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Article

Determining the Contribution of Osmotic Stress to the Antibacterial Properties of Honey

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SUMMARY

Researchers have repeatedly shown that honey possesses distinctive antimicrobial properties; however, there is uncertainty over which compounds in honey are responsible for these properties. In this research study, we sought to quantify the role of osmotic stress in honey and determine the efficacy of two types of honey: Manuka and raw pasture honey. Bacteria were sequentially cultured in sublethal concentrations of Manuka and raw pasture honey for five days. The role of osmotic stress as a contributor to the antibacterial properties of Manuka and raw pasture honey was quantified in the first culture and over five serial cultures. The growth levels of bacteria in honey were compared to growth levels in glucose, an osmotic control, to quantify the role of osmotic stress in the two types of honey. The results of this study indicate that in the first culture, the antibacterial impacts of Manuka and raw pasture honey were primarily attributable to osmotic stress. However, over five days of sequential transfers, both raw pasture honey and Manuka honey showed significant antibacterial properties beyond osmotic strength. It was established that the antibacterial properties of honey cannot be investigated based solely on the first culture. Serial transfers over several days should be employed to investigate the efficacy of honey as an antibacterial substance.

INTRODUCTION

Over the past century, antibiotics have saved countless lives; however, the recent rise of antibiotic resistance has established a critical need for the use of novel antibacterial agents. Moreover, few new antibiotics are being developed by pharmaceutical companies, which further increases this need (1). This quandary has instigated a re-examination of several ancient plant-based remedies, such as honey, to assess their potential to be used therapeutically (2).

Honey has been used as a traditional remedy for centuries (3), yet its use in contemporary medicine has been limited due to a lack of scientific support (4). Recently, however, researchers have been re-evaluating honey as a potential therapeutic treatment, and several studies have reported its antibacterial properties (1, 3, 5, 6). Honey has been shown to possess antibacterial effects against microorganisms, such as *Salmonella*, *Shigella*, *Escherichia coli*, and *Helicobacter pylori*, according to several laboratory and clinical investigations (3).

Honey has been reported to contain about 200 different substances. Sugar and water are the two main constituents, and fructo-oligosaccharides, minerals, enzymes, and various amino acids are some of the other components of honey (3). Honey is also hygroscopic, meaning it absorbs moisture from its environment, which partially contributes to its antimicrobial effects (2). In addition to the poor environment honey creates for bacteria, many phytochemical factors have been found in honey, which contribute to its antibacterial effects (3).

Certain types of honey are more effective than others. Commercially available honeys differ in their antibacterial activity compared to newly identified medical-grade honey, which may be used therapeutically in the future (7).

Manuka honey (*Leptospermum scoparium*), a monofloral honey from New Zealand, is distinctive for its potent and broad-spectrum antibacterial properties (8). This honey has been proven to be effective against pathogenic bacteria such as *S. aureus* and *H. pylori*, making it a possible effective treatment for wounds or stomach ulcers (2). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *S. aureus* that is resistant to most of the modern-day antibiotics, rendering it a pertinent threat to worldwide health, often leading to high treatment costs and several deaths over the past several decades (9). In conjunction with oxacillin, a penicillin antibiotic, Manuka honey has been shown to restore the susceptibility of MRSA to oxacillin (10).

While the antibacterial effects of several honeys are solely due to the production of hydrogen peroxide (8), Manuka honey has been shown to exhibit other antibacterial effects. Methylglyoxal (11) and leptosin (12) have already been identified as some of the compounds responsible for its unique antibacterial effect. Despite the lack of notable antibacterial compounds, raw pasture honey may still be beneficial as it is more accessible than medical-grade honeys in local communities.

Since sugar is the most abundant component of honey, it is expected that some of the antibacterial effects of honey are due to the high osmotic stress (3). However, it is unclear what the contribution of osmotic stress is to the antibacterial properties of Manuka or other commercial types of honey. The purpose of this study was to investigate the antibacterial effects of Manuka honey and raw pasture honey. By quantifying

the impact of osmotic stress on the antibacterial properties of Manuka and raw pasture honey, the other antibacterial effects of honey beyond osmotic stress can be discerned. A native strain of *S. aureus*, a gram-positive bacterium, was used. *S. aureus* has a thick cell membrane with a large amount of peptidoglycan, which is a mesh layer that preserves the shape of the bacteria and allows it to better endure intracellular pressure (13). We hypothesized that both Manuka and raw pasture honey would exhibit unique antibacterial properties beyond osmotic stress. Instead of just a single culture, this study employed multiple serial transfers to assess the efficacy of different concentrations of honey. Bacteria were serially transferred into sublethal concentrations of Manuka and raw pasture honey for a total of five cultures to evaluate the efficacy of a range of honey concentrations.

RESULTS

Determining Treatment Concentrations to Use in Serial Transfers

In a brief pilot study, bacteria were first grown in Manuka honey concentrations of 50, 60, 70, 80, 90, 100, and 110 mg/mL for 24 hours. The growth levels of these cultures are shown in **Figure 1**. Concentrations of Manuka honey of 90 mg/mL and greater were lethal in the first culture, and thus, they were not used in serial transfers. Sublethal concentrations of Manuka honey between 50 and 80 mg/mL were utilized in serial transfers.

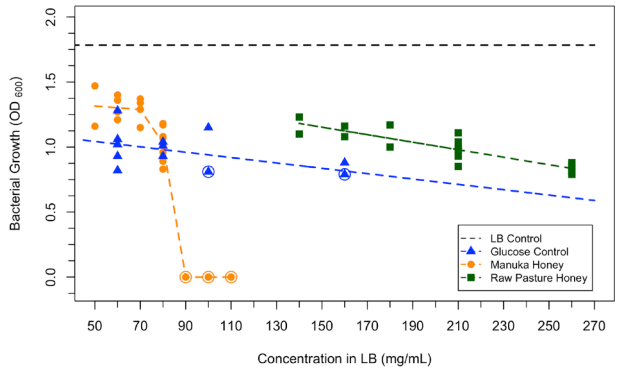

Figure 1. Dose-response curves for Manuka honey and raw pasture honey. Manuka honey concentrations from 50–80 mg/mL and raw pasture honey concentrations from 140–260 mg/mL were sublethal upon first exposure. Multiple Manuka honey culture losses are indicated by outer circles around points at an optical density of 0. The dotted black line represents the average growth level of bacteria grown in LB under no stress in the first culture. The dotted blue line represents growth levels in glucose concentrations in the first culture with circles around points representing multiple points with the same value.

Bacteria were also grown in raw pasture honey concentrations of 140, 160, 180, 210, and 260 mg/mL. All concentrations of raw pasture honey tested were sublethal upon initial exposure as shown in **Figure 1**, and therefore, all tested concentrations of raw pasture honey were utilized in serial transfers. Note that the range of effective concentrations used for raw pasture honey was drastically higher than the range of effective concentrations used for Manuka honey.

Effective honey concentrations used in this study and concentrations of glucose, ranging from 0–200 mg/mL, were measured with an osmometer. Glucose concentrations measured were 0, 60, 80, 100, 120, 150, and 200 mg/mL; **Figure 2** is a plot for glucose concentrations between 0 and 200 mg/mL with a regression line for osmolality on concentration included. As an osmotic control, concentrations of glucose were chosen to mimic the osmolality of the highest concentrations of each honey that permitted growth in the first culture. A 60 mg/mL glucose concentration was found to have a similar osmolality to Manuka honey at 80 mg/mL (765 mOsm/kg) and was chosen as a glucose concentration for the experiment. A 160 mg/mL glucose concentration had a similar osmolality to raw pasture honey at 260 mg/mL (1577 mOsm/kg); thus, it was chosen as another glucose concentration for the experiment.

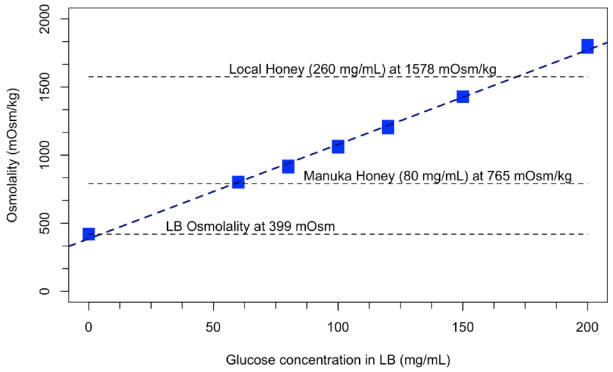

Figure 2. Osmolalities of seven different concentrations of glucose in LB, shown as a linear relationship. Marked on the graph are the osmolalities of Manuka honey (80 mg/mL), raw pasture honey (260 mg/mL), and LB (0 mg/mL glucose). The dotted line is the regression line of osmolality on concentration of glucose. Regression line equation is $\hat{O} = 385.98 + 6.93C$, where $\hat{O}$ is predicted osmolality on the line and C is glucose concentration (in mg/mL).

Bacteria were serially transferred into 60 and 160 mg/mL glucose growth media for five days, similar to honey. Osmotic stress was clearly effective in lowering bacterial growth, as both glucose cultures grew to nearly half the growth level of bacteria grown in sterile LB. Both concentrations showed stable growth throughout the five culture days. Although the 160 mg/mL glucose concentration was slightly more effective in inhibiting bacterial growth than the 60 mg/mL glucose, on average, the difference between the growth levels of bacteria cultured in these two media was relatively small (OD_{600} = 0.1887 ± 0.0335, $p < 0.001$).

Serial Transfers are Essential in Evaluating the Efficacy of Honey

A majority of the concentrations which were sublethal in the first culture did not permit growth upon later transfers

into fresh honey-containing media. Concentrations that did not permit detectable growth upon further transfers were tested several times to ensure that this loss of growth was not coincidental. It was confirmed that Manuka honey (70 and 80 mg/mL) and raw pasture honey (180, 210, and 260 mg/mL), which were sublethal in the first culture, were in fact lethal when bacteria were retransferred into them (**Figure 3**).

Lethal concentrations of Manuka honey (70 and 80 mg/mL) showed no detectable growth after the first transfer, and were thus, discontinued from further serial transfers. Sublethal concentrations of Manuka honey (50 and 60 mg/mL) maintained stable growth for all five culture days. Raw pasture honey concentrations of 210 and 260 mg/mL did not permit growth in the second culture, while 140 and 160 mg/mL raw pasture honey concentrations showed lower levels of growth. After the second sequential transfer, 140 and 160 mg/mL raw pasture honey strains returned to the growth levels of day one and sustained stable growth for the remaining culture days.

Osmotic Stress Fully Accounts for Antibacterial Effects of Sublethal Concentrations of Honey in the First Culture

In model equation [1], treatment-concentration factor $(TC)_i$ was leveled to have the LB control as the reference level to verify that each bacterial culture was placed under significant stress. Relative to the LB control, all treatment-concentrations showed significantly lower growth levels upon initial exposure (**Table 1**). This verified that Manuka honey, raw pasture honey, and glucose exhibited significant antibacterial effects.

Table 1 clearly shows that both Manuka and raw pasture honey cultures grew at a significantly lower level than the LB control bacteria, and the following results quantified how much of this inhibitory effect was due to osmotic stress. The efficacy of Manuka and raw pasture honey was evaluated by comparing the growth of bacteria in honey versus glucose, an osmotic control.

The osmolalities of honey and glucose were then used as grounds for comparing osmotic strength. The osmolality of glucose and honey concentrations were measured three times with an osmometer, and their average osmolalities are given in **Figure 2** and **Table 2**, respectively.

The effect of osmotic stress on the growth levels of bacteria in the first culture was quantified using statistical model [2], verifying several things about sublethal honey concentrations in the first culture, which are summarized in **Table 3**.

The effect of osmolality on bacterial growth had a significant negative slope, verifying that osmotic stress effectively depressed the growth of bacteria. In addition, treatment effects, T_i, were accounted for in the model to detect other antibacterial properties beyond osmotic stress in each treatment. Both Manuka and raw pasture honey had nonsignificant positive estimates relative to glucose (Raw pasture honey OD_{600} = 0.0304 ± 0.0719, p = 0.6738; Manuka honey OD_{600} = 0.0164 ± 0.0650, p = 0.8025), indicating that when osmotic stress was accounted for, the antibacterial properties of Manuka and raw pasture honey in the first culture did not differ significantly from glucose. In the first culture of bacteria into sublethal concentrations of honey, neither Manuka nor raw pasture honey exhibited substantial antibacterial properties beyond osmotic stress; therefore, the inhibitory effects of honey in the first culture seemed to be primarily attributable to osmotic impact.

While the unique antibacterial properties of sublethal honey concentrations beyond osmotic impact are not detected in the first culture, further serial transfers will reveal

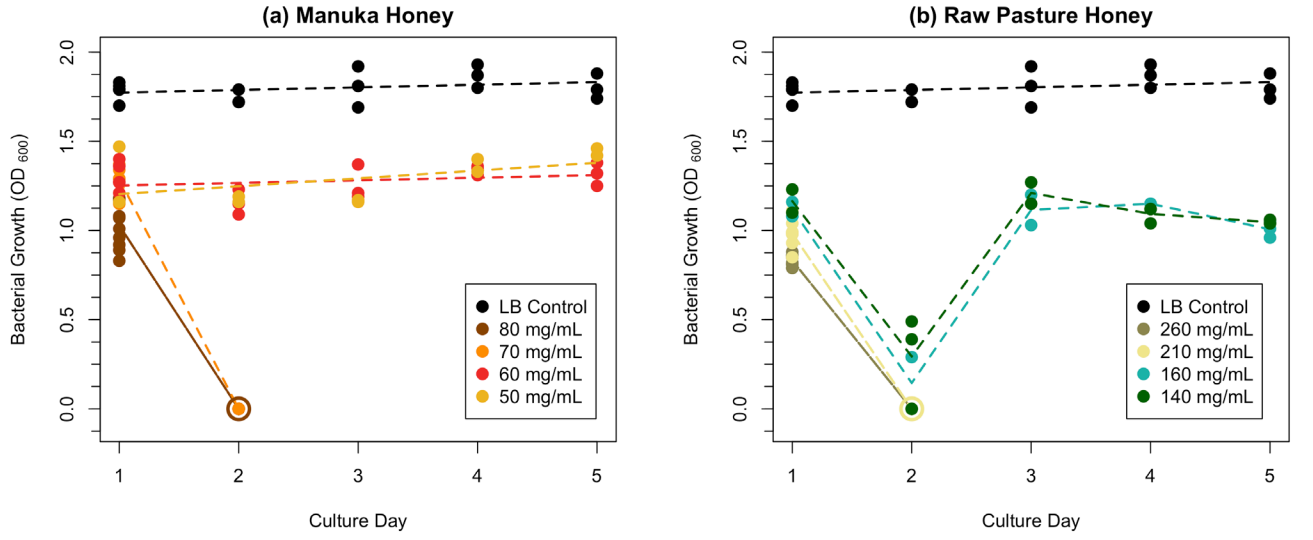

Figure 3. (a) Growth levels of bacterial strains grown in concentrations of Manuka honey ranging from 50 to 80 mg/mL and LB control. Bacteria grown in 70 and 80 mg/mL Manuka honey did not show visible growth upon second exposure. **(b) Growth levels of bacterial strains grown in concentrations of raw pasture honey ranging from 140 to 260 mg/mL and LB control.** Bacteria grown in 210 and 260 mg/mL raw pasture honey did not show visible growth upon second exposure. Multiple culture losses are indicated by outer circles around points at OD_{600} = 0.

Treatment	Concentration (mg/mL)	OD_{600} Estimate	SE[b]	*p*	Significance[c]
Overall mean ($\hat{\mu}$)[a]	---	1.8233	0.0417	< 0.001	***
Day ($\hat{\alpha}$)[a]	---	-0.0393	0.0139	0.0071	**
Glucose	160	-0.9247	0.0657	< 0.001	***
	60	-0.6680	0.0657	< 0.001	***
Raw pasture honey	140	-0.3045	0.1334	0.0274	*
	160	-0.3888	0.1221	0.0027	**
	180	-0.3845	0.1334	0.0061	**
	210	-0.5742	0.0944	< 0.001	***
	260	-0.8930	0.0525	< 0.001	***
Manuka honey	50	-0.2331	0.1113	0.0420	*
	60	-0.2461	0.1003	0.0182	*
	70	-0.3388	0.0787	< 0.001	***
	80	-0.7157	0.0502	< 0.001	***

a. Note: $\hat{\mu}$ and $\hat{\alpha}$ are estimates for overall mean and regression coefficient of OD on day.
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Day-adjusted estimates of first-day cultures studied relative to an LB control strain grown with no antibacterial additives. All strains studied were put under stress as shown by their significantly lower growth levels, relative to the LB control strain. LB was the first level of the treatment factor, hence, estimates of all other levels were obtained relative to LB.

a more comprehensive picture about the efficacy of these honey concentrations.

The antibacterial effects of Manuka and raw pasture honey in the first culture were verified using an alternative method of analysis involving contrasts of statistical model [1]. The growth levels of bacteria in certain concentrations of honey were compared against growth in solutions of glucose with similar osmolality measures.

First, the growth levels of bacteria in 80 mg/mL Manuka honey were compared to the growth levels of bacteria in 60 mg/mL glucose. A 60 mg/mL glucose solution and 80 mg/mL Manuka honey have similar osmolality measures (**Figure 2**). Bacterial growth in 80 mg/mL Manuka honey and 60 mg/mL glucose was not significantly different in the first culture

Treatment	Concentration (mg/mL)	Osmolality (mOsm/kg)	Standard Deviation
LB	0	398.7	2.52
Raw pasture honey	140	992.67	8.74
	160	1090.00	9.64
	180	1167.33	6.66
	210	1323.33	5.13
	260	1577.67	10.69
Manuka honey	50	605.00	7.21
	60	671.00	7.94
	70	699.67	10.97
	80	765.33	1.53

Table 2. Average osmolality of effective sublethal concentrations of Manuka and raw pasture honey used in this study along with standard deviation.

Effect	OD_{600} Estimate	SE[b]	*p*	Significance[c]
Overall mean ($\hat{\mu}$)[a]	1.3623	0.1444	< 0.001	***
Day ($\hat{\alpha}$)[a]	0.0196	0.0082	0.0214	*
Raw pasture honey	0.0264	0.0703	0.7085	
Manuka honey	0.0117	0.0650	0.8577	
Osmolality ($\hat{\beta}$)[a]	-0.0004	0.0001	< 0.001	***

a. Note: $\hat{\mu}$, $\hat{\alpha}$, and $\hat{\beta}$ are estimates for μ, α, and β, respectively, in statistical model [2].
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3. The effect of treatment and osmolality on growth level of bacteria in the first culture, relative to glucose. Manuka and raw pasture honey exhibited nonsignificant antibacterial effects beyond the contribution of osmotic stress in the first culture. Therefore, osmotic impact was the primary antibacterial effect of honey in the first culture.

(OD_{600} = -0.0477 ± 0.0579, $p = 0.4149$). This affirms the results in **Table 3**: the impact of sublethal concentrations of honey in the first culture was not significantly different from glucose solutions with equivalent osmolalities.

However, the antibacterial effects of Manuka honey, as an overall treatment in the first culture, were not the same as those of glucose. It is important to emphasize that concentrations of Manuka honey above 80 mg/mL were completely lethal, but concentrations of glucose greater than 60 mg/mL (i.e. 160 mg/mL glucose) were not, thus nullifying the impression that Manuka honey does not differ significantly from glucose as an overall treatment in the first culture.

Next, to verify whether raw pasture honey has antibacterial properties other than osmotic stress in the first culture, another contrast was used for statistical model [1], with 160 mg/mL glucose as a reference. A 160 mg/mL glucose solution and 260 mg/mL raw pasture honey had similar osmolalities (**Figure 2**), and their growth levels were not significantly different from each other in the first culture (OD_{600} = 0.0317 ± 0.0583, $p = 0.5900$). These results are consistent with **Table 3**: the inhibitory effects of sublethal concentrations of honey in the first culture did not differ significantly from glucose concentrations with similar osmolalities.

The first culture of bacteria into honey did not provide a comprehensive picture of the efficacy of either raw pasture honey or Manuka honey. To truly evaluate the efficacy of honey, the role of osmotic stress was quantified over five subsequent cultures.

Osmotic Stress of Honey has Nonsignificant Contribution on Bacterial Growth Beyond the First Culture

Similar procedures to the analysis for first culture were utilized to assess the role of osmotic strength in each treatment for five cultures. The same treatment-concentrations used in the first culture analysis were also used in the five-culture-day analysis.

Statistical model [1] was once again used with the data over all five culture days to verify that each treatment-concentration used in this experiment was in fact inhibitory. By

comparing the growth level of bacteria in honey and glucose to growth levels in the LB control, it was affirmed that each treatment-concentration studied was inhibitory. All bacterial cultures in this study were placed under stress, as indicated by their significantly lower growth levels, relative to the LB control (**Table 4**).

The role of osmotic strength in honey and glucose over five cultures was then quantified. Due to the change in the behavior of bacteria in response to further transfers into fresh honey-containing media, as shown in **Figure 3**, it was necessary to evaluate the efficacy of honey over several serial transfers. The role of osmotic stress in Manuka and raw pasture honey was quantified over five bacterial cultures using statistical model [2] with data now spanning five cultures. The results of this analysis, with glucose as the reference level, are summarized in **Table 5**.

The effect of osmolality had a nonsignificant impact on the growth levels of bacteria in honey over five culture days (slope of OD_{600} on osmolality = -0.0003 ± 0.0002, $p = 0.1259$). Both Manuka and raw pasture honey had significant negative estimates relative to glucose (Raw pasture honey OD_{600} = -0.4512 ± 0.1147, $p < 0.001$; Manuka honey OD_{600} = -0.3391 ± 0.1180, $p = 0.0047$), indicating that when osmotic stress was accounted for, the growth levels of bacteria in sublethal concentrations of both Manuka and raw pasture honey over five cultures were significantly lower than in glucose. Thus, Manuka and raw pasture honey exhibited antibacterial properties beyond osmotic stress when bacteria were serially transferred over five cultures.

The results of statistical model [2] with bacterial growth over five cultures were different from those of the first-culture. The first culture gave an insufficient representation of how bacteria responded to honey; concentrations which were sublethal in the first culture were actually lethal in later cultures. Due to the incomplete depiction of the efficacy of honey in the first culture, it is imperative to evaluate the antibacterial activity of honey over several serial transfers, as this study has done.

Treatment	Concentration (mg/mL)	OD_{600} Estimate	SE[b]	*p*	Significance[c]
Overall mean ($\hat{\mu}$)[a]	---	1.8287	0.1008	< 0.001	***
Day ($\hat{\alpha}$)[a]	---	-0.0165	0.0124	0.1839	
Glucose	160	-0.8566	0.1150	< 0.001	***
	60	-0.6679	0.1150	< 0.001	***
Raw pasture honey	140	-0.7174	0.1285	< 0.001	***
	160	-0.7440	0.1311	< 0.001	***
	180	-1.1101	0.1161	< 0.001	***
	210	-1.3631	0.1096	< 0.001	***
	260	-0.3879	0.1317	0.0037	**
Manuka honey	50	-0.4062	0.1082	< 0.001	***
	60	-1.0938	0.1295	< 0.001	***
	70	-1.2729	0.1123	< 0.001	***
	80	-0.7157	0.0502	< 0.001	***

a. Note: $\hat{\mu}$, $\hat{\alpha}$, and $\hat{\beta}$ are estimates for μ, α, and β, respectively, in statistical model [1].
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4. Estimates of cultures studied throughout the five cultures relative to an LB control strain grown under no stress. All strains studied were put under stress as shown by their significantly lower growth levels, relative to the LB control strain.

Effect	OD_{600} Estimate	SE[b]	*p*	Significance[c]
Overall mean ($\hat{\mu}$)[a]	1.1094	0.2631	< 0.001	***
Day ($\hat{\alpha}$)[a]	0.0553	0.0136	< 0.001	***
Raw pasture honey	-0.4594	0.1126	< 0.001	***
Manuka honey	-0.3414	0.1195	0.0049	**
Osmolality ($\hat{\beta}$)[a]	-0.0003	0.0002	0.1310	

a. Note: $\hat{\mu}$, $\hat{\alpha}$, and $\hat{\beta}$ are estimates for μ, α, and β, respectively, in statistical model [2].
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 5. The effect of treatment and osmolality on growth level of bacteria over five cultures, relative to glucose. Manuka and raw pasture honey exhibited significant antibacterial effects beyond osmotic stress over five cultures.

The antibacterial properties of Manuka honey over five cultures were confirmed by comparing the growth of bacteria in 80 mg/mL Manuka honey with bacterial growth in a glucose concentration of similar osmolality, 60 mg/mL glucose. While bacteria grown in 60 mg/mL glucose maintained stable growth throughout the five culture days, bacteria grown in 80 mg/mL Manuka honey did not show detectable growth after the first culture. This loss of viability in bacteria clearly suggests that Manuka honey exhibits antibacterial properties other than just osmotic stress. Although both 80 mg/mL Manuka honey and 60 mg/mL glucose had similar osmolalities, bacteria were only able to sustain stable growth in glucose, which confirms that sublethal concentrations of Manuka honey had antibacterial properties beyond osmotic stress (**Table 5**).

The antibacterial properties of Manuka honey were detected by using serial transfers. The first culture provided evidence to suggest that Manuka honey was only effective due to its high osmotic impact; however, further cultures revealed the antibacterial effects of Manuka honey. This further demonstrates the importance of using serial transfers when evaluating honey.

The antibacterial properties of raw pasture honey were verified next. The growth of bacteria in 260 mg/mL raw pasture honey was compared against glucose solution with a similar osmolality, 160 mg/mL glucose. Raw pasture honey at 260 mg/mL did not permit detectable growth beyond the first culture, whereas 160 mg/mL glucose permitted bacterial growth throughout all five culture days. Although both 260 mg/mL raw pasture honey and 160 mg/mL glucose had the same osmotic impact on the growth of bacteria, bacteria behaved substantially poorer in 260 mg/mL raw pasture honey, which suggests that raw pasture honey exhibited antibacterial properties other than causing osmotic stress.

DISCUSSION

The difference in the role of osmotic strength in honey in the first culture versus over five cultures suggests that the efficacy of honey cannot be evaluated without the use of several serial transfers. Bacteria behaved drastically differently upon further exposures into honey; thus, the first culture provides a deficient depiction of how bacteria behave in honey. For example, if the efficacy of the sublethal Manuka honey concentrations studied here had only been evaluated in the first culture, there would be flawed evidence to suggest that Manuka honey is only effective because of the high osmotic environment, which contradicts the literature (2, 3, 5, 8, 11, 12, 14).

Although several concentrations of Manuka and raw pasture honey were sublethal in the first culture of bacteria and seemed appropriate to use throughout the study, further serial transfers revealed a more comprehensive picture about the behavior of bacteria in response to honey. The loss of bacteria grown in concentrations which were sublethal in the first culture emphasizes the importance of utilizing serial transfers not only when selecting honey concentrations, but also when measuring the effectiveness of honey. Data from the initial 24-hour incubation of bacteria in a certain honey concentration provided insufficient information about the behavior of bacteria in that medium.

The results of this study indicate that honey has unique antibacterial properties beyond the osmotic impact in the first culture. Even when honey is administered at low concentrations that have no impact beyond causing osmotic stress in the first culture, bacterial populations seem to be transformed in future generations to make them more vulnerable upon further treatments with honey. The unique effects of sublethal concentrations of honey only became noticeable upon further transfers into honey, which further necessitates the use of serial transfers when evaluating the efficacy of honey.

It is important to emphasize that the analysis did not include lethal concentrations of honey (those that did not permit growth in the first cultures). Because only sublethal concentrations of honey were included, results cannot be generalized about the effectiveness of honey as an overall treatment in the first culture. The antimicrobial effects of lethal honey concentrations are attributable to factors beyond osmotic impact starting right in the first culture, e.g., Manuka concentrations of 90, 100, and 110 mg/mL (**Figure 1**).

Several authors have described the antibacterial properties of honey based only upon the first culture of bacteria. Osato, Reddy, and Graham sought to evaluate the efficacy of U.S.-produced honey (14). Similar to this study, the authors compared the growth of bacteria in honey to growth in a sugar solution and found no significant antibacterial properties in their U.S.-produced honey. This is consistent with the results of this study during the first culture. Since only the first culture of bacteria into honey was used, accurate conclusions regarding the antibacterial properties of honey cannot be made.

There are several other methods of evaluating the efficacy of honey which are employed in the literature; however, these methods did not account for the behavior of honey beyond the first culture. Perhaps the most common methods of evaluating honey involve determining the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentration (MBC) of honey. To determine the MIC, as described by Mohapatra, Thakur, and Brar, bacteria are cultured using the broth dilution method into several concentrations of honey growth medium; the lowest concentration showing no observable growth after the incubation period is considered the MIC (6). To determine the MBC, microorganisms from the test tubes that did not permit visible growth are streaked onto a sterile nutrient agar plate. After incubation of the plates, the lowest concentration that shows no growth is considered the MBC (6). A plethora of studies have used the MIC or MBC to determine the efficacy of different types of honey (2, 6, 15, 16). However, they did not consider the fact that bacterial response to honey varies over several serial exposures.

It is important to emphasize that the results of this study do not necessarily apply to all concentrations of Manuka and raw pasture honey. Only concentrations of honey which permitted growth in the first culture were utilized in data analysis. Lethal concentrations of honey, which did not permit growth in the first culture, were not included in the analysis. This study quantified the role of osmotic stress in the antibacterial properties of honey; it also emphasized the importance of serial transfers in evaluating honey as an antibacterial agent.

METHODS

The effect of osmotic stress in honey was statistically separated from the effect of other antibacterial compounds in honey. Osmotic strength was quantified in the first culture of bacteria into honey as well as over five serial cultures in sublethal honey concentrations.

Bacteria

The role of osmotic strength in honey-containing media on the growth level of bacteria was quantified twice throughout this experiment. First, the osmotic strength of honey was quantified in the first culture of Manuka and raw pasture honey. An amount of 100 µL of *S. aureus* bacterial broth was cultured into 5 mL of growth media containing sublethal concentrations of Manuka honey, raw pasture honey, and glucose, as well as an LB control. Three to five replicates of each treatment-concentration were prepared. The test tubes with inoculated growth media were placed in a VWR Incubating Orbital Shaker at 37°C and a speed of 200 RPM for 24 hours. To measure the growth levels in each strain and evaluate the efficacy of each treatment, a Varian Cary 50 Ultraviolet–Visible spectrophotometer (Agilent Technologies,

Santa Clara, CA) was utilized to measure the optical density of each culture at 600 nm, which is the standard wavelength used to measure bacterial cultures. The extent to which osmotic strength contributes to the inhibitory properties of Manuka and raw pasture honey in the first culture was quantified.

Treatments

Manuka honey, raw pasture honey, and glucose were utilized as the three treatments in this experiment. First, Manuka was Kiva certified with Unique Manuka Factor (UMF) of 20+. UMF rating is a measure of the valuable contents in Manuka honey which guarantees its quality and purity (17). This specific honey is medical-grade with a methylglyoxal (MGO) of 825+. Second, raw pasture honey was polyfloral honey harvested in Northeast Wisconsin. Neither of the honeys were purified nor heated so as to avoid damaging or deactivating their antibacterial properties. Heating honey has been shown to reduce its antibacterial properties (18). Third, glucose was utilized as an osmotic control. Glucose inhibits bacterial growth by creating a poor environment for bacteria (19, 20). Finally, bacteria were grown in a sterile LB growth medium with no antibacterial additives. This culture served as an LB-only negative control and represented maximum growth for each day of the experiment.

Concentrations of Manuka honey from 50–80 mg/mL, raw pasture honey from 140–260 mg/mL, and glucose from 0–400 mg/mL were prepared. Osmolality values for all concentrations were measured by a Wescor vapor pressure Osmometer.

Serial Transfers

Serial transfers of bacteria into honey and glucose took place over a total of six days. Viable bacterial strains from the first culture were transferred into fresh medium with the same treatment-concentration the following day. 100 µL of the previous day's growth were transferred into the same concentration of growth medium in a new, sterile test tube. This procedure was performed for a total of five sequential cultures. After each bacterial strain was incubated for 24 hours each day, the growth level was measured with the spectrophotometer. Throughout the experiment, concentrations which did not permit visible growth were discontinued and excluded from further serial transfers to the next day. After all growth measurements were taken, the impact of osmotic stress on the antibacterial properties of Manuka and raw pasture honey over five subsequent culture was quantified.

Statistical Analysis

The impact of osmotic stress on the antibacterial properties of honey in the first culture was quantified by comparing the growth levels of bacteria in honey with growth levels in glucose, an osmotic control. Concentrations of honey which were lethal at the first culture were excluded from data analysis; only concentrations of honey which showed growth in the first culture were used. Growth levels from Manuka honey concentrations of 50, 60, 70, and 80 mg/mL and raw pasture honey concentrations of 140, 160, 180, 210, and 260 mg/mL in the first culture were utilized to quantify the role of osmotic strength in honey. R statistical software (21) was used for data analysis.

Model [1] was fit to verify that each bacterial culture was placed under significant stress relative to the LB control. Model [1] equation is:

$$OD_{ij} = \mu + (TC)_i + \alpha D + e_{ij} \qquad [1]$$

where OD_{ij} is the j^{th} replicate of the optical density measurement associated with treatment-concentration i; μ is an overall mean for optical density across all treatment concentrations, $(TC)_i$ is the i^{th} treatment-concentration, which is one of LB or several Manuka honey, raw pasture honey, or glucose concentrations; D is the day on which the optical density measure was taken, α is the regression coefficient of OD_{ij} on D, and e_{ij} is the random residual component of the model. In this experiment, not all cultures of bacteria were grown on the same day; therefore, day was considered as a covariate in model [1] to adjust for random day differences.

To quantify the contribution of osmotic stress on inhibiting bacterial growth and to separate it from other antibacterial effects of honey, the osmolality of all honey concentrations was accounted for as a covariate in model [2]. The following model fits optical density against treatment, day, and osmolality:

$$OD_{ij} = \mu + T_i + \alpha D + \beta O + e_{ij} \qquad [2]$$

where OD_{ij} is the j^{th} replicate of the optical density measurement associated with treatment i; μ is an overall mean for optical densities across treatments; T_i is the i^{th} treatment which is one of glucose, Manuka honey, and raw pasture honey; D is the day on which the OD_{ij} measure was taken; O is the osmolality associated with T_i; α and β are regression coefficients of OD_{ij} on D and O, respectively; and e_{ij} is the random residual component of the model.

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