

Characterization of the Gain and Loss of Resistance to Antibiotics versus Tolerance to Honey as an Antimutagenic and Antimicrobial Medium in Extended-Time Serial Transfer Experiments

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ABSTRACT

Background: Honey contains several substances with antimicrobial properties that appear more recalcitrant to generating bacterial-evolved resistance than traditional antibiotics. **Objectives:** This study seeks to characterize the evolution of bacteria grown for successive generations in honey as an antimutagenic medium versus ampicillin.

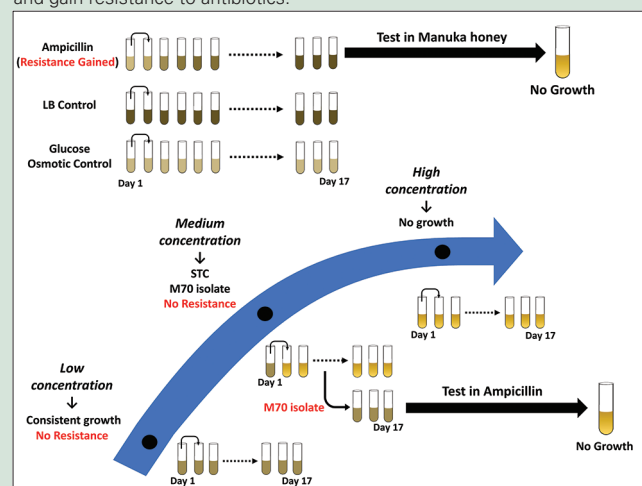
Materials and Methods: A naive strain of *Staphylococcus aureus* was serially cultured for 17 days in Lysogeny broth (LB) containing sublethal concentrations of medical-grade Manuka honey, polyfloral honey, and ampicillin. Glucose as an osmotic control and pure LB were included for comparison. A portion of each culture was removed every 24 h to (a) determine the amount of growth that occurred during the previous 24 h and (b) use as a genetic stock in serially transferred tubes containing the same inhibitory compound as used previously. **Results:** As indicated by an increase in growth over sequential 24-h period, bacteria rapidly gained resistance to ampicillin in a step-wise pattern. However, bacteria grown in Manuka and polyfloral honey never exceeded their initial growth levels. Bacteria grown in relatively high concentrations of honey for a single 24-h period consistently lost viability after one transfer, a phenomenon that has not been reported in the literature before and which indicates the inability of bacteria to adapt to honey as an antimutagenic medium. While bacteria grown in honey did not evolve the ability to grow at higher concentrations, a single isolate grown in Manuka honey gained tolerance to Manuka by successfully surviving and proliferating beyond second transfers. Bacteria that developed antibiotic resistance were found to remain sensitive to honey. Moreover, bacteria lost their resistance to ampicillin upon a single exposure to Manuka honey.

Key words: Antibiotic resistance, antimutagenicity, honey, Manuka, polyfloral, second transfer crash

SUMMARY

- Bacteria were serially cultured over hundreds of generations in inhibitory concentrations of antibiotics and honey. Adaptability of bacteria in antimutagenic media such as Manuka and polyfloral honeys versus mutagenic media such as amoxicillin was characterized with new findings reported. Statistical approaches to validate trends in survival fitness over multiple days were introduced. A new phenomenon, named second transfer crash (STC), in which

bacteria that survived first treatment of honey lost adaptability to subsequent treatments was discovered after repeatedly losing the strain in its second transfer culture in honey. STC was validated experimentally and explanations to interpret it were discussed. Cross-culturing challenge experiments validated the importance of substances such as honey with antimutagenic and anti-quorum-sensing properties in combating the ability of bacteria to adapt and gain resistance to antibiotics.



Abbreviations used: LB: Lysogeny broth; STC: Second transfer crash; OD: Optical density.

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INTRODUCTION

Through the process of mutation, adaptation, and natural selection, pathogenic bacteria often gain resistance to antimicrobial agents, threatening the treatment of many human health conditions. However, due to the speed at which micro-organisms are evolving resistance to antibiotics and the cost of the development of alternatives, many pharmaceutical companies are halting the development of new antibiotics or searching for new ways to combat pathogenic bacteria.^[1]

Although the medicinal benefits of honey have been documented in ancient civilizations, it has been researched in clinical and laboratory settings just recently. The antimicrobial properties of raw honey and its naturally derived components provide novel, effective antimicrobial

compounds that are less inclined to promote microbial resistant strains.^[1] Moreover, raw honey inhibits the growth of many bacteria,

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including *Salmonella*, *Shigella*, *Escherichia coli*, and *Helicobacter pylori* (*H. pylori*).^[1-5]

Along with the unfavorable environment, honey creates for bacteria, several phytochemical factors for antibacterial activity have been identified in honey.^[6] Manuka honey (*Leptospermum scoparium*), a monofloral honey from New Zealand, is highly effective at inhibiting the growth of pathogenic bacteria such as *Staphylococcus aureus* (*S. aureus*) and *H. pylori*, making it a potential treatment of wounds and stomach ulcers.^[2] This honey displays relatively potent and broad-spectrum antibacterial properties regardless of osmotic stress and sugar and hydrogen peroxide content and has proven effective even when diluted, thus demonstrating that it is not solely osmotic stress that inhibits microbial growth.^[3,7,8]

Studies have shown that Manuka honey is a complex antimutagenic medium with individual variation among other honeys in their degree of effectiveness.^[9,10] Manuka honey was found to possess strong antiproliferative mechanisms that prevent bacterial cells from cleaving their fully formed septa at the point of cell division. Henriques *et al.*^[11] reported that Manuka targets a site on the *S. aureus* genome involved in cell division.

As Gram-positive bacteria, *S. aureus* has thick cell walls composed of peptidoglycan, a mesh layer that preserves the shape of the cell and allows it to better endure intracellular pressures. Ampicillin is a β -lactam antibiotic that binds to and inactivates penicillin-binding proteins, which are enzymes involved in the synthesis of peptidoglycan in the periplasmic space, thus leading to the inhibition of cell wall synthesis and lysis of the cell. *S. aureus* gains resistance by modifying the target or destroying the bullet within the bullet-target framework.^[12] Bacteria may genetically alter their penicillin-binding proteins (modifying the target) to reduce affinity with β -lactam antibiotics or by gaining the genetic ability to produce β -lactamases which inactivate β -lactam antibiotics (destroying the bullet). Thus, the combination of ampicillin and *S. aureus* was appropriately chosen for this study. *S. aureus* may evolve to become methicillin-resistant *S. aureus* (MRSA) which is a multidrug-resistant strain associated with growing health concerns.^[13]

The objectives of the current study include the characterization of the evolutionary process of bacteria in their development and loss of resistance to antibiotics. A naive strain of *S. aureus* was repeatedly cultured in Lysogeny broth (LB) agar with four substances, ampicillin, Manuka honey, polyfloral honey, and glucose, for several days. Each culture was analyzed daily to quantify bacterial growth. In addition, an ampicillin-resistant strain was exposed to Manuka as an antimutagenic medium, and the impact on resistance was assessed. Finally, the ability of bacteria to adapt and gain tolerance to Manuka and polyfloral honey was investigated and bacteria that developed tolerance to Manuka honey were evaluated for their subsequent adaptive ability to ampicillin.

MATERIALS AND METHODS

This study spanned three experimental phases. In Phase I, sublethal concentrations of ampicillin, Manuka and polyfloral honey were determined based on initial pilot experiments of culturing naive bacteria, *S. aureus* subsp. *aureus* (ATCC 35553), in a range of concentrations over two incubation periods of 24 h and each. The 2-day trials were essential for honey concentrations that were found in this study to be sublethal to bacteria on first but lethal upon second exposure.

In the next phase, the same naive strain of bacteria was serially cultured for 17 days in LB agar containing the predetermined sublethal concentrations of ampicillin and honey with bacterial growth evaluated daily. In Phase II, sublethal concentrations of ampicillin at 1.49 μ g/mL,

Manuka at 60 and 70 mg/mL, polyfloral honey at 140 and 160 mg/mL, and glucose at 6% and 16% were utilized. Finally, to assess if resistance gained to antibiotics impacted sensitivity to honey or vice versa, a series of cross-challenge experiments were performed in experimental phase III by growing resistant and tolerant strains in solutions containing inhibitory concentrations of substances that they were not exposed to before.

Treatments

Three substances, with different antibacterial mechanisms, were used in this experiment: medical-grade Manuka honey, polyfloral honey, and ampicillin. Certified, Unique Manuka Factor (UMF) 20+, Manuka honey harvested in New Zealand was obtained. UMF is a measure for unique Manuka markers, mainly leptosperin, and is used to authenticate Manuka honey.^[14] A second type of honey, polyfloral honey with nectar sourced from an assortment of plants, was obtained and used in this experiment to compare its antibacterial properties against those of Manuka honey. This was honey harvested in Wisconsin, United States of America. To preserve their original antimicrobial properties, both polyfloral and Manuka honeys were not filtered or heated. As a control for the impact of osmolality on bacterial growth, glucose dissolved in LB was used at concentrations mimicking the highest sublethal honey osmolality. Finally, ampicillin was used in this experiment as the antibiotic to which bacteria were trained to gain resistance.

For Manuka, a stock solution of 350 mg/mL was first prepared by adding 0.443 g of Manuka to each 1 mL of LB. Since dissolving this weight of Manuka in LB increased each 1 mL of the mixture to 1.266 mL, the final stock solution had Manuka concentration of about 350 mg/mL (443 mg/1.266 mL = 349.9 mg/mL). Multiple media concentrations were then prepared from the same stock solution every day, for example, a 70 mg/mL growth medium was prepared every day by pipetting 1 mL of Manuka stock to 4 mL of sterile LB.

A stock solution of 632 mg/mL was prepared for polyfloral honey by mixing 0.959 g of polyfloral honey in each 1 mL of LB. After dissolving this amount of polyfloral honey in LB, each 1 mL of the mixture increased to 1.518 mL, creating a final solution with honey concentration of about 632 mg/mL (959 mg/1.518 mL = 631.8 mg/mL).

Osmolality of glucose concentrations from 0% to 40% was measured by using a Wescor Vapor Pressure Osmometer (Logan, UT). Specific values measured were 0%, 6%, 8%, 10%, 12%, 16%, 20%, and 40%. Osmolality of Manuka honey with concentration of 80 mg/mL and polyfloral honey with concentration of 260 mg/mL were also measured and their glucose equivalents within the 0%–40% range were determined.

Three to five replicates of 5 mL growth media were prepared daily for each treatment throughout the experiment. Inoculated test tubes were incubated in a VWR Incubating Orbital Shaker at 37°C and a speed of 200 RPM for 24 h. This procedure was consistently performed for 16 consecutive days with final set of measurements taken on day 17.

To measure the effectiveness of the treatment in each test tube, a Varian Cary 50 ultraviolet-visible spectrophotometer (Agilent Technologies, Santa Clara, CA) was utilized to measure the optical density (OD) of each culture after 24 h at 600 nm, which is the standard wavelength measurement used for bacterial cultures.

Degradation of incubated Manuka honey

It was observed in the current study that the highest sublethal concentrations of honey were lethal to bacterial cells whose ancestors were previously cultured in the same honey medium. In developing strains to tolerate Manuka at 70 mg/mL for multiple exposures, inoculated honey was incubated for longer periods, 48 instead of 24 h, to allow for

gradual degradation of honey and to give room for bacteria to adapt. To validate that honey partially degraded over the extended incubation period, sterile LB broth medium with Manuka honey at 70 mg/mL was incubated for 24 h at 37°C [Figure 1]. On the next day, incubated honey was inoculated from a strain previously exposed to Manuka at the same concentration and was incubated for a second 24-h period. In addition, fresh test tubes with the same Manuka medium were inoculated from the same strain and incubated for 24 h. Incubated bacteria in old honey grew in 24 h, whereas bacteria in fresh honey required 48 h of incubation to show growth. The same process was performed twice with multiple replicates in each trial. Figure 1 presents a schematic of each trial. These trials also validate the antiproliferative effects of honey as will be discussed later in the discussion section.

Cross-culture experiments

As will be explained in more detail in the results section, repeated culturing in experimental media resulted in an ampicillin-resistant strain and a Manuka-tolerant isolate (M70.iso) which tolerated multiple sequential transfers into Manuka at 70 mg/mL. To study if tolerance to Manuka transcended to resistance to ampicillin and to determine if the antibacterial properties of Manuka were as effective with antibiotic-resistant as with naive bacteria, cross-culturing experiments took place. Serially grown cultures in Manuka at 70 mg/mL and ampicillin at 1.49 µg/mL were subjected to a series of cross-challenge experiments by growing them in solutions containing inhibitory concentrations of substances that they were not previously exposed to.

In synchronization with the ongoing daily serial cultures, cross-culturing from the ampicillin-resistant strain, Manuka-tolerant isolate and LB control strain into Manuka medium at 70 mg/mL, and ampicillin at 1.49 µg/mL took place over a period of 3 days toward the end of the experiment. The LB control strain was cultured into Manuka and ampicillin as a baseline for all other cultures on the same day. Growth levels of all strains cross-cultured into Manuka and ampicillin were compared based on their optical densities after 24 h of incubation.

Exposure to honey and the loss of antibiotic resistance

Manuka honey was shown to have antimutagenic properties.^[9,10,15] Since reducing mutation rate impacts the ability of bacteria to adapt to adverse environments and gain antibiotic resistance, the impact of Manuka on ampicillin-resistant bacteria was tested. To test whether antibiotic-resistant bacteria lose resistance upon exposure to Manuka, resistant bacteria grown and transferred for several days in ampicillin were cultured in Manuka honey at 70 mg/mL and incubated for 24 h. After 24 h, bacteria from Manuka were cultured back into ampicillin at 1.49 µg/mL and incubated for a second 24-h period. The outcome was compared against ampicillin-resistant bacteria grown in ampicillin throughout the experiment [Figure 2].

RESULTS

Identification of selective conditions

To determine the appropriate conditions for the antibiotic-resistance and honey-tolerance selection experiments, the growth of *S. aureus* cultures was measured in LB solutions containing various concentrations of honey and ampicillin. Manuka concentrations between 50 and 110 mg/mL [Figure 3] and polyfloral honey concentrations between 140 and 260 mg/mL were tested [Figure 4] over two consecutive transfers to determine sublethal concentrations of Manuka and polyfloral honey that allowed repeated transfers. Glucose concentrations were chosen to

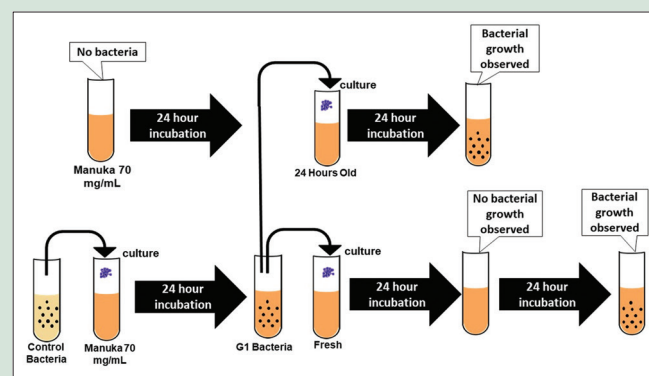


Figure 1: Experimental procedures to validate Manuka degradation over time and train bacteria to tolerate concentrations that were lethal upon the second exposure (70 mg/mL). G1 bacteria were a population exposed to Manuka only once. Only 24 h of incubation in 24-h-old Manuka was needed to observe growth, while 48 h of incubation was required to observe growth in fresh Manuka, validating partial degradation of honey during incubation. (Full page width)

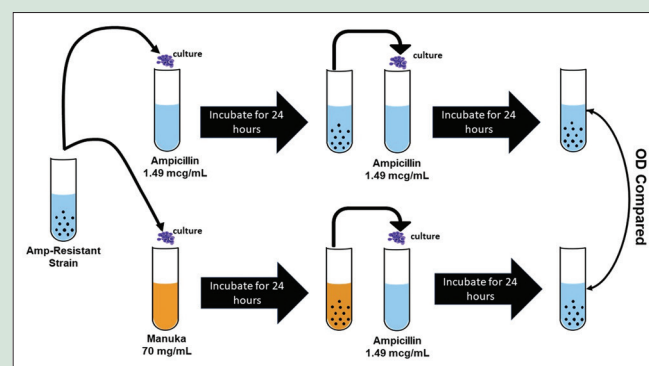


Figure 2: The effect of exposure to honey on ampicillin-resistant bacteria. An antibiotic-resistant strain was grown in Manuka honey for 24 h and then cultured back into ampicillin. Final growth level was compared to that of the original ampicillin-resistant strain. (Full page width)

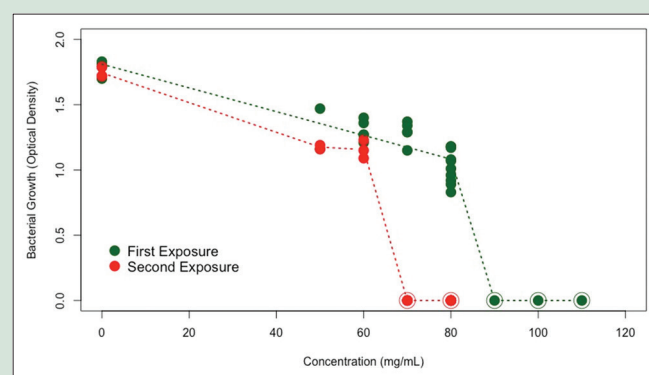


Figure 3: First two consecutive exposures to Manuka at concentrations of 50, 60, 70, and 80 mg/mL. For inhibitory concentrations 70 and 80 mg/mL, bacteria grew during the first 24 h of incubation, but no growth was observed in the second exposure after transfer and another incubation period of 24 h. Concentrations >80 mg/mL were lethal at the first exposure. Multiple losses were indicated by the outer circles at optical density of 0. (Column width)

mimic the osmolality of the highest viable honey concentrations. Glucose concentrations of 6% and 16% were found to contain similar osmotic strengths to Manuka and polyfloral honey solutions with concentrations of 80 mg/mL and 260 mg/mL, respectively.

Concentrations of Manuka and polyfloral honey solutions up to 80 mg/mL and 260 mg/mL, respectively, were found to be sublethal to the naive strain of *S. aureus*. However, progeny from cultures grown overnight in Manuka at 70 and 80 mg/mL and in polyfloral honey between 160 and 260 mg/mL were not viable when transferred and grown overnight in new solutions with identical concentrations [Figures 4 and 5]. Testing Manuka and polyfloral honey concentrations over two transfers were repeated several times on different days to confirm the second exposure loss. The normal growth of bacteria in their first culture into honey and their loss upon the second exposure is an indication of the inability of bacteria to adapt over multiple generations to honey as an antimutagenic medium, a phenomenon that was not reported in the literature before.

Further experiments confirmed that Manuka at or below 60 mg/mL and polyfloral honey at or below 160 mg/mL were capable of sustaining growth for repeated transfers. Manuka concentration of 60 mg/mL and polyfloral honey concentrations of 140 and 160 mg/mL were then utilized for honey-tolerance selection experiments.

Ampicillin concentrations from 0.75 to 1.49 µg/mL were tested over 3 distinct days with 2–5 tubes of LB control treatment included on every day. A slight noise was introduced by the day factor but was taken out by adjusting for day according to linear model equation (1) where OD_{ijk} is the OD measurement associated with concentration i on day j ; μ is an overall mean; C_i is the i^{th} concentration with $C_i \in \{0.0, 0.75, 1.0, 1.3, 1.4, 1.49\}$; D_j is the j^{th} day with $j = 1-3$ and e_{ijk} is the error term.

$$OD_{ijk} = \mu + C_i + D_j + e_{ijk} \quad (1)$$

All ampicillin concentrations were inhibitory as they allowed growth that was significantly lower than LB control. The highest ampicillin concentration of 1.49 µg/mL was chosen for the ampicillin-resistance experiment as it reduced bacterial growth by approximately 70% which gave room for bacteria to gain resistance by showing increased growth as the experiment progressed. After adjusting for the day, ampicillin concentration of 1.49 µg/mL was estimated to depress growth in the first 24 h by 1.266 below LB control ($0.121, P < 0.001$). For the sake of comparison, the lowest concentration of ampicillin, 0.75 µg/mL, inhibited growth by 0.532 ($\pm 0.14, P = 0.004$), after adjusting for the day.

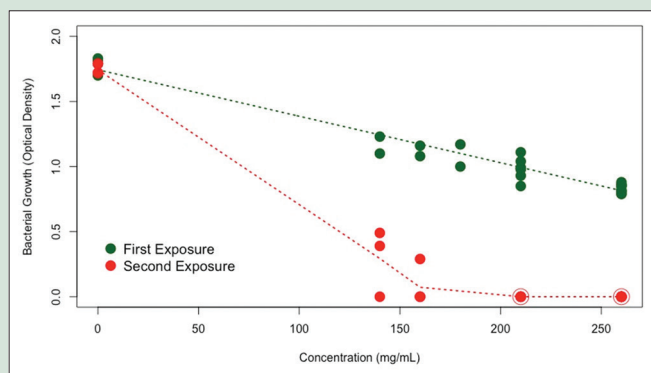


Figure 4: First two consecutive exposures to Lysogeny broth treated with polyfloral honey at concentrations of 140–260 mg/mL. For inhibitory concentrations 260 and 210 mg/mL, bacteria grew upon first exposure, but no growth was observed in the second exposure. Multiple second-exposure losses were indicated by the outer circles at optical density of 0. (Column width)

Evolution of bacteria grown in antimicrobial treatments

The evolutionary ability of bacteria to gain resistance to ampicillin and tolerance to honey was studied through serial culturing and transferring over 16 consecutive days. Results of each treatment are presented next.

Resistance gained to ampicillin

Bacteria evolved quickly to adapt to the medium treated with 1.49 µg/mL of ampicillin [Figure 5]. To assess daily growth, optical densities for ampicillin replicates were fit against day according to linear model equation (2) where OD_{ij} is the measurement of the j^{th} replicate on day i ; μ is an overall mean; and D_i is change in bacterial growth on the i^{th} day with $i = 1-17$.

$$OD_{ij} = \mu + D_i + e_{ij} \quad (2)$$

Bacteria doubled their level of growth in the first 4 days going from an average OD of 0.59 on day 1–1.21 on day 4 ($\pm 0.044, P < 0.001$). Stagnant change in bacterial growth then persisted for a few days before bacteria started to develop additional resistance to ampicillin by a second upward increase in their level of growth toward the end of the experiment. OD difference between day 1 and 17 was 0.66 ($\pm 0.051, P < 0.001$) with a potential for the step-wise trend of Figure 5 to continue until full resistance is attained.

Ampicillin time trend line in Figure 5 was based on a version of model (2) which expressed day as a third-degree polynomial, i.e.,

$$OD_{ij} = \mu + aD_i + bD_i^2 + cD_i^3 + e_{ij} \quad (3)$$

Terms in model equation (3) were defined as those in (2) but because day was fit as a numeric covariate in (3) as opposed to a class factor in (2), only four unknowns were estimated in (3), namely, the overall mean $\hat{\mu}$ and linear, quadratic and cubic components of day ($\hat{a}$, $\hat{b}$ and $\hat{c}$). Statistical analysis found the three components of day to be significantly different from 0, confirming the step-wise shape of the trend. Table 1 lists estimates, standard errors and P values for the overall mean and the time trend components that determined the shape of resistance to ampicillin. Based on the outcomes of models (2) and (3), ampicillin resistance of *S. aureus* appears to be acquired through both simple and complex loci, where complex loci required more time for mutation and adaptation.

Outcomes of sequential transfers in Manuka

As reported above, Manuka concentrations of 70 mg/mL consistently

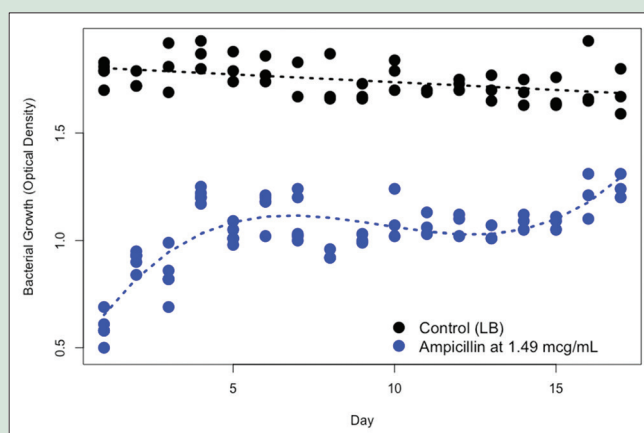


Figure 5: Daily measurements for optical density of bacteria sequentially cultured in ampicillin at 1.49 µg/mL. Dotted lines represent time trends observed over the experiment. Bacteria gained initial rapid resistance to ampicillin, followed by a period of no apparent loss or gain of resistance and in days 15–17 bacteria developed more resistance to ampicillin. (Column width)

inhibited the growth of naive *S. aureus* but yielded a viable population after 24 h of incubation. However, all second exposures to LB with fresh Manuka at the same concentration failed to grow after 24 h of incubation [Figure 3]. When the incubation period was extended, bacteria showed observable growth after 48 h because of partial degradation of Manuka as validated by procedures described in Figure 1. Bacteria then consistently showed observable growth every 48 h for multiple transfers (M70.48), until a more tolerant strain that developed the ability to reach observable growth in only 24 h was isolated (M70.iso). M70.iso was then defined as a Manuka-tolerant strain that reached levels of growth comparable to those of first exposure within 24 h of incubation after each transfer. Although the strain adapted gradually while attaining increased levels of growth for multiple transfers as Figure 6 shows, M70.iso never exceeded levels of growth of first exposure as was observed with the ampicillin-resistant strain.

To statistically characterize trends of bacterial growth in Manuka over the course of the experiment, the following model was fit.

$$OD_{ijk} = \mu + S_i + a_i D_{ij} + e_{ijk} \quad (4)$$

Where OD_{ijk} is the OD of strain i on day j for replicate k ; μ is an overall mean; S_i is the average growth of strain i ; D_{ij} is day j nested within strain i ; a_i is the slope of day within strain i ; and e_{ijk} is the error term.

Bacteria survived serial cultures in Manuka with a concentration of 60 mg/mL with no upward or downward trends throughout the experiment [Figure 6]. The slope of day within strain was not significantly different from 0 for bacteria grown in 60 mg/mL [Table 2], ruling out any development of honey resistance; where resistance is defined in the current experiment as elevated growth levels exceeding those attained by naive bacteria upon the first exposure to the same medium. Table 2 shows a positive slope estimate for day within M70.iso which was significantly >0 . The positive slope indicates gradual gain of tolerance to Manuka at 70 mg/mL.

Polyfloral honey time trends

Growth levels for bacteria grown in polyfloral honey at concentrations of 140 and 160 mg/mL are shown in Figure 7. A distinctive observation is the partial loss of viability upon second exposure to polyfloral honey followed by a recovery to the original level of growth of naive bacteria. Strain and day nested within strain were fit as explanatory variables against OD as the response variable. For the first 3 culture days, day as a second-degree polynomial was fit within strain to account for the quadratic component associated with the viability loss then recovery

occurring on day 2 and 3, respectively (5a). For days following the second exposure, day was fit within strain (5b).

$$OD_{ijk} = \mu + S_i + a_i D_{ij} + b_i D_{ij}^2 + e_{ijk}, \text{ if } j \leq 3 \quad (5a)$$

$$OD_{ijk} = \mu + S_i + a_i D_{ij} + e_{ijk}, \text{ if } j \geq 3 \quad (5b)$$

Terms in model equations (5a) and (5b) are defined similar to the terms of equation (4) and b_i is defined as the quadratic effect of day within strain i . As summarized in Table 3, statistical analysis showed that the two strains did not significantly differ from each other neither in (5a) nor in (5b). Only the quadratic components in (5a) were significant; linear components were not significant, validating that culture days 1 and 3 attained similar levels of growth. Finally, slope of day within strain in (5b) was not significantly different from 0, validating no gain in tolerance or development of resistance against these concentrations of polyfloral honey [Table 3].

Glucose time trends

To statistically characterize trends of bacterial growth in glucose over the course of the experiment, the following model was fit

Table 1: Linear, quadratic, and cubic components of time trend needed to determine the shape of resistance to ampicillin

	Estimate	SE	P	Significance*
Overall mean ($\hat{\mu}$)	0.4345	0.0597	<0.001	***
Trend components				
Linear ($\hat{a}$)	0.2459	0.0290	<0.001	***
Quadratic ($\hat{b}$)	-0.0281	0.0038	<0.001	***
Cubic ($\hat{c}$)	0.0010	0.0001	<0.001	***

The three trend components were all significantly different from 0, confirming the step-wise gain in ampicillin resistance observed in the current study. *The three stars indicate highly significant effects (near-zero P values). SE: Standard error

Table 2: Day trends of bacteria grown in Lysogeny broth with Manuka at 60 and 70 mg/mL

Trend*	Estimate	SE	P	Significance
M60	0.002	0.005	0.688	
M70.48	0.007	0.005	0.189	
M70.iso	0.092	0.022	<0.001	***

Time trend of M70.iso was positive and significantly different from 0 showing gradual adaptation to tolerate Manuka at 70 mg/mL. Time trend within other strains had 0 slopes. *Trend expressed as the slope of day within strain. SE: Standard error

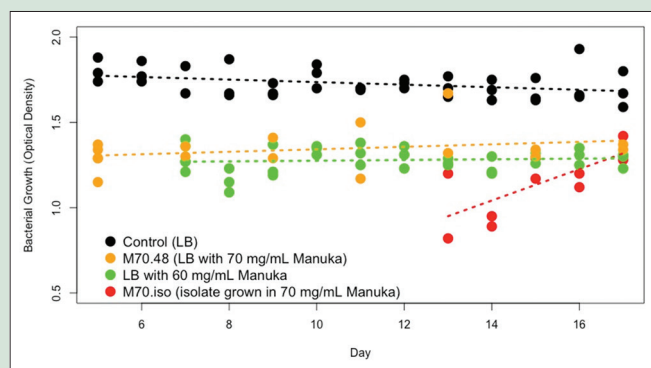


Figure 6: Daily optical density measurements of bacteria cultured in Lysogeny broth with 60 and 70 mg/mL Manuka. Dotted lines represent time trend within strain. The M70.48 and 60 mg/mL strains showed no significant upward or downward trend, but the M70.iso strain showed a positive linear slope. The M70.iso strain developed gradual tolerance to Manuka at 70 mg/mL. (Full page width)

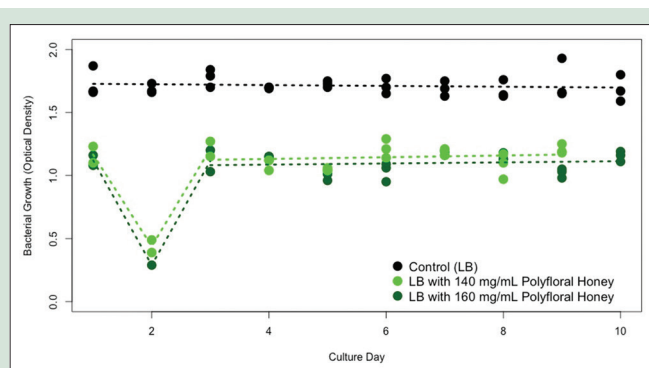


Figure 7: Daily optical density measurements of bacteria cultured in Lysogeny broth with 140 and 160 mg/mL of polyfloral honey. Dotted lines represent time trend within strain. Recovery from partial loss of viability on the second exposure to polyfloral honey was observed and zero slopes for growth across culture days 3–10 were estimated within the 140 and 160 mg/mL polyfloral honey strains. (Column width)

$$OD_{ijk} = \mu + C_i + a_i D_{ij} + e_{ijk} \quad (6)$$

where OD_{ijk} is the OD of strain i on day j for replicate k ; μ is an overall mean; C_i is the effect of concentration i on bacterial growth; D_{ij} is day j nested within concentration i ; a_i is the slope of day within concentration i ; and e_{ijk} is the error term.

Osmotic stress was effective in depressing bacterial growth. Glucose concentrations of 6% and 16% showed levels of growth that were nearly as half as this of control bacteria cultured in LB. Although the 16% glucose concentration was more impactful than the 6% concentration, the difference was relatively small. No time trend was observed for either concentration. Slope estimates of day within concentration were not significantly different from 0 as summarized in Table 4.

Note that osmotic stress was not as effective as honey with equivalent osmolality. Osmolality of the 6% glucose was equivalent to this of Manuka at a concentration of 80 mg/mL and osmolality of the 16% glucose was equivalent to this of polyfloral honey at a concentration of 260 mg/mL; both Manuka and polyfloral honey were lethal upon second exposure at the aforementioned concentrations.

A minor but non-significant loss of viability of the LB control strain was observed over the 17 days as indicated by the control time trend slope in Figures 6-8. However, none of days 2-17 was significantly different from day 1.

Outcomes of cross-culture experiments

Viability of resistant and tolerant strains in Manuka honey

Ampicillin-resistant and Manuka tolerant bacteria performed at an equivalent level to that of LB control bacteria when cultured in Manuka

honey at 70 mg/mL [Figure 8a], indicating the effectiveness of Manuka honey against ampicillin-resistant bacteria. It is important to emphasize here that second exposure to Manuka honey at 70 mg/mL results in loss of the strain or limited growth only after 48 h of incubation. The tolerant isolate, on the other hand, survived multiple cultures in Manuka at 70 mg/mL with successful growth every 24 h. The following model was fit to estimate and test differences in OD between tolerant and resistant strains relative to naive bacteria,

$$OD_{ijk} = \mu + S_i + D_j + e_{ijk} \quad (7)$$

where OD_{ijk} is the k^{th} replicate of OD measurement associated with strain i and day j ; μ is an overall mean; S_i is the i^{th} strain which is one of ampicillin-resistant, Manuka-tolerant, or LB control; D_j is the j^{th} day; and e_{ijk} is the error term. Cross-cultures were performed over the last 3 days and day was adjusted for as a class variable or factor.

Day-adjusted OD of the ampicillin-resistant bacteria relative to this of first exposure growth of the LB control strain was not significantly different from 0 [Figure 8]. The estimate for the difference was -0.015 ($P = 0.82$). Manuka-tolerant strain differed from control by an estimate of -0.075 , which was not significantly different from 0 ($P = 0.256$). The cross-culturing into Manuka honey was done over 3 distinct days and days 2 and 3 were not significantly different from day 1.

Viability of resistant and tolerant strains in ampicillin

Cross-cultures into ampicillin at 1.49 $\mu\text{g/mL}$ from LB control and Manuka-tolerant strains were evaluated. Cultures from ampicillin (i.e., ampicillin-resistant strain) on the same days were used for comparison. A model that accounted for source strain and day of culture was fit and included terms similar to those of model equation (7).

As shown in Figure 8b, ampicillin-resistant strain performed at a growth level significantly higher than that of the LB control strain by 0.389 ± 0.054 ($P < 0.001$). Manuka-tolerant strain showed no resistance to ampicillin. The Manuka isolate, M70.iso, and the LB control strain were not significantly different from each other when cultured in ampicillin at 1.49 $\mu\text{g/mL}$; relative to the LB control strain, the OD estimate of M70.iso was -0.010 ± 0.047 ($P = 0.832$).

Exposure to Manuka and loss of ampicillin resistance

Ampicillin-resistant bacteria lost its resistance when cultured in Manuka honey at 70 mg/mL for 24 h. Figure 9 shows that ampicillin-resistant bacteria previously cultured in Manuka for 24 h performed significantly

Table 3: Day trends within the 140 and 160 mg/mL polyfloral honey strains

	Estimate	SE	P	Significance
Culture days 1-3				
Polyfloral 160- polyfloral 140	-0.092	0.054	0.1478	
Linear components				
Polyfloral 140	0.064	0.124	0.6291	
Polyfloral 160	-0.007	0.124	0.9567	
Quadratic components				
Polyfloral 140	1.104	0.112	0.0002	***
Polyfloral 160	1.222	0.145	0.0004	***
Culture days ≥ 3				
Polyfloral 160- polyfloral 140	-0.035	0.086	0.6850	
Day within strain				
Polyfloral 140	0.007	0.010	0.4840	
Polyfloral 160	0.004	0.008	0.5900	

Strains had similar growth across all culture days with no upward or downward trends for growth. Only the quadratic components for days 1-3 were significant indicating loss of viability on the second culture day followed by quick recovery on the third exposure. SE: Standard error

Table 4: Daily trend and average optical density differences between bacteria cultured in Lysogeny Broth with 6% and 16% glucose

	Estimate	SE	P	Significance
Overall mean	0.901	0.033	<0.001	***
Glucose (6%-16%) ^a	0.190	0.047	<0.001	***
Trend of glucose 16% ^b	-0.002	0.004	0.606	
Trend of glucose 6% ^b	0.001	0.004	0.871	

The 6% glucose strain grew significantly higher than the 16% strain. No daily trends were found with glucose as indicated by the nonsignificant day within strain slopes. ^aAverage growth level relative to glucose 16%, ^bTrend expressed as the slope of day within glucose concentration. SE: Standard deviation

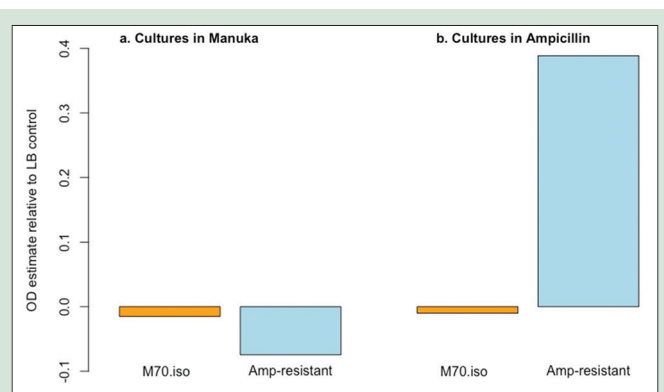


Figure 8: (a and b) Cultures of Manuka-tolerant isolate (M70.iso) and ampicillin-resistant strain in Manuka at 70 mg/mL and ampicillin at 1.49 $\mu\text{g/mL}$. Bars are day-adjusted estimates of optical density relative to the Lysogeny broth control strain cultured in the same medium. Manuka was effective in inhibiting the growth of ampicillin-resistant strain to levels similar to those of naive bacteria, and the Manuka-tolerant strain remained sensitive to ampicillin. (Column width)

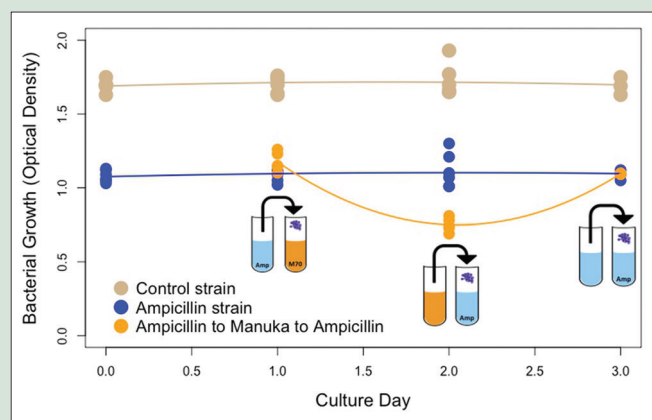


Figure 9: Antibiotic-resistant bacteria lost resistance to ampicillin when cultured in Manuka at 70 mg/mL for 24 h followed by a quick recovery of resistance after culturing into ampicillin. A single transfer of ampicillin-resistant bacteria into Manuka for 24 h lowered its resistance by 0.37. (Full page width)

poorer than the same bacteria cultured in ampicillin over the same period. On culture day 2 of Figure 9, average OD of bacteria sourced from Manuka into ampicillin was 0.37 (± 0.06) below ampicillin-resistant bacteria ($P < 0.001$). Performance of the LB control strain cultured in LB on the same days was also shown in Figure 9.

Antimutagenic properties of Manuka play an important role here as the honey reduces the expected rate of cell mutation after exposure. A resulting population of bacteria with lower mutation abilities is expected to be less capable of adapting to adverse environments, for example, antibiotics, than a population with higher rates of mutation.

DISCUSSION

Antimutagenic and antiproliferative properties of honey

Antimutagenic properties of honey were a major obstacle against bacteria to gain resistance to honey in the current study. Manuka honey has been shown to exhibit antimutagenic properties against bacteria.^[9,15,16] Ahmed and Othman^[15] found that Manuka honey was both antimutagenic and antiproliferative against cancer cells. They showed several mechanisms, such as the induced apoptosis and antimutagenesis, that Manuka honey employs to suppress mutation in eukaryotic cancer cells. It is important to note that although cancer cells are eukaryotic cells, it is well documented that the majority of carcinogens can also be identified using bacterial cells. McCann *et al.*^[16] tested 300 compounds and was able to identify 90% of carcinogens by testing their mutagenicity in the simple *Salmonella*/Ames test.

The current study showed that Manuka honey at 70 mg/mL slowed down cell division as opposed to killing bacteria. After the first exposure, bacteria required 48 instead of 24 h to reach observable growth. By extending incubation periods and allowing honey to sufficiently degrade, bacterial growth was eventually observed, which validates the antiproliferative properties of Manuka honey, documented in the literature.^[11,17,18] Henriques *et al.*^[11] found that cells treated with Manuka honey accumulated with completely formed septa at the point of cell division without dividing, suggesting that cells do not progress normally through the cell division cycle. They found that Manuka honey targeted a site on the *S. aureus* genome involved with cell division mechanism. Jenkins *et al.*^[18] reported that murein hydrolase, an enzyme produced to cleave the peptidoglycan in the cell wall at the end of the cell division, was undetectable in both cell-free and extracellular extracts of MRSA

treated with Manuka honey. This led to the failure of manuka-treated MRSA to carry out and complete the cell cycle.

Second transfer crash and anti-quorum sensing

Honey in general exhibits anti-quorum-sensing activities which may be independent of the nectar source.^[19] Quorum sensing is defined as the regulation of gene expression based on the density of bacteria in the surrounding environment.^[20] Jenkins *et al.*^[18] found a decreased level of transcription of three genes within the accessory gene regulator operon, which is a quorum sensing gene cluster that controls the production of a peptide which regulates the expression of virulence genes.

In evaluating concentrations of Manuka and polyfloral honey, bacteria were either unsuccessful to grow or had poor growth upon the second exposure to high concentrations of honey. This phenomenon, identified as second transfer crash (STC), could be attributed to anti-quorum-sensing activities of honey which causes bacteria to proliferate while completely unaware of the surrounding cell density. This results in the loss of cells with high division rates when density reaches critical levels toward the end of the first 24 h, leaving bacteria with lower rates of division which were also the most efficient to survive nutrient scarcity. Thus, at the end of the first 24 h, OD was high, mainly due to dead cells that had high division rates. On the second transfer, a minority live cells with low rates of division could not restore the population back to its first exposure OD level.

Honey has also been shown to impair division of bacterial cells, resulting in large undivided cells.^[11] Such large undivided cells would be more common for bacteria with higher division rates. These large undivided cells may contribute to OD in the first 24 h but will not contribute to population growth when transferred for a second 24-h period.

STC was observed in the current study with both Manuka and polyfloral honey, but it was more severe in Manuka which showed STC occurrences at much lower concentrations than polyfloral honey. The greater severity of STC in Manuka is attributed to its strong antimutagenic activity. It is already established in the literature that selection for mutator genes not only confers selective advantage against subsequent treatments of the same drug but also of other drugs.^[21] This also means that selection against mutator genes reverses the process and reduces bacterial ability to survive subsequent treatments. Manuka, as an antimutagenic medium, reduces the ability of surviving bacteria to mutate and adapt to stressful environments. Therefore, initial treatment failure with Manuka leaves bacteria with inferior mutation and adaptation capabilities which reduce their ability to survive subsequent treatments with the same concentration. Manuka not only prevented bacteria from developing adaptive mutations, but it also selected against mutator genes. Therefore, upon the second transfer, bacteria possessed neither enough adaptive mutations to survive fresh honey nor the mutation engine to generate them.

Manuka-tolerant strain

A Manuka-tolerant strain was isolated on day 12 [Figure 6]. This strain was able to show growth every 24 instead of 48 h, implying that tolerance to honey was gained without the need for degradation. The isolate was obviously successful in overcoming STC. However, the tolerance this strain gained was limited because its growth level never exceeded original growth level of M70.48 nor M60 strain. An increasing pattern of gaining resistance above original level of growth, such that observed with ampicillin, was not found here.

Cooper *et al.*^[22] reported a few strains of bacteria that had a reduced susceptibility in Manuka honey. They conducted three procedures of sequential exposure to sublethal concentrations of Manuka in continuous and step-wise training for short and long-term exposure.

They were unsuccessful in developing a strain of *S. aureus* that showed significant reduction in susceptibility to Manuka. Moreover, for four clinical isolates, the authors found significant decrease in susceptibility to Manuka over a period of 28-day resistance training, corroborating the findings of the current study.

Several studies have affirmed that developing resistant strains of bacteria to Manuka honey was near impossible due to quorum-sensing inhibition by Manuka.^[19,23,24] These studies found that sensitivity to Manuka and lack of biofilm formation was attributed to Manuka's anti-quorum-sensing activities. Note that quorum sensing is not essential for survival which means that strategies to inhibit it would reduce virulence while minimizing selection for resistance.^[19,23,24] Despite the evidence of tolerance provided by our study, resistance defined as enhanced growth above the first exposure levels was not found during resistance training under any concentration of Manuka or polyfloral honey.

CONCLUSION

This research highlights important new findings capitalizing on the impact of honey, as an antimutagenic, anti-quorum-sensing agent, on the development and loss of resistance to antibiotics. While the study was not pursuing the excellence of honey, compounds found in honey provide important insight into what strategies and tactics may be effective in limiting and eventually combating resistance. A phenomenon in which honey attacked the adaptive capability of bacteria was reported. The phenomenon was investigated after numerous second exposure losses with honey occurred. The phenomenon was not previously reported in the literature perhaps because the loss might look like naturally occurring random loss. This initial reporting should give impetus to further research and strategies to circumvent the ability of bacteria to develop antimicrobial resistance. In line with this finding, the outcomes of experiments in which exposure to honey resulted in the loss of antibiotic resistance were reported and discussed. Finally, although honey is known to be more recalcitrant to generating bacterial evolved resistance than traditional antibiotics, a strain that tolerated honey was isolated. An experimental strategy to train bacteria to tolerate honey was introduced.

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Conflicts of interest

There are no conflicts of interest.

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