in 0.05 mM Tris buffer pH 7.2 containing 10 % (w/v) manuka honey for 4 hours at 37^0C observed by SEM using 12,000 times magnification

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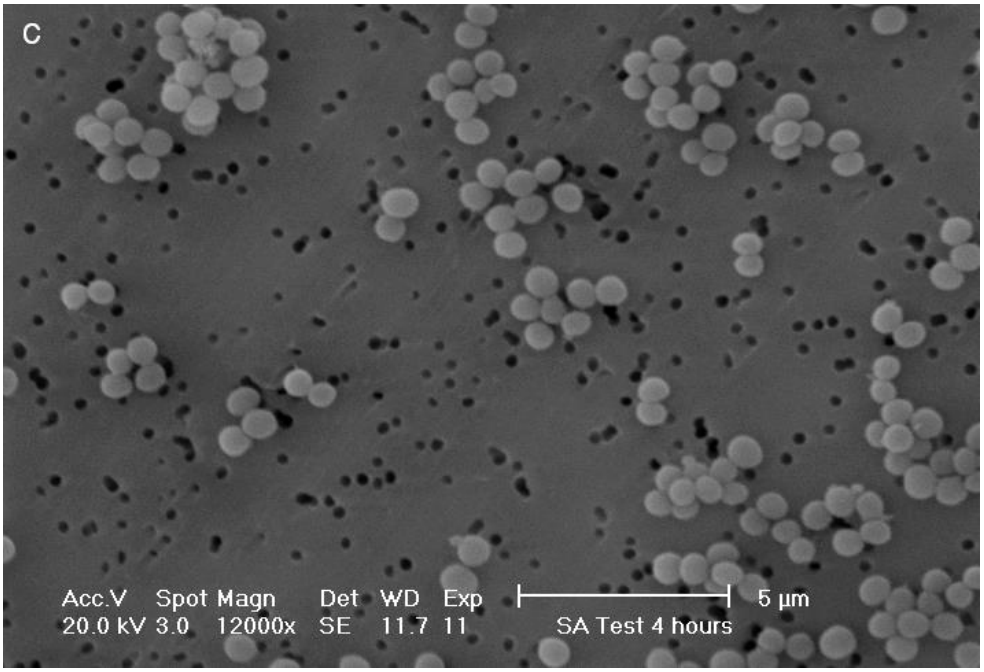
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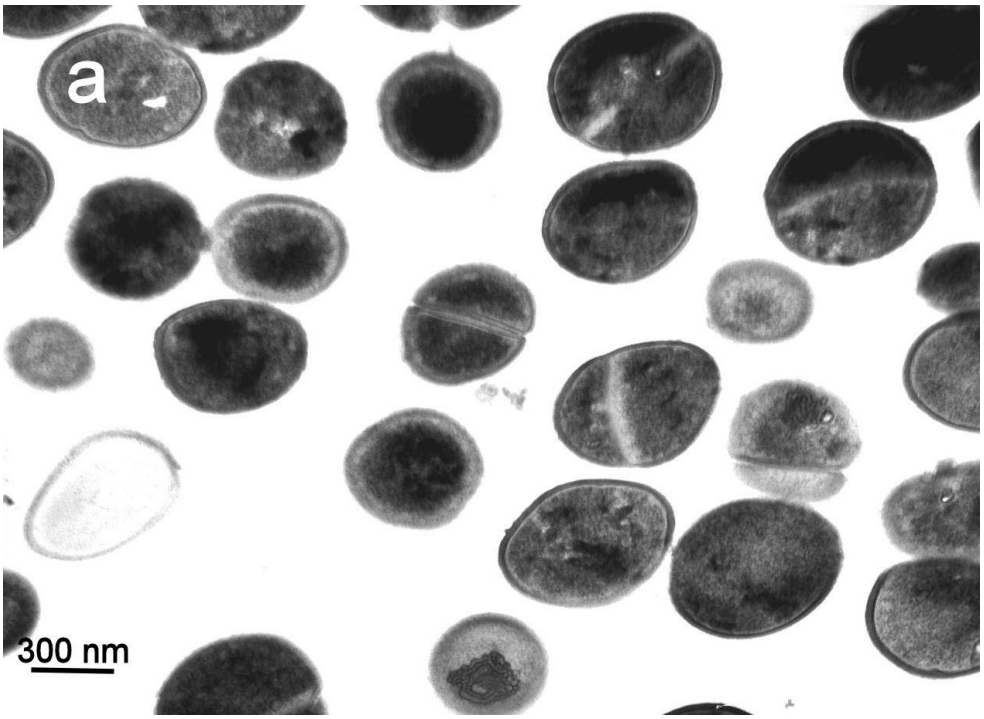
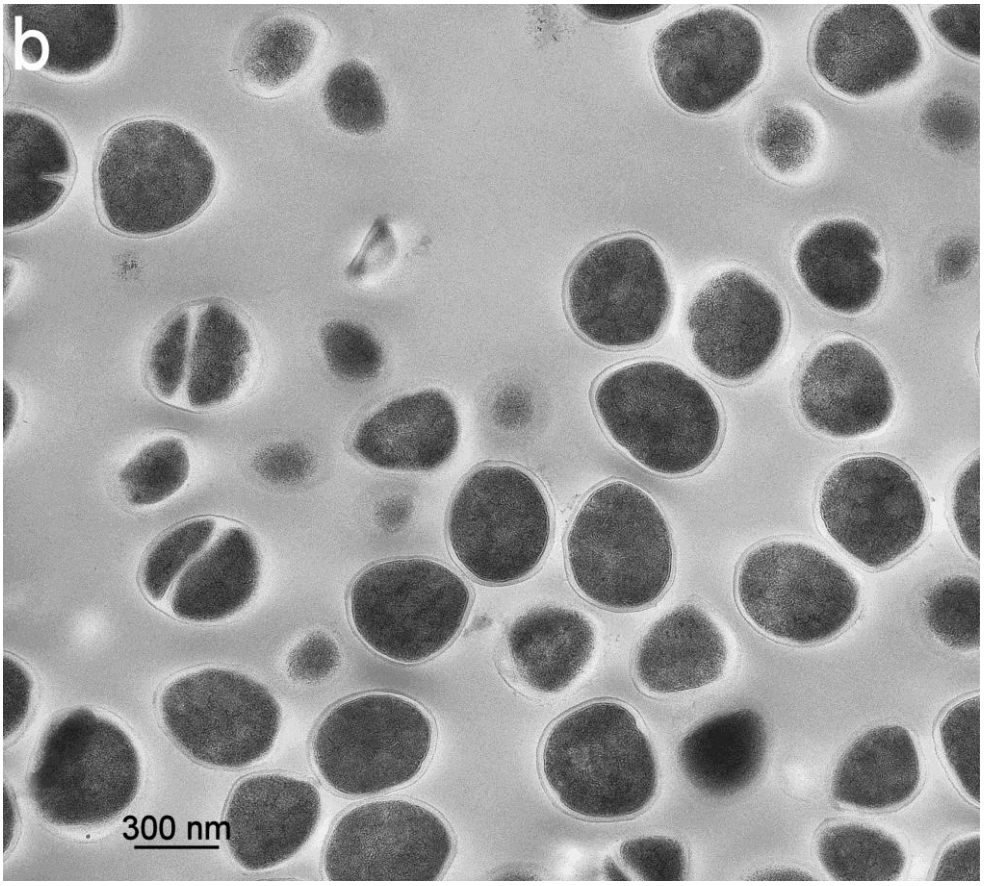
Figure 3: The effect of manuka honey on exponential phase *Staphylococcus aureus* as seen by transmission electron microscopy

3a) Exponential cells of *Staph. aureus* incubated in 0.05 mM Tris buffer pH 7.2 for 4 hours at 37^0C observed by TEM using 20,000 times magnification

(b) Exponential cells of *Staph. aureus* incubated in 0.05 mM Tris buffer pH 7.2 containing 10% (w/v) artificial honey for 4 hours at 37^0C observed by TEM using 20,000 times magnification

(c) Exponential cells of *Staph. aureus* incubated in 0.05 mM Tris buffer pH 7.2 containing 10 % (w/v) manuka honey for 4 hours at 37^0C observed by TEM using 20,000 times magnification





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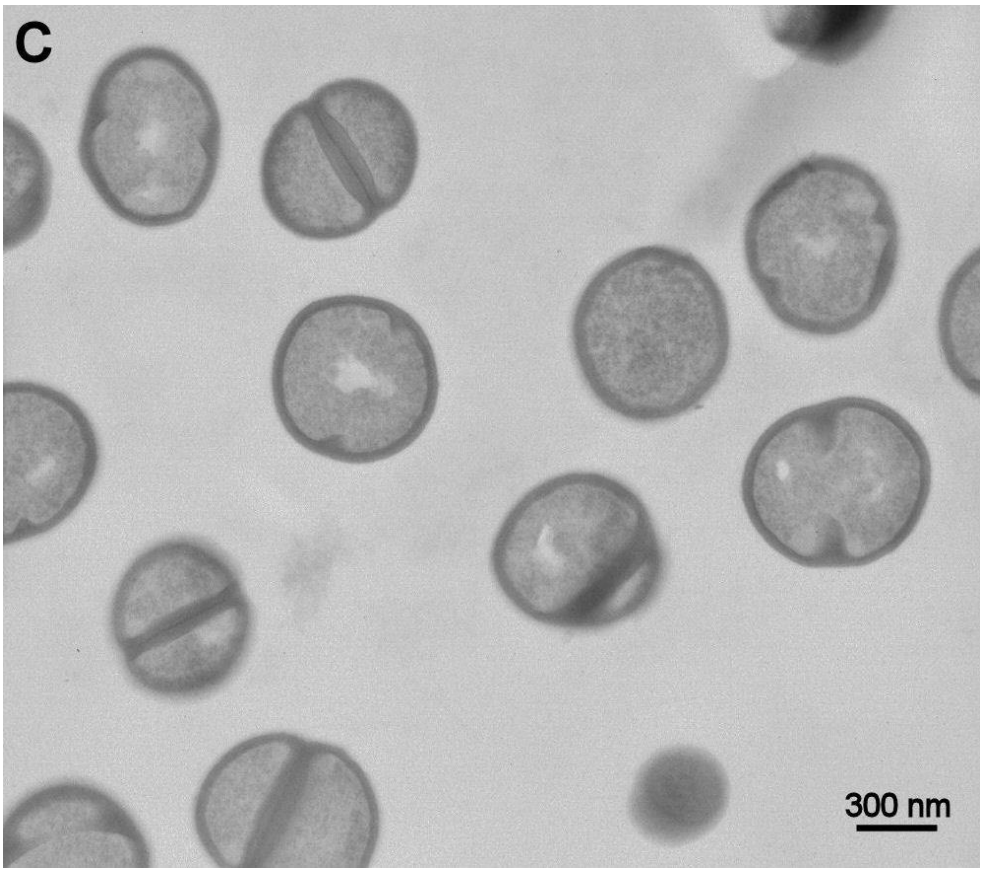

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