Manuka-type honeys can eradicate biofilms produced by *Staphylococcus aureus* strains with different biofilm-forming abilities

Chronic wounds are a major global health problem. Their management is difficult and costly, and the development of antibiotic resistance by both planktonic and biofilm-associated bacteria necessitates the use of alternative wound treatments. Honey is now being revisited as an alternative treatment due to its broad-spectrum antibacterial activity and the inability of bacteria to develop resistance to it. Many previous antibacterial studies have used honeys that are not well characterized, even in terms of quantifying the levels of the major antibacterial components present, making it difficult to build an evidence base for the efficacy of honey as an antibiofilm agent in chronic wound treatment. Here we show that a range of well-characterized New Zealand manuka-type honeys, in which two principle antibacterial components, methylglyoxal and hydrogen peroxide, were quantified, can eradicate biofilms of a range of a Staphylococcus aureus strains that differ widely in their biofilm-forming abilities. Using crystal violet and viability assays, along with confocal laser scanning imaging, we demonstrate that in all S. aureus strains, including methicillin-resistant strains, the manuka-type honeys showed significantly higher anti-biofilm activity than clover honey and an isotonic sugar solution. We observed higher anti-biofilm activity as the proportion of manuka-derived honey, and thus methylglyoxal, in a honey blend increased. However, methylglyoxal on its own, or with sugar, was not able to effectively eradicate S. aureus biofilms. We also demonstrate that honey was able to penetrate through the biofilm matrix and kill the embedded cells in some cases. As has been reported for antibiotics, sub-inhibitory concentrations of honey improved biofilm formation by some S. aureus strains, however, biofilm cell suspensions recovered after honey treatment did not develop resistance towards manuka-type honeys. New Zealand manuka-type honeys, at the concentrations they can be applied in wound dressings are highly active in both preventing S. aureus biofilm formation and in their eradication, and do not result in bacteria becoming resistant. Methylglyoxal

requires other components in manuka-type honeys for this anti-biofilm activity. Our findings support the use of well-defined manuka-type honeys as a topical anti-biofilm treatment for the effective management of wound healing.

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8   **Introduction**

9   Chronic wounds currently affect 6.5 million people in the US. These wounds are difficult to treat

10 and estimated to cost in excess of US $ 25 billion annually, with significant increases expected in

11 the future (Sen et al. 2009). A wound is generally considered chronic if it has not started to heal

12 by four weeks or has not completely healed within eight weeks (McCarty et al. 2012). Such

13 prolonged, non-healing wounds are caused by a variety of factors, with bacterial infection being a

14 significant contributor.

15 In chronic wounds, as with everywhere on earth, bacterial cells predominantly exist as biofilms,

16 where cells are embedded within a matrix of polysaccharides and other components. This matrix

17 affords resistance to environmental stresses such as altered pH, osmolarity, and nutrient limitation

18 (Fux et al. 2005). The matrix also limits access of antibiotics to the biofilm embedded cells

19 (Ranall et al. 2012), which are up to 1,000 times more recalcitrant to these compounds than

20 planktonic cells (Hoyle & Costerton 1991). Planktonic bacteria may also contribute to

21 pathogenesis, as their release from biofilms has been proposed to maintain the inflammatory

22 response within the wound (Ngo et al. 2012; Wolcott et al. 2008), as well as allowing seeding to

23 other areas (Battin et al. 2007; Costerton et al. 2003). Along with the difficulties of treating

24 biofilm infections, the emergence of resistance to multiple antibiotics has exacerbated the

25 problem of chronic wound treatment (Engemann et al. 2003; Projan & Youngman 2002). Thus,

26 there is an increasing need for new approaches to combat bacterial biofilms in chronic wounds.

27   Honey has been used to treat acute and chronic wound infections since 2500 BC (Forrest 1982;

28   Molan 1999; Simon et al. 2009). Honey possesses a number of antimicrobial properties including

29   high sugar content, low pH, and the generation of hydrogen peroxide by the bee-derived enzyme

30   glucose oxidase (Stephens 2009). However, not all honeys are the same and their antimicrobial

31   properties vary with floral source, geographic location, weather conditions, storage (time and

32   temperature) and various treatments, such as heat (Al-Waili et al. 2013; Allen et al. 1991; Molan

33   1999; Sherlock et al. 2010). These factors lead to differences in the levels of various antibacterial

34   components. Manuka honey is derived from *Leptospermum scoparium* bush and is particularly

35   potent (Adams et al. 2008; Allen et al. 1991; Kwakman et al. 2011). This is believed to be largely

36   due to the high levels of the reactive dicarbonyl methylglyoxal (MGO) (Adams et al. 2008;

37   Mavric et al. 2008), which is highly inhibitory to bacterial growth (Lu et al. 2013). Other

38   antimicrobial compounds in honeys include bee defensin-1 (Kwakman et al. 2010; Kwakman &

39   Zaat 2012), various phenolic compounds and complex carbohydrates (Adams et al. 2008; Gresley

40   et al. 2012; Mavric et al. 2008; Molan 1999; Weston et al. 2000). The combination of these

41   diverse assaults may account for the inability of bacteria to develop resistance to honey (Blair et

42   al. 2009; Cooper et al. 2010), in contrast to the rapid induction of resistance observed with

43   conventional single-component antibiotics (Colsky et al. 1998; Cooper 2008).

44   A few studies have examined the effect of manuka honey on biofilms, showing it to be active

45   against a range of bacteria, including Group A *Streptococcus pyogenes*, S*treptococcus mutans,*

46   *Proteus mirabilis*, *Pseudomonas aeruginosa, Enterobacter cloacae* and *Staphylococcus aureus*

47   (Alandejani et al. 2008; Maddocks et al. 2013; Maddocks et al. 2012; Majtan et al. 2013).

48   However, the levels of reported anti-biofilm activity are not consistent among these studies. This

49   is highly likely to be at least in part due to differences in the levels of the principle antibacterial

50   components in the honey, MGO and hydrogen peroxide, which varies with the floral and

51   geographic source of nectar, the honey storage time and conditions, and any possible other

52   treatments that may have occurred. All these conditions affect the antimicrobial activity of honey

53   (Adams et al. 2008; Al-Waili et al. 2011; Sherlock et al. 2010; Stephens 2009), but are often not

54   reported. Importantly, medical-grade honeys, while often composed primarily of manuka, can

55   also contain honey derived from other flora sources, which can alter the levels of various

56   antimicrobial components. Therefore, it is imperative to use well-characterized honeys to enable

57   both accurate comparisons among studies, and the rigorous assessment of the potential of

58   medical-grade honey to be used in wound treatment in the clinic.

59   Here we have performed biomass and viability assays, as well as confocal scanning light

60   microscopy to examine the anti-biofilm activity of four NZ manuka-type honeys, clover honey

61   and an isotonic sugar solution on a range of *S. aureus* strains that differ widely in their biofilm-

62   forming ability. These honeys have been well characterized in terms of their geography, floral

63   source and the level of the two principal antibacterial components found in honey, MGO and

64   hydrogen peroxide. We demonstrate that the manuka-type honeys are highly active in both the

65   prevention and elimination of methicillin-sensitive and methicillin-resistant *S. aureus* biofilms.

66   The antibiofilm activity was highest in the honey blend that contained the highest level of

67   manuka-derived honey; although the same level of MGO, with or without sugar, could not

68   eradicate biofilms. This suggests that additional factors in these manuka-type honeys are

69   responsible for their potent anti-biofilm activity; and emphasise the importance of characterizing

70   honey in order to understand and choose the best honey product for wound management.

Materials and Methods

71

Honey Samples

72

73   The New Zealand (NZ) honey samples used in this study are listed in Table 1, and include

74   monofloral manuka honey, Medihoney (a manuka-based medical grade honey; Comvita Ltd), a

75   manuka/kanuka blend, and clover honey (a white New Zealand honey). All honey samples were

76   supplied by Comvita New Zealand Ltd. (Te Puke, New Zealand). The harvesting and geographic

77   information for these honeys, as well as the levels of the three major antimicrobial components:

78   methylglyoxal (MGO), di-hydroxyacetone (DHA) and hydrogen peroxide, are listed in Table 1.

79   All samples were stored in the dark at 4 ºC and were freshly diluted in Tryptone Soya Broth

80   (TSB) immediately before use in assays. All honey concentrations are expressed as % w/v.

Other Tested Solutions

81

82   A series of other solutions were included for investigation alongside the honey samples: i) a sugar

83   solution designed to mimic the concentration and composition of honey sugars (45% glucose,

84   48% fructose, 1% sucrose) diluted as above for honey; ii) MGO diluted in TSB to concentrations

85   similar to those present in the manuka-type honeys (100 mg/kg, 700 mg/kg and 900 mg/kg honey)

86   to assess the effect of MGO alone on bacterial growth; iii) MGO diluted in sugar solution to the

87   same concentration as (ii). MGO was obtained as a ~40% (w/w) solution in water (Sigma-

88   Aldrich Co., MO, USA).

Hydrogen Peroxide Assay

89

90   The level of hydrogen peroxide produced by the NZ honey samples was determined using a

91   hydrogen peroxide/peroxidase assay kit (Amplex Red, Molecular Probes, Life Technologies

92   Corp., Carlsbad, CA, USA) as previously reported (Lu et al. 2013).

93   ***Bacterial Strains and Growth Conditions***

94   Four strains of *S. aureus* were examined. These include two laboratory reference strains: NCTC

95   8325 (National Collection of Type Cultures) (Stepanovic et al. 2000) and ATCC 25923

96   (American Type Culture Collection) which are methicillin-sensitive, and two clinical isolates:

97   MW2 (Hospital-Acquired Methicillin-resistant *Staphylococcus aureus,* HA-MRSA) (Baba et al.

98   2002) and USA300 (Community-Acquired Methicillin-resistant *Staphylococcus aureus*, CA-

99   MRSA) (Kazakova et al. 2005). All *S. aureus* strains were grown in TSB at 37ºC. For optimal

100   biofilm formation, 1% (w/v) glucose was added to this medium (TSBG) except for strain NCTC

101   8325 which was found to produce optimal biofilm in the absence of added glucose.

102   ***Susceptibility* of *S. aureus* to NZ Honeys: *Growth Response Assays***

103   In this study, growth response assays were carried out to assess whether the NZ honeys affected

104   cell growth of the different strains of *S. aureus* (at concentrations of 1-32%; prepared as serial 2-

105   fold dilutions in TSB(G)). Details of the growth assay methods are described in our previous

106   publication (Lu et al. 2013). TSB(G) media without honey was included as a control. Unless

107   otherwise stated, all assays were performed with three biological replicates and three technical

108   repeats of each replicate.

109   ***Biofilm Formation Assays***

110 The effects of NZ honeys and other solutions on *S. aureus* biofilm formation were determined

111 using crystal violet static biofilm formation assays in microtitre plates according to published

112 studies with the following modifications (Christensen et al. 1985; Stepanovic et al. 2000). Crystal

113 violet stains all biomass including live and dead cells and the biofilm matrix. *S. aureus* strains

114 were cultured in 2 mL of TSB(G) with shaking (250 rpm) overnight at 37°C . A suspension from

115 the overnight culture was then diluted to a cell density of approximately 10^7 CFU/mL in fresh

116 TSB containing the appropriate test solution (honey, sugar solution, MGO or MGO in

117 combination with sugar) to give a final volume of 150 μL. The suspension was added to each well

118 of a 96-well tissue-culture treated microtitre plate (BD Falcon, NJ, USA). Media-only and media

119 with the appropriate test solution without *S. aureus* inoculation were included as negative

120 controls. The microtitre plates were sealed with AeraSeal (Excel Scientific, CA, USA) and

121 incubated in a humidified incubator for 24 h at 37°C. Following this, planktonic cell growth was

122 assessed by transferring the planktonic phase into a new 96-well microtitre plate and reading the

123 optical density at 595 nm with a microplate reader (VersaMax, Molecular Devices, California,

124 USA). This step was required as *S. aureus* forms biofilms on the bottom of the microtitre plate

125 wells, which interferes with optical density readings of the planktonic culture. The microtitre

126 plates with residual biofilm were then washed three times with sterile phosphate buffered saline

127 (PBS) to remove unattached cells and air-dried at 65 $^{\circ}$C for 1 h, to fix the *S. aureus* biofilm to the

128 bottom of the well surface. The plate was then stained with 0.2% (w/v) crystal violet at room

129 temperature for 1 h, excess crystal violet solution was decanted and the plates were washed as

130 above with PBS. Stain that was bound to the adherent biomass was resolubilized with 200 μL

131 33% acetic acid and transferred into a new 96-well microtitre plate to measure the OD_{595}.

132 *Biofilm Elimination Assays*

133 *S. aureus* biofilms were first formed in the wells of a 96-well microtitre plate for 24 h at 37°C as

134 described above. Biofilms were then washed three times with PBS. Various concentrations (0% -

135 32% in two-fold serial dilutions) of honey and other test solutions were then added to the

136 established *S. aureus* biofilms. The assay plates were then incubated for a further 24 h at 37°C,

137 and planktonic cell growth and biofilm mass were quantified as described above.

138 *Determination of Bacterial Cell Viability in Biofilms*

139 Crystal violet stains all the components of the biofilm (Bauer et al. 2013). To quantify the

140 viability of cells within the *S. aureus* biofilms following honey treatment, we used a BacTitre Glo

141 Microbial Cell Viability Assay Kit (Promega, WI, USA), which measures ATP levels as a proxy

142 for viability. The assay reagents lyse the bacterial cells to release intracellular ATP, the levels of

143 which are quantified via a luminescence-based luciferase activity assay (Haddix et al. 2008;

144 Junker & Clardy 2007). The BacTitre Glo protocol involved the same steps as crystal violet

145 staining (above), however, instead of drying and staining the biofilms, plates were incubated with

146 BacTitre Glo reagent in TSB(G) for 10 minutes at 37 °C in the dark. The contents of each well

147 were then transferred into white solid-bottom 96-well microtitre plates (Cellstar, Greiner Bio-one,

148 France) for luminescence measurement. Luminescence, which is proportional to the amount of

149 ATP produced by metabolically active cells, was recorded using a 96-well microplate reader

150 (TeCan, Infinite 200Pro, Männedorf, Switzerland).

151 To ensure the validity of this assay, a standard curve was constructed to assess the correlation

152 between bacterial cell numbers and the luminescent signal in the biofilm. This was performed on

153 the untreated control (containing *S. aureus* in TSB(G) only). Biofilms produced as above were

154 washed and cells within the biofilm dispersed using a small probe sonicator (Sonics and

155 Materials VC-505) to enable quantification by direct enumeration (Merritt et al. 2005). The

156 recovered cell suspension was serially diluted 10-fold and a 20μL aliquot was plated on Tryptone

157 Soya Agar (TSA) for CFU determination. Luminescence of cells in the remaining suspension was

158 assessed using the BacTitre Glo kit. From this, a correlated standard curve was constructed

159 between calculated CFU/well and the relative luminescence readings. According to the standard

160 curve shown in Figure 1, the detection limit of the BacTitre Glo is at a luminescence reading

161 below 1,000, which is equivalent to 10^3 CFU/well (linear range from 10^3-10^7 CFU/well). An upper

162 limit was not detected.

163

164 ***Visualizing live/dead stained* S. aureus *biofilms using confocal***

165   ***laser scanning microscope (CLSM)***

166 *S. aureus* biofilms were treated with TSB containing 1%, 2%, 16%, and 32% NZ honeys or sugar

167 solution for 24 h in black polystyrene 96-well microtitre plates with μClear bottoms (Cellstar,

168 Greiner Bio-One, France) as described above, except the biofilm mass was not fixed by air-

169 drying. The treated biofilm mass was washed three times with PBS and cells within the biofilm

170 structure were fluorescently stained with 2.5 μM Syto9 (Invitrogen, CA, USA) and 4.3 μM

171 propidium iodide (PI) (Becton Dickinson, NJ, USA), which identify live and dead cells in the

172 biofilm structure, respectively. After 30 min of incubation in the dark at room temperature, the

173 wells were washed thoroughly with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich,

174 MO, USA) for 15 minutes. The wells were then rinsed and stored in PBS for imaging. Biofilms

175 were imaged using confocal laser scanning microscopy imaging (CLSM) on a Nikon A1 confocal

176 microscope. The Syto9 and PI fluorophores were excited at 488 nm and 561 nm, and the

177 emissions were collected at 500-550 nm and 570-620 nm, respectively. For quantitative analysis,

178 at least eight separate CLSM image stacks of each NZ honey treated biofilms were acquired with

179 a resolution of 512 x 512 pixels. Biofilm biomass was calculated using COMSTAT (Heydorn et

180 al. 2000) and is expressed as volume of the biofilm over the surface area ($\mu m^3/\mu m^2$).

181 Representative presentation image stacks of each treatment were acquired at a resolution of 1024

182 x 1024 pixels and three dimensional biofilm images reconstructed using NIS-elements

183 (Version10, Nikon Instruments Inc., USA). It should be noted that due to the incomplete

184 displacement of Syto9 by propidium iodide in dead cells that there will remain some Syto9

185 staining of dead cells. Therefore the absolute level of live cells detected in the Syto9 channel will

186 be somewhat overestimated using this co-staining method (Stocks 2004).

187 *Assaying Honey Resistance in Cells Recovered from Biofilms*

188 The development of resistance is a great concern in clinical settings, where bacteria can become

189 resistant to inhibitory compounds after exposure to sub-inhibitory concentrations (Cars &

190 Odenholt-Tornqvist 1993; Pankuch et al. 1998). Planktonic cells that appeared after 24 h manuka-

191 type honey treatment of established biofilms were assumed to have been released from the

192 biofilm matrix. Cells recovered from biofilms treated with sub-eliminatory concentrations of

193 manuka-type honeys were collected and tested for their ability to grow and form biofilms under

194 the static growth conditions described above. Cell growth and biofilm formation were defined as

195 not detected when the $OD_{(x)} - OD_{(media\ only\ blank)} \leq 0.1$. Each experiment was performed with three

196 biological replicates and three technical repeats of each biological replicate.

197 ***Statistical Analysis***

198 Statistical analysis to determine significant differences between treatments and among honey

199 samples were performed. First, data sets were checked for normality (Gaussian Distribution)

200 using the D'Agostino-Pearson normality test (alpha = 0.05) in GraphPad Prism (versions 5 and 6).

201 Once normality was confirmed, significant differences between treatments and among honey

202 samples were performed using One-Way ANOVA with Tukey Test using the same software.

203 Statistical significance was set at $p < 0.05$.

Results

The Effect of NZ Manuka-Type Honeys on the Planktonic Growth of *S. aureus*

Planktonic growth and biofilm mass were assessed to examine the ability of four NZ honeys,

three manuka-types and one clover, and a sugar solution to prevent biofilm formation by different

strains of *S. aureus*. Following 24 h incubation under static conditions, *S. aureus* cells formed

biofilms at the bottom of microtitre plates, with very little or no planktonic growth detected,

indicating that the concentration of honey needed to prevent *S. aureus* planktonic cell growth

could not be calculated under these conditions. Shaking broth cultures were instead used to assess

the effect of the treatments on planktonic growth. The results are shown in Table 2. Planktonic

growth of the four *S. aureus* strains, NCTC 8325, ATCC 25923, MW2 and USA300, was

completely inhibited by 8% manuka honey and Medihoney, 16% manuka/kanuka honey and 32%

clover honey. The 32%, sugar solution was only effective at inhibiting growth of the MRSA strain

MW2, with no inhibition of growth of the other strains at the concentrations tested (1-32%).

These data are in agreement with the results of our previous study using the standard *S. aureus*

reference strain, ATCC 25923, which used a similar suite of honey types and the same

experimental conditions (Lu et al. 2013).

The Effect of NZ Manuka-Type Honeys on *S. aureus* Biofilm Formation

All strains of *S. aureus* were assessed for their biofilm-forming ability after 24 h and 48 h. Under

static conditions, biofilm-forming ability varied between strains, with NCTC 8325 forming the

most robust biofilms, and generating significantly more biofilm mass than the other three tested

strains (Figure 2A and Figure 2B). This was followed by ATCC 25923 and USA300, with MW2

forming the thinnest biofilms (Figure 2A and 2B; $p < 0.05$). The effects of the four NZ honeys

226   and the sugar solution on biofilm formation of strain NCTC 8325 are shown in Figure 3A.

227   Manuka honey was the most effective at preventing biofilm formation by *S. aureus* NCTC 8325,

228   resulting in ~95% reduction ($p < 0.001$) in biofilm formation at 8% (Figure 3A) compared to the

229   untreated (0%) control. At this concentration, the other honeys and the sugar solution did not

230   significantly reduce biofilm formation. Medihoney and manuka/kanuka honey were highly

231   effective at 16%, preventing biofilm formation by ~95% ($p < 0.001$). Clover honey was much less

232   active and was less able to prevent biofilm formation than the sugar solution, even at the highest

233   concentration used (32%).

234   For NCTC 8325, the addition of sub-inhibitory concentrations of manuka and manuka/kanuka

235   honey significantly enhanced biofilm formation, increasing it by 1.5- and 2-fold, compared to the

236   untreated control ($p < 0.001$). In contrast, Medihoney, clover honey and the sugar solution did not

237   enhance biofilm formation by strain NCTC 8325 at any concentration tested.

238   *S. aureus* strain ATCC 25923, which is a standard clinical reference strain, produced similar

239   results to NCTC 8325, including the enhancement of biofilm formation following sub-inhibitory

240   honey treatment (Figure 3B). The hospital-acquired MRSA strain MW2 – the weakest biofilm

241   former out of all four tested strains (Figure 2A and 2B; $p < 0.05$) - displayed a very sensitive

242   profile to all of the NZ honeys and the sugar solution at all tested concentrations (Figure 3C).

243   Even with only 1% honey or sugar solution, a ~50% reduction in biofilm formation was observed

244   for MW2 ($p < 0.001$). At higher concentrations ($\geq 8\%$), all four NZ honeys were significantly

245   more effective than the sugar solution at preventing MW2 biofilms. The other MRSA strain, USA

246   300, responded similarly to NCTC 8325, with approximately the same concentrations of manuka-

247   type honey being required to reduce biofilm formation by ~95%. However, unlike NCTC 8325,

248   sub-inhibitory concentrations of manuka-type honey reduced biofilm formation of USA 300

249   rather than enhancing it (e.g. 4% manuka-type honeys exhibited ~50-80% biofilm inhibition of

250   USA 300). Moreover, in USA300, biofilm formation was not affected by the sugar solution at any

251   tested concentration.

252   The results above can be summarize as follows: i) all three manuka-type honeys are effective at

253   inhibiting biofilm formation of a range of of MSSA and MRSA strains; with (monofloral)

254   manuka honey being generally more effective than the other maunka-type honeys; and ii) the

255   manuka-type honeys are generally more effective than clover honey and the isotonic sugar

256   solution, although clover honey was just as inhibitory as the manuka-type honeys for the weakest

257   biofilm former, *S. aureus* MW2.

258   **The Effect of MGO on *S. aureus* Biofilm Prevention**

259   MGO is a principle antibacterial component of manuka honey responsible for its inhibitory

260   effects on the growth of *S. aureus* and other bacterial species. This is evidenced by the correlation

261   between the MGO level and the proportion of manuka-derived honey in a honey blend (Jervis-

262   Bardy et al. 2011; Lu et al. 2013). To determine whether MGO is solely responsible for the

263   inhibitory effect of the three manuka-type honeys on *S. aureus* biofilm formation, biofilm assays

264   were performed using MGO at equivalent concentrations to those present in each of the manuka-

265   based honey samples, with and without the addition of the sugar solution (Figure 4). *S. aureus*

266   NCTC8325 biofilm formation was not significantly ($p > 0.05$) affected by MGO at concentrations

267   equivalent to that present in 1- 16% manuka-type honeys. MGO at medium (700 mg/kg) and high

268   (900 mg/kg) levels at the equivalent concentration to 32% manuka-kanuka honey and Medihoney

269   prevented approximately 50% and 75% biofilm formation, respectively ($p < 0.05$). The addition of

270   the sugar solution to MGO at the same levels present in 16% of all three manuka-type honeys, led

271   to a dramatic decrease (~95%) in biofilm formation.

272   **The Effect of NZ Manuka-Type Honeys on Established *S. aureus* Biofilms**

273   Bacterial biofilms are usually already established in open, chronic wounds prior to presentation to

274   the clinic for medical treatment. We therefore assessed the ability of the four NZ honeys to

275   remove established biofilms produced by the four strains of *S. aureus.* These results are presented

276   in Figure 5, with coloured lines showing biofilm mass present following treatment with different

277   concentrations of the various honey types. While there was variation among the *S. aureus* strains

278   in their response to the different honeys, there are some important general trends. First, manuka

279   honey was consistently the most effective at removing biofilm, eliminating almost all of the

280   established *S. aureus* biofilms at concentrations of 16%–32%, ($p < 0.001$ compared to the

281   untreated control sample; Figure 5 top panel, orange lines). Second, Medihoney and

282   manuka/kanuka honey were also effective at these concentrations for some *S. aureus* strains, but

283   only consistently effective across all four strains at 32% (Figure 5, blue and green lines). Third,

284   both the clover honey and the sugar solution did not significantly reduce ($p > 0.5$) established

285   biofilm mass until their concentration reached 32%. However, the sugar solution did not remove

286   the USA 300 biofilm, with no significant reduction in biofilm mass at 32% (Figure 5, purple

287   line).

288   NCTC 8325, the most efficient biofilm former out of all tested strains, gave a slightly different

289   response toward honey treatment compared to the other three strains. Significant biofilm

290   enhancement occurred in this strain at sub-inhibitory concentrations of manuka honey (1-2%) and

291   manuka/kanuka honey (1-4%) ($p < 0.001$; Figure 5). In addition, this strain was the least sensitive

292   to the manuka-type honeys. For example, at 8% manuka honey treatment, the NCTC 8325

293   biofilm mass remained similar to the untreated control ($p > 0.05$; Figure 5), while the biofilms

294   produced by the other three strains were significantly reduced at this concentration ($p < 0.001$;

295   Figure 5).

296   **The Effect of NZ Manuka-Type Honeys on Cell Viability within *S. aureus* Biofilms**

297   Elimination of biofilm mass was assessed using crystal violet, a cationic dye that stains all the

298   components of the biofilm. However, this assay cannot assess the viability of cells remaining

299   within the biofilm structure (Bauer et al. 2013). To determine this, we used a BacTitre Glo assay,

300   which measures ATP levels as a proxy for viability. Side-by-side CFU measurements showed that

301   the level of ATP detected in these assays was proportional to the count of viable cells per well

302   (ranging from 10^3-10^7 CFU/well) (Figure 1).

303   The viability of cells remaining in the biofilm after the various treatments is shown in Figure 5.

304   In general, cell viability decreased in proportion to the elimination of biofilm biomass (Figure 5,

305   black lines). However, several exceptions to this general trend were observed. In some cases,

306   biofilm biomass increased but cell viability did not, e.g. NCTC 8325 biofilms with low

307   concentrations of manuka (2%) and manuka/kanuka honey (1-4%) (Figure 5). In others, biofilm

308   biomass remained relatively constant while cell viability increased, e.g. NCTC 8325 with 1-4%

309   Medihoney ($p < 0.05$; Figure 5), and ATCC 25923 with 4% and 8% clover honey. Another

310   deviation from the general trend was a significant reduction of cell viability while biofilm

311   biomass remained unaffected, seen for NCTC 8325 and USA 300 with 4% and 8% manuka honey

312   treatment (Figure 5; $p < 0.05$). This emphasizes the importance of assessing viability alongside

313   crystal violet assays for biofilm assessment.

314   Overall, at concentrations easily attainable in the clinic, the tested four NZ honeys were effective

315   at eliminating biofilm biomass and at killing both MSSA and MRSA *S. aureus* cells in the

316   residual biofilm. Among the honey types, manuka honey was the most effective, where the

317   elimination of biofilm biomass largely paralleled the reduction in viability. Following treatment

318   with 8% manuka honey only ~10% of cells were viable in the remaining ATCC 25923 and USA

319   300 biofilms, compared to the untreated control (i.e. 0% honey), and no generation of ATP could

320   be detected from MW2 (Figure 5). This is similar to the degree of biofilm biomass removal,

321   where 85-98% of biofilm biomass was removed following 8% manuka honey treatment. Although

322   NCTC 8325 biofilm biomass was seemingly unaffected at 8% manuka honey compared to the

323   untreated control (Figure 5), the number of viable cells detected within this biofilm was

324   drastically reduced by approximately 80% ($p < 0.001$; Figure 5).

325   **The Effect of MGO on Established *S. aureus* Biofilms**

326   To assess the contribution of MGO alone, as well as MGO plus sugar, to biofilm removal, these

327   components were tested on established *S. aureus* NCTC 8325 biofilms (Figure 6). MGO levels

328   equivalent to the presence of 1-8% manuka/kanuka honey (Table 1) caused biofilm biomass to

329   increase approximately 2-fold, relative to the untreated control ($p < 0.001$). However, the

330   established biofilm biomass was not reduced significantly ($p > 0.05$), for any of the tested

331   concentrations (1-32%) of MGO by itself, or in combination with the sugar solution. Thus,

332   neither MGO nor the combination of MGO with sugar is solely responsible for the elimination of

333   biofilms observed with these manuka-type honeys.

334   **Visualizing the Effects of NZ Manuka-Type Honeys on Established *S. aureus* Biofilms**

335   To assess the effect of the NZ honeys on *S. aureus* NCTC 8325 biofilms at the cellular level, we

336   used confocal laser scanning microscopy (CLSM) of biofilms stained with fluorescent dyes for

337   the detection of live and dead bacteria. This allows both the visualization of individual cells

338   within the biofilm in three dimensions and the effect of treatments on cell viability to be

339   determined. Treatment by sub-inhibitory (1% and 2%) and inhibitory (16% and 32%)

340   concentrations of NZ honeys was visualized by viewing fluorescently-labelled live (Syto9; green)

341   and dead (propidium iodide; red) cells. Representative images of each treatment are presented in

342   Figure 7and quantification of live and dead cell biofilm biomass for several samples for each

343   treatment is shown in Figure 8. In general, the established biofilm biomass decreased with

344   increasing concentrations of manuka-type honey. More specifically, manuka honeys were

345   effective in reducing the live cells in established *S. aureus* biofilms. Sub-inhibitory concentrations

346   of all the manuka-type honeys (1% and 2%) and the sugar solution did not reduce the amount of

347   biomass compared to the non-treated control cells (Figure 8). This is shown in Figure 7 where

348   the untreated control cells displayed a green (live-cell) lawn that covered nearly the entire surface

349   and this remained following treatment with 1% and 2% manuka-type honeys. At concentrations

350   of 16% and 32%, the manuka-type honeys substantially reduced the density and depth of the

351   biofilm, along with the amount of live cells, compared to the untreated control (Figure 7 and

352   Figure 8). For example, the 32% manuka honey significantly reduced the Syto9 stained (live)

353   biofilm biomass to 10% ($p < 0.001$) compared to the non-treated live biofilm biomass (Figure 8).

354   Only small micro-colonies were present following treatment with 32% manuka honey, and the

355   colour of the biofilms was predominantly yellow (where both the green and red dye were retained

356   within cells), indicating mostly dead cells. In contrast, 32% clover honey and sugar solution

357   reduced the total biomass by a maximum of 30% ($p < 0.001$) compared to the non-treated control

358   (Figure 8). This result corresponds to the 3D reconstructed images, where the Syto9 stained cells

359   remained dominant after treatment (Figure 7).   At 32%, clover honey or sugar solution, a

360   substantially larger Syto9 stained (live) lawn remained in comparison to the 32% manuka-type

361   honeys, although the biomass was less confluent than in the untreated control. These results are

362   consistent with the results obtained with the crystal violet stained biofilm biomass and ATP

363   viability assays.

364   **Assessing the Development of Resistance to Manuka-type Honeys in *S. aureus* Biofilms**

365   Bacteria that are exposed to sub-inhibitory concentrations of antimicrobial agents generally

366   develop resistance to these agents (Braoudaki & Hilton 2004; Davies et al. 2006). The ability of

367   cells released from *S. aureus* NCTC 8325 biofilms to develop resistance after exposure to sub-

368   inhibitory concentrations of honey was investigated, and the results are summarized in Table 3.

369   All cells recovered from the *S. aureus* biofilm after 24 h with 8% of all three manuka-type honeys

370   were viable and able to form biofilms in media (TSB). However, they were unable to grow

371   planktonically when subsequently exposed to 8% manuka, or 16% Medihoney and

372   manuka/kanuka honey (the MIC levels for these honeys). Biofilm formation was also inhibited by

373   8% manuka honey, and by 16% Medihoney and manuka/kanuka honey. These growth- and

374   biofilm-inhibitory concentrations of manuka-type honeys are the same as those observed for cells

375   that had not previously been treated with these honeys. These results indicating that planktonic

376   cells released from the biofilms with exposure to sub-inhibitory concentrations did not acquire

377   resistance to the same honey treatment.

378   **Discussion**

379   Chronic wounds are costly and difficult to treat (Hoyle & Costerton 1991; Ranall et al. 2012; Sen

380   et al. 2009), and bacterial biofilms are important contributors to the delay in healing. Honey is a

381   promising alternative treatment for these wounds, and studies have indicated that it is able to

382   prevent bacterial biofilms and eliminate established biofilms *in vitro* (Alandejani et al. 2008;

383   Maddocks et al. 2013; Maddocks et al. 2012; Majtan et al. 2013). However, the effective

384   concentration of honey reported by these studies varies significantly, making it hard to establish a

385   foundation for the efficacy of honey on chronic wound-associated bacterial biofilms in the clinic.

386   This is probably largely due to the fact that, in most of these studies, very little information is

387   reported on the honey itself, including the floral source, geographic location, storage conditions,

388   and the level of the two principle antibacterial components, MGO and hydrogen peroxide. Here

389   we utilize a suite of well-defined NZ honeys, including manuka-type honeys (manuka,

390   Medihoney and manuka/kanuka honey) and clover honey, to investigate their anti-biofilm activity

391   on a range of *S. aureus* biofilms that differ in their ability to form biofilms. We show that

392   manuka-type honeys can be used to kill all MSSA and MRSA cells when present as a biofilm in a

393   chronic wound, supporting the use of this honey as an effective topical treatment for chronic

394   wound infections.

395 Our study has shown that prevention of *S. aureus* biofilm formation occurred at honey

396 concentrations that also inhibit planktonic growth (Figure 3A-D; Table 2), suggesting that biofilm

397 prevention was a consequence of planktonic growth inhibition, as opposed to any specific effects

398 on biofilm development. Other studies have also shown that manuka-type honeys can inhibit

399 bacterial biofilm formation, however, the concentrations required were higher than those reported

400 to inhibit growth (Alandejani et al. 2008; Maddocks et al. 2013; Maddocks et al. 2012; Majtan et

401 al. 2013).

402 We found that higher concentrations of all honeys were necessary to eliminate established

403 biofilms compared to those needed for prevention, as assessed by both quantification of biofilm

404 biomass and cell viability. Manuka honey was the most effective, closely followed by both

405 Medihoney and manuka/kanuka honey. Elimination of biofilms was visually confirmed using

406 CLSM of fluorescently-stained live and dead cells. The sugar content of honey clearly mediates

407 some effect, as sugar solution and clover honey were able to eliminate established biofilms at

408 high concentrations (32%), as has been shown in other studies (Chirife et al. 1983; Chirife et al.

409 1982). However, manuka-type honeys consistently achieved biofilm elimination at lower

410 concentrations, suggesting that components specifically within manuka-type honeys contribute

411 towards biofilm elimination. The concentrations of manuka-type honeys that show significant

412 anti-biofilm activity are easily achievable in the clinic, since honey dressings typically contain

413 >80% honey (Cooper et al. 2010).

414   The use of assays for total biofilm biomass and cell viability to examine the effects of the various

415   treatments on biofilm elimination afforded some other interesting observations. We observed that

416   in some cases, sub-inhibitory concentrations of two of the manuka-type honeys enhanced biofilm

417   formation; however, cell viability did not increase. This could be due to a stress response, as has

418   been previously observed when bacteria are exposed to sub-inhibitory concentrations of

419   antibiotics (Haddadin et al. 2010; Kaplan et al. 2012; Mirani & Jamil 2011; Subrt et al. 2011). In

420   other cases, no reduction of biofilm biomass was observed but cell viability was significantly

421   reduced. This suggests that unlike antibiotics, the manuka-type honeys (or active components

422   therein) are able to penetrate through the biofilm matrix, killing the bacterial cells whilst leaving

423   intact matrix.

424   It is believed that MGO is the primary component in manuka-type honeys responsible for its anti-

425   biofilm activity (Jervis-Bardy et al. 2011; Kilty et al. 2011). The effectiveness of the different

426   manuka-type honeys tested here did increase with MGO content. However, the same degree of

427   biofilm prevention and elimination could not be reproduced with equivalent amounts of MGO

428   either alone or in combination with sugar. In the case of prevention, MGO alone was generally

429   ineffective, although a significant amount of biofilm prevention was achieved in combination

430   with sugar. This suggests that the MGO and sugar do contribute to biofilm prevention, but their

431   effects are not as strong as those observed with manuka honey.

432

433   Unlike the three NZ manuka-type honeys, neither MGO alone nor MGO with sugar at honey-

434   equivalent concentrations showed significant *S. aureus* biofilm elimination. This indicates that

435   the ability of manuka-type honeys to eliminate biofilms of this organism is due to one or more

436   components present in the honey other than MGO and sugar, such as low pH, hydrogen peroxide,

437   phenolics and other unknown components (Jagani et al. 2009; Jervis-Bardy et al. 2011; Kilty et al.

438   2011; Zmantar et al. 2010). Interestingly, while the kanuka/manuka honey had a relatively high

439   rate of hydrogen peroxide production compared to the manuka and Medihoney (Table 1), but low

440   MGO levels, it was not any more active against biofilms of *S. aureus*. This suggests that, at least

441   for this organism, hydrogen peroxide within these manuka-type honeys does not provide

442   significant anti-biofilm activity.

443   **Conclusions**

444   This study is the first to use a suite of well-characterized manuka-type honeys against a range of

445   strains of *S. aureus* that differ in their ability to form biofilms. We demonstrate that: 1) at very

446   low levels, some honeys can enhance biofilm formation, presumably by evoking a stress response

447   similar to that seen with some antibiotics; 2) the ability to prevent or eliminate biofilms is

448   influenced by MGO levels and the presence of sugar, but these alone do not account for all of the

449   anti-biofilm effect; 3) honey is able to reduce biofilm mass and also to kill cells that remain

450   embedded in the biofilm matrix; and 4) planktonic cells released from biofilms following honey

451   treatment do not have elevated resistance to honey. Taken together our results show that if used at

452   an appropriate therapeutic level, manuka-type honey can be used to kill *S. aureus* when present as

453   a biofilm in a chronic wound, supporting the use of this honey as an effective topical treatment for

454   chronic wound infections.

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Harvesting and chemical information for the tested NZ honey samples

Honey type	Harvest period	Area	Floral source	Major Antimicrobial Composition		
				DHA [a]	MGO [a]	H_2O_2 [b]
Manuka	Spring 2010	Hokianga, Northland, NZ	*Leptospermum scoparium var. incanum*	4277	958	0.34
Medihoney	Spring 2010	Northland, NZ	*Leptospermum scoparium var. incanum + Kunzea ericoides*	883	776	0.31
Manuka/kanuka	Summer 2010/11	Hokianga, Northland, NZ	*Leptospermum scoparium var. incanum + Kunzea ericoides*	652	161	0.68
Clover	N/A*	Balcutha, Otago, NZ	*Trifolium spp.*	< 20	< 10	0.11

[a] MGO (methylglyoxal) levels were analyzed against di-hydroxyacetone (DHA) and expressed as mg MGO per kg of honey.

[b] Rate of production of H_2O_2 (hydrogen peroxide) is expressed as μmol/h in 1 mL of 10% w/v honey.

* Information not available

Concentration of Honey Required to Inhibit *S. aureus* Growth

Honeys	NCTC 8325	ATCC 25923	MW2	USA300
Manuka honey	8*	8	8	8
Medihoney	8	8	8	8
Manuka/kanuka honey	16	16	16	16
Clover honey	32	32	32	32
Sugar solution	>32	>32	32	>32

1 * All numbers in the table are honey concentrations (%, w/v).

Resistance of *S. aureus* Cells Recovered from Biofilms after 8% Manuka Honey Treatments[a]

Honey (%)	Type of assay	Manuka honey	Medihoney	Manuka/kanuka honey
0	Growth	✓	✓	✓
	Biofilm formation	✓	✓	✓
8	Growth	✗	✗	✓
	Biofilm formation	✗	✓	✓
16	Growth	✗	✗	✗
	Biofilm formation	✗	✗	✗

2   [a] A tick means that there was normal growth or biofilm formation and a cross means that there

3   was no growth or no bioflm formation.

Figure 1

Correlation of levels of intracellular ATP to colony forming units (CFU) in static biofilms of *S. aureus*

Static biofilms of *S. aureus* were formed in the wells of a microtitre plate for 48 h (with media replenishment at 24 h). After removal of the biofilm from the wall of each well, intracellular ATP levels were measured by the BacTitre Glo Viability Kit and CFU were determined for each well. The intracellular levels of ATP are plotted as a function of CFU and validate that the BacTitre Glo Viability Kit can be used as a surrogate measure of biofilm cell viability in subsequent assays.
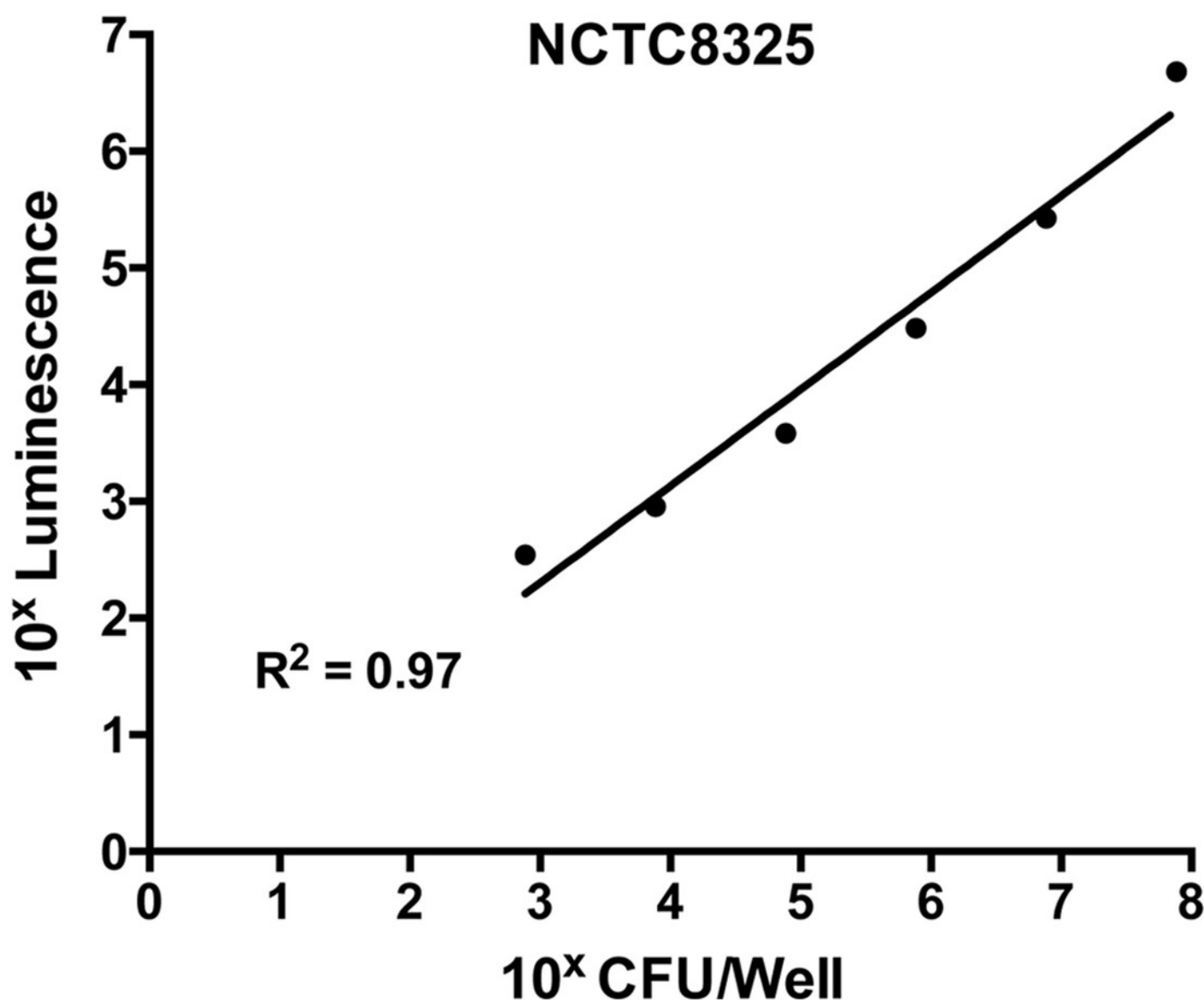
Figure 2

Quantification of Biofilm Formation by Different Strains of *S. aureus*

The ability of different strains of *S. aureus* to form biofilms on the plastic surface of tissue-culture treated 96-well microtitre plate was assessed in TSB(G) at 24 h and 48 h. Biofilm adherence was determined using a static biofilm formation assay over 24 h (A) and 48 h (with media replenished after 24 h incubation) (B). Biofilm formation was quantified by staining with 0.2% crystal violet solution and measured at an optical density of 595 nm. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate, *** represents $p < 0.001$, compared to NCTC 8325 , as assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.
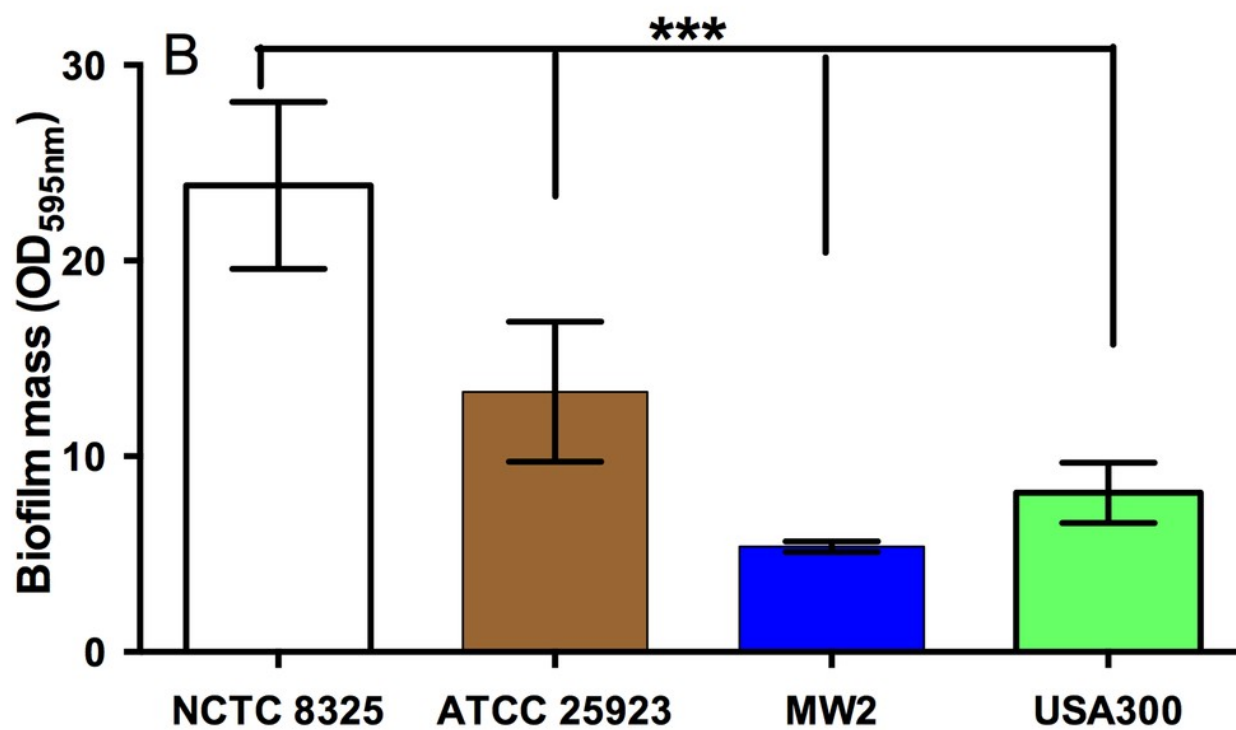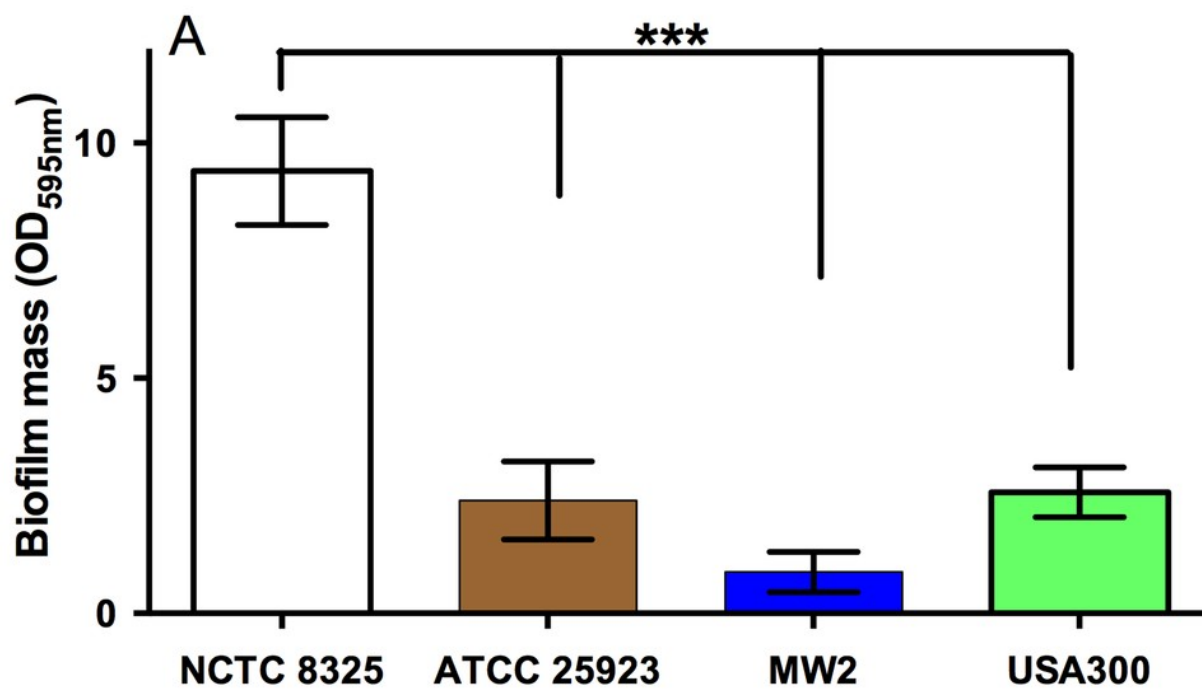
Figure 3

Effects of NZ honeys and sugar on *S. aureus* biofilm formation

S. aureus biofilms were allowed to form in the presence of four different NZ honey types (manuka, Medihoney, manuka/kanuka or clover) or a sugar solution. Biofilm formation was assessed using a static biofilm formation assay with crystal violet staining to quantify biomass. *S. aureus* strains are: (A) NCTC 8325; (B) ATCC 25923; (C) MW2 (HA-MRSA) and (D) USA300 (CA-MRSA). Biofilm formation is expressed as a percentage relative to that produced by the untreated control, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.
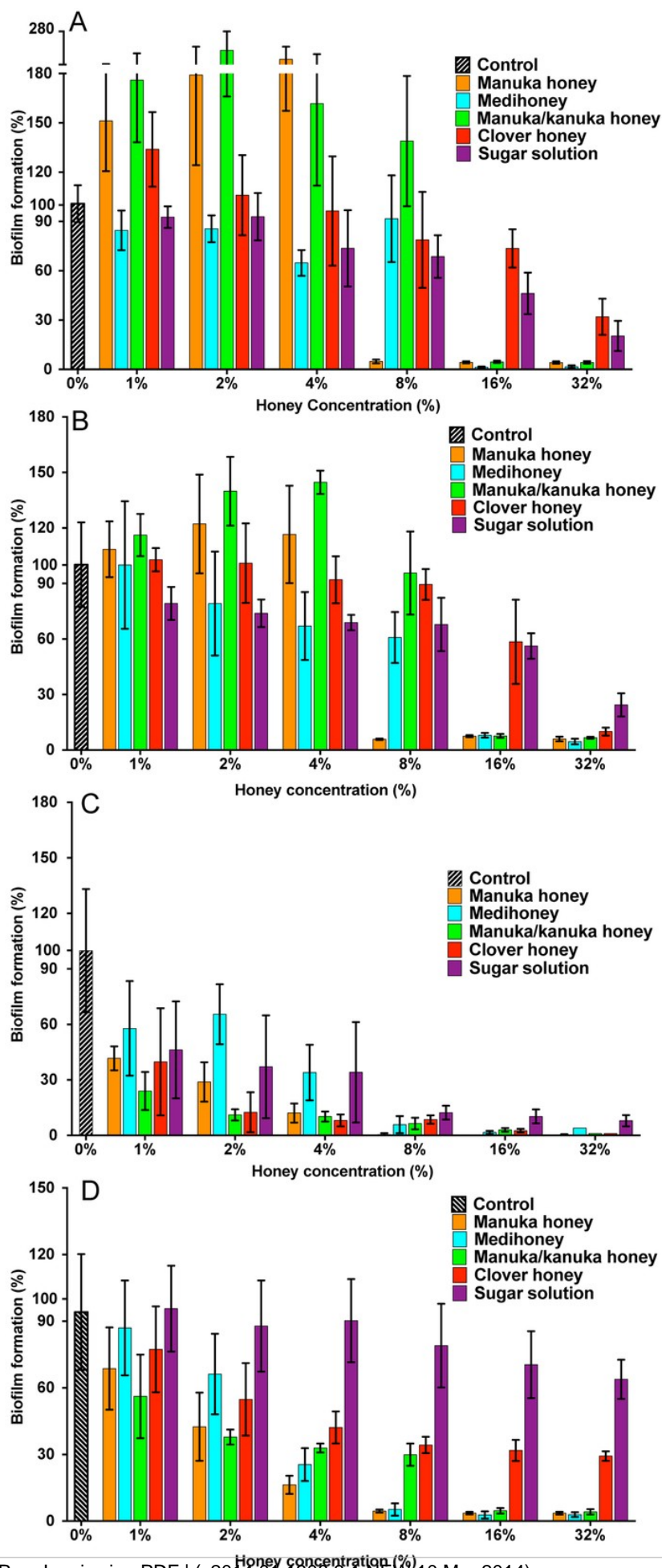
Figure 4

Effects of MGO on *S. aureus* biofilm formation

Biofilm formation by *S. aureus* NCTC 8325 grown in the presence of MGO and MGO plus sugar solution. MGO stock solutions were prepared to correspond to the MGO levels in undiluted manuka-type honeys (100 mg/kg of manuka/kanuka honey, 700mg/kg of Medihoney, and 900 mg/kg of manuka- honey; Table 1). Biofilm formation was assessed using the described static assay with crystal violet staining to quantify biomass. Biofilm formation is expressed as a percentage relative to the untreated control, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate. Statistical significance (p<0.05) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.
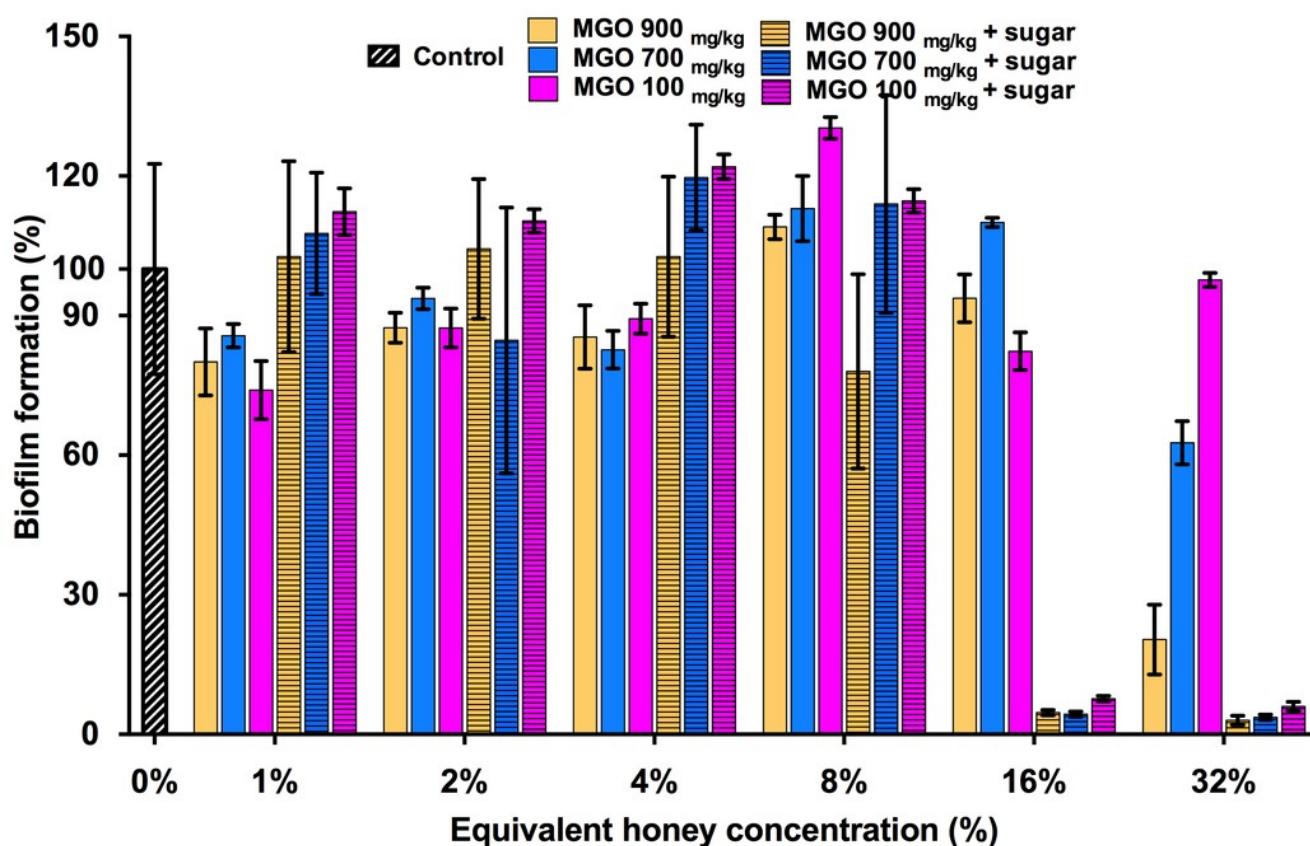
Figure 5

Effects of NZ honeys on established *S. aureus* biofilms and cell viability within the biofilms

Established *S. aureus* NCTC 8325, ATCC 25923, MW2 and USA300 biofilms were treated with NZ honeys – manuka, Medihoney, manuka/kanuka, clover, and a sugar solution. The remaining biofilm masses were quantified using crystal violet staining (left y-axis) and cell viability within these remaining biofilms were assessed using the BacTitre Glo Viability Kit (right y-axis). Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate Statistical significance (p<0.05) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.
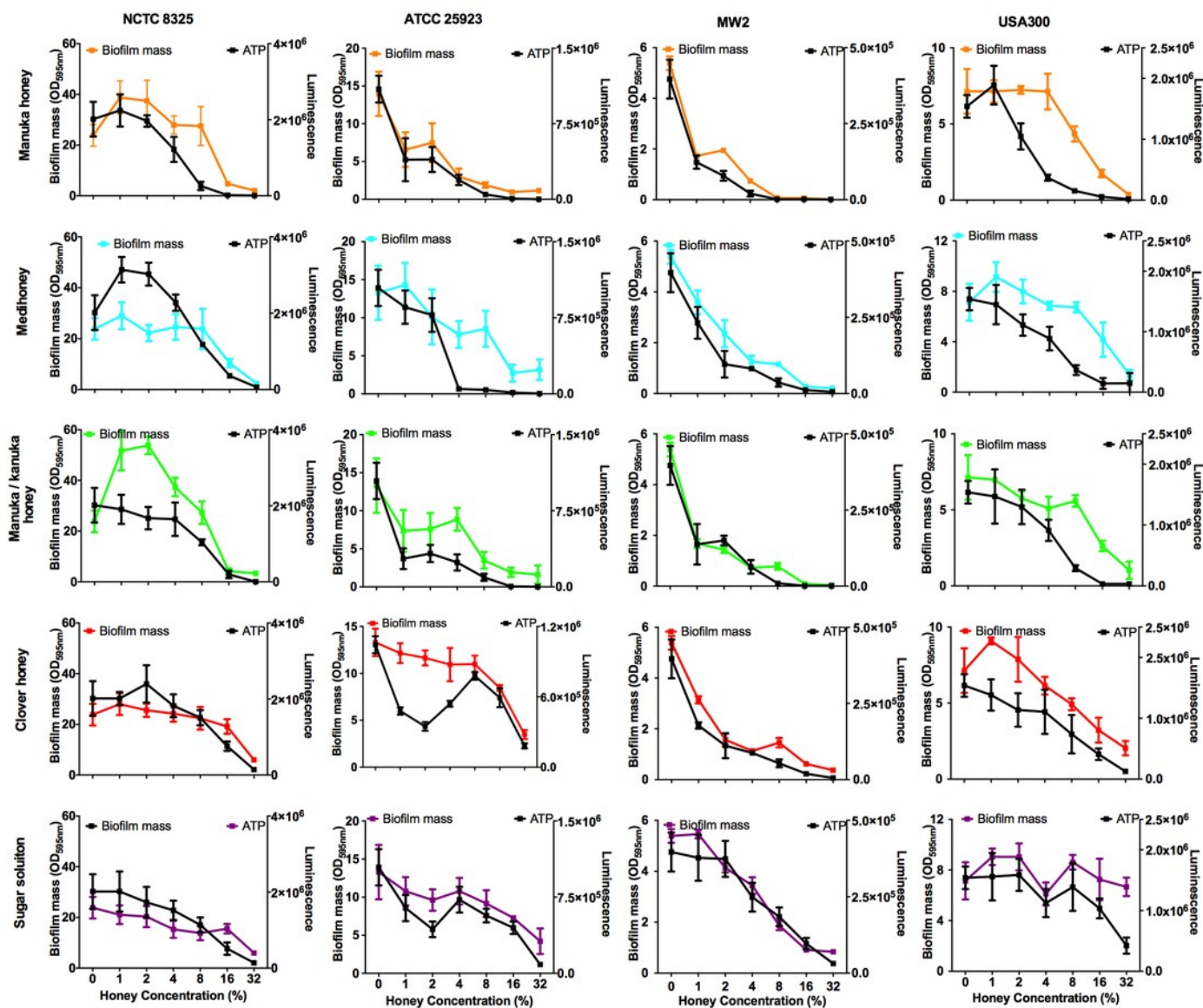
Figure 6

Effects of MGO on established *S. aureus* biofilms

S. aureus NCTC 8325 biofilms were treated with MGO and a combination of MGO and the sugar solution. MGO stock solutions were prepared to correspond to the MGO levels in undiluted honey (100 mg/kg of manuka/kanuka honey, 700mg/kg of Medihoney, and 1,000 mg/kg of manuka honey; Table 1). The crystal violet stained residual biofilm mass after 24 h treatment was quantified using optical density (OD595nm). Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate. Statistical significance (p<0.05) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.
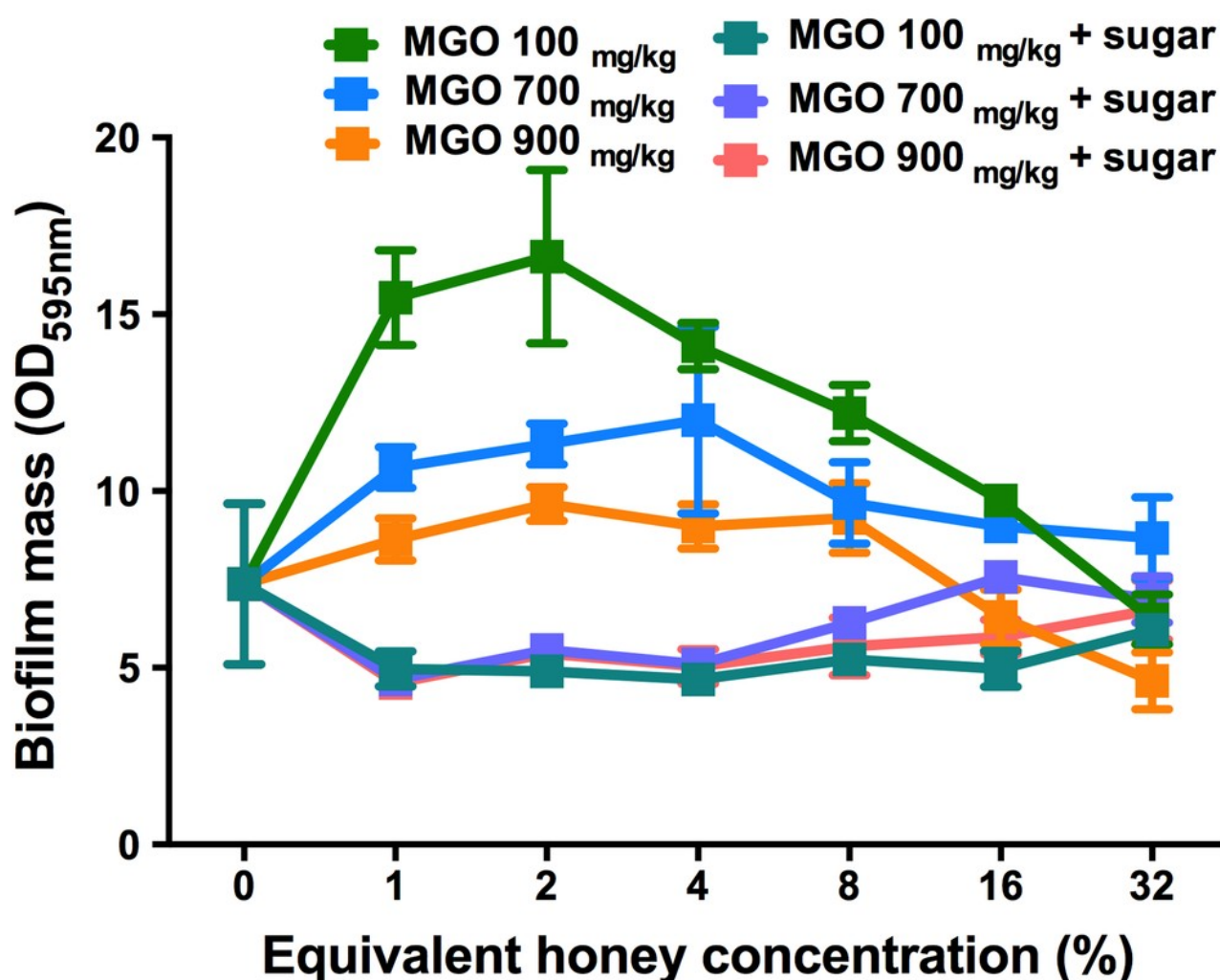
Figure 7

Live/dead staining of different honey treated established biofilms

Established biofilms produced by *S. aureus* NCTC 8325 were treated with TSB containing honey (manuka, Medihoney, manuka/kanuka or clover) or sugar solution at 1%, 2%, 16%, and 32% (w/vol). Syto9 (green; viable cells) and propidium iodine (red; dead cells) stained images were acquired using Nikon A1 Confocal Laser Scanning Microscope. The 3D- images were reconstructed using NIS-elements (version 10). Scale bar represents 50 μm.

No-honey control
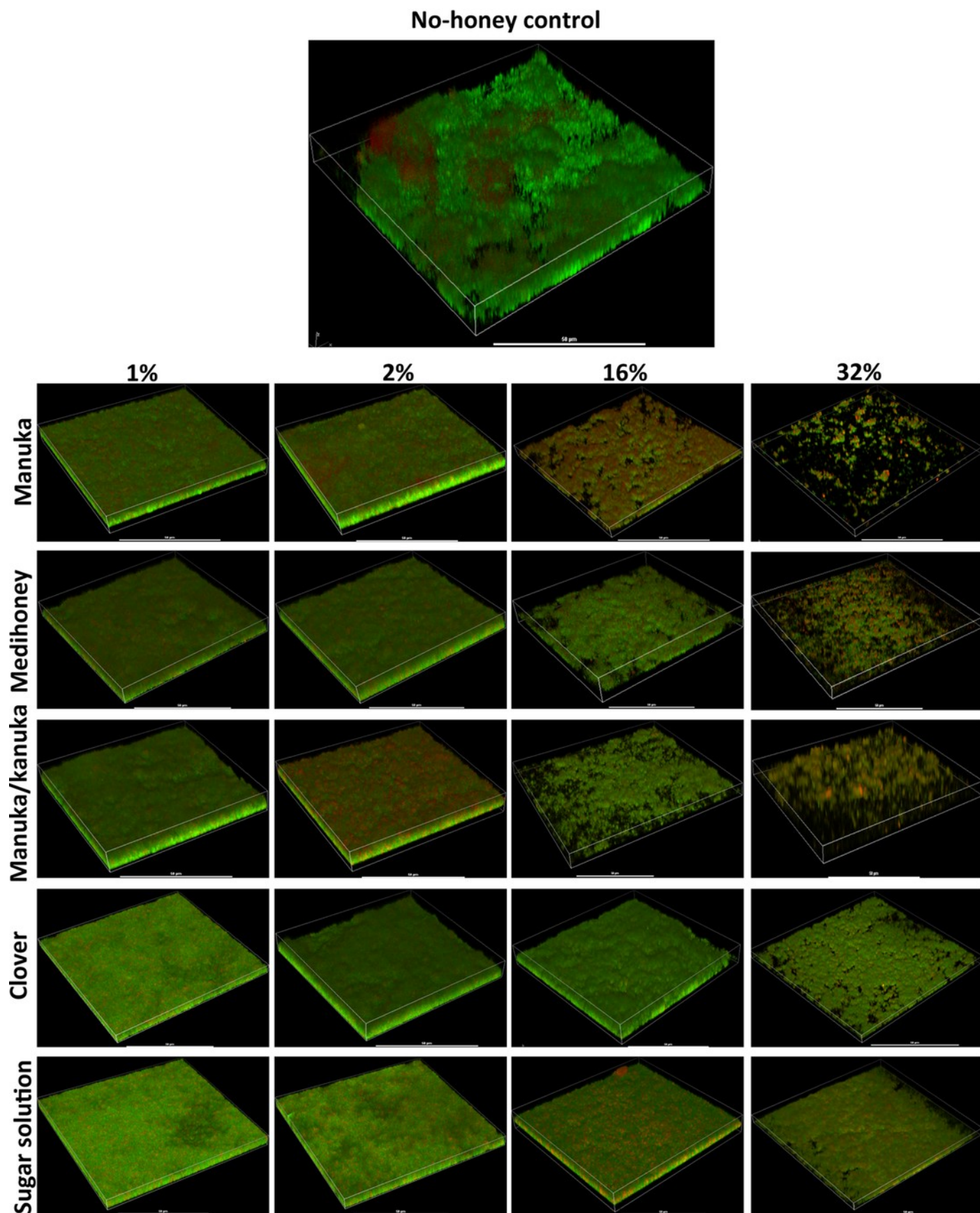
Figure 8

Quantitative analysis of live/dead stained honey treated biofilms

The established *S. aureus* NCTC 8325 biofilm was treated with New Zealand honeys (manuka honey, Medihoney, manuka/kanuka honey, and clover honey) and a sugar solution at 1%, 2%, 16%, and 32% (w/v) concentrations. Biofilms were co-stained with Syto9 (S, viable cells) and propidium iodide (P, dead cells) and analyzed using COMSTAT. The estimated live (S) and dead (P) biomass (volume of the biofilm over the surface area (μm3/μm2)) are expressed as a percentage of the non-treated control live and dead biomass, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples where eight representative images were acquired. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.