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Growth Inhibition of *Escherichia coli* and *Staphylococcus epidermidis* from Various Types of Honey

Garrett Lemmen, Alyssa Hudson, Joe Rasmus

Williamston High School, Williamston, MI, USA
Email: galem25@hotmail.com, alyssahudson42408@gmail.com, mr.rasmus314@gmail.com

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Abstract

Honey has long been considered a wound treatment used to keep cuts and other epidermal injuries clean. This study tested that claim by comparing manuka honey used in medicine today, local unprocessed honey taken straight from a hive, and pasteurized honey found at a store, on strains of *E. coli* and *S. epidermidis*. The study evaluated the effects these honeys had on bacterial growth to determine which had the greatest inhibition of bacterial growth. To determine this, plates streaked with strains of *E. coli* or *S. epidermidis* bacteria and agar wells filled with one of the honeys were incubated and subsequently the diameter of the zone of inhibition was measured. After 20 trials using each honey and bacteria type, manuka and unprocessed were shown to have a statistically significant advantage over the pasteurized honey at inhibiting the growth of *E. coli* and *S. epidermidis*, though it was variable whether manuka had an advantage over the unprocessed honey.

Keywords

Honey, Inhibition, Natural Antibiotic, *S. epidermidis*, *E. coli*

1. Introduction

Since the 1940s, the number of drug-resistant bacteria has risen dramatically. When the first strain of penicillin-resistant *Staphylococcus aureus* was discovered, scientists began researching stronger antibiotics capable of killing this new strain of bacteria, and all others that followed. Unfortunately, with every new antibiotic discovered, the bacteria evolved to resist the drug, and with every new antibiotic, the time for the bacteria to develop resistance to it decreased. The time for *Staphylococcus aureus* to evolve into a phage to resist penicillin on a large scale took

nearly 15 years, but the time to resist methicillin on the same scale was only two years [1]. In an attempt to combat antibiotic resistance, scientists have worked to expand their horizons for new potential antibiotic bases. One of these bases is honey [2]. In more recent times, a type of manuka honey has been standardized for medical uses with gamma radiation and filtration to amplify its antimicrobial effects in a way that is replicable and consistent [3]. Our research sought to determine potential medicinal use of honey by comparing how effective different honeys are at killing bacteria commonly found in skin infections when applied topically. The honeys used in the experiment have multiple compounds that kill bacteria. With hydrogen peroxide, methylglyoxal, and bee defensin-1 all attacking different parts of the bacterial cell, making it difficult for the bacteria to grow and evolve to resist all factors of antimicrobial activity presently found in honey [4]-[7].

This *in vitro* study focused on the use of three different types of honey: unprocessed, pasteurized, and manuka honey, and their effectiveness as topical, antibacterial treatments for wounds [8]. Specifically, our research sought to determine how well these honeys work as antibiotics when applied directly to colonies of *Staphylococcus epidermidis* and *Escherichia coli* [8]. Our goal for this research was to open up the doors for standardized honey to be widely used as a wound cleaner and antibiotic.

In this study, the independent variables were the type of honey used in our experiment and the bacteria tested against the honey. The honey was applied to the petri dish in an agar well to mimic topical application on the skin. The dependent variable of our experiment was the measurements of inhibited bacterial growth by taking the diameter of the zone of inhibition (ZOI) from our manuka, unprocessed, and pasteurized honeys.

Before our experiment, we predicted manuka honey to display the largest diameter of inhibition against the bacteria because it contained the highest concentration of hydrogen peroxide, methylglyoxal, bee defensins. After manuka, we hypothesized the unprocessed honey to perform better, because it still contains all the natural compounds that kill bacteria. The pasteurized honey has many of the factors that contribute to honey's natural antibacterial properties removed during the pasteurization process, so we thought that this honey would perform the weakest.

2. Materials and Method

Our study tested the antibacterial efficacy of various honeys on strains of *Staphylococcus epidermidis* and *Escherichia coli*. In order to conduct the experiment, we acquired *E. coli* (Carolina Biological Supply Company), *S. epidermidis* (Carolina Biological Supply Company), manuka honey (Better Health Market), pasteurized honey (Meijer), and unprocessed honey straight from a hive [8]. Throughout our study, we had to obtain agar plates and syringes, but other materials, such as inoculating loops and candles, were previously supplied. Before any true trials

were conducted, we confirmed that both of the bacteria types were capable of growth and uncontaminated for our tests.

2.1. Process

E. coli and *S. epidermidis* were tested separately. Each trial was defined as a set of three petri dishes, with the first plate containing manuka honey, the next having unprocessed honey, and the third being split into pasteurized honey and negative control. Our process utilized a sterile inoculating loop streaking one of the bacterial species completely across all three plates. After the bacteria were inoculated in a petri dish, we used a 6 mm agar hole puncher to punch a circular hole into the agar, creating a well for the honey, in the center of each plate except for the pasteurized plate where a second hole was punched in for the negative control [9]. Then we measured 0.05 millimeters of honey, with one type of honey in each syringe, and filled their respective wells, with the last well remaining blank as a control. We repeated this procedure with both bacteria, producing six total plates inoculated with different bacteria species with honey filled wells in the agar. The plates were placed in the incubator and left to incubate at 37˚C. After the incubation period, visible colonies formed on the plate, creating a layer of bacterial growth across the plate. *E. coli* was incubated for 24 hours and *S. epidermidis*, for 48 hours. This was based on the amount of growth we observed after 24 hours. Looking at the *E. coli* trials, 24 hours covered the plate in enough bacteria to easily determine the diameter of the ZOI, but for *S. epidermidis*, that amount of time only resulted in a few small colonies that could not be used to measure a zone of inhibition.

2.2. Data Collection

For each trial completed, we measured the diameter of the zone of inhibition (ZOI) around each of the honey-filled wells with a digital caliper in millimeters [6] [10]. A greater ZOI indicated a stronger antibacterial effect that honey displayed against the bacteria. At the end of the study, the data was transferred from the spreadsheets into the Kruskal Wallis test. Running the test twice (one for each bacteria) compared the honeys to determine if there was any statistically significant group. Furthermore, the same data was then run through the Mann Whitney U test at $p < 0.05$ to determine which group of honey was statistically significant for each bacteria at inhibiting growth.

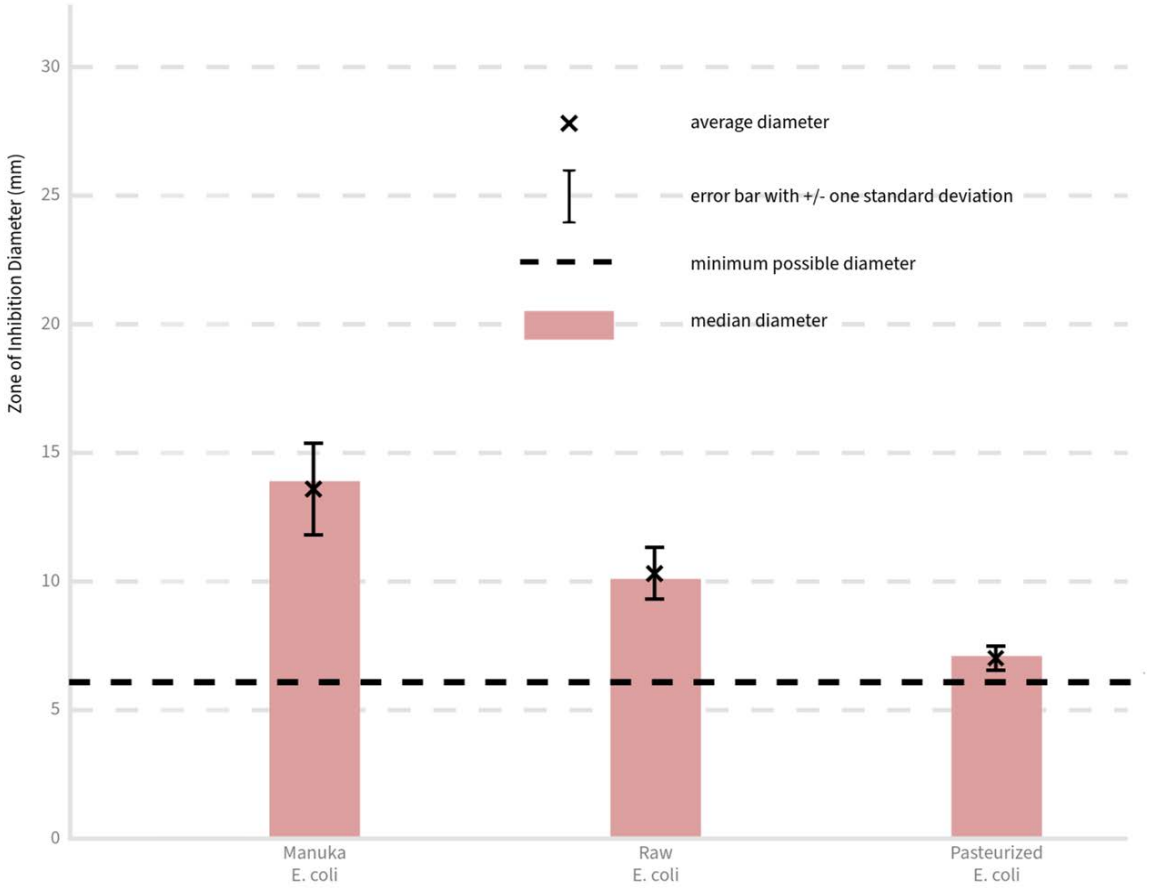
3. Results

Three varieties of honey were studied, measuring inhibition of *E. coli* and *S. epidermidis* colonies around agar wells. After incubating the plates for 24 and 48 hours respectively, and repeating the process 20 times, we graphed the mean, median, and standard deviation diameters of the ZOI, shown in **Figure 1** and **Figure 2**.

The minimum diameter of the zone of inhibition is 6mm, signified by the

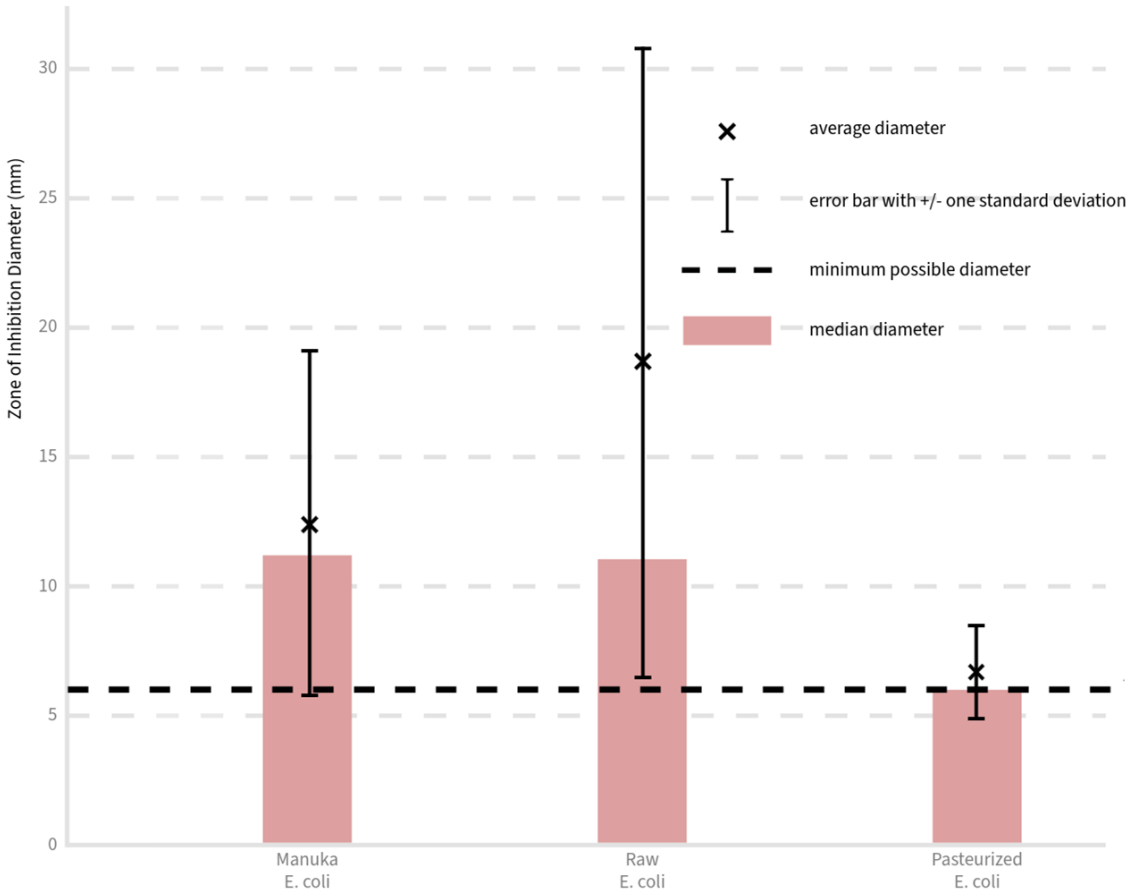
dashed line. The means calculated varied between the types of honey. Manuka honey had means of 13.625 and 12.605, unprocessed honey's average means were 10.34 and 18.955, and pasteurized honey had means of 6.97 and 6.755 for *E. coli* and *S. epidermidis,* respectively. However, 75% of the data was non-normal, so the data was calculated primarily with the median to avoid outliers that would otherwise affect the mean. Against *E. coli,* the median for manuka honey was 13.9 mm, for unprocessed honey, it was 10.1 mm, and for pasteurized honey, it was 7.1 mm. Against *S. epidermidis*, the median for manuka honey was 11.2 mm, for unprocessed honey, it was 11.05 mm, and for pasteurized honey, it was 6 mm.

After computing the data above, we calculated the standard deviation of the diameters of ZOIs. The results found from data against *E. coli* were very consistent, and therefore resulted in small standard deviations of 1.827 mm, 1.053 mm, and 0.495 mm for manuka, unprocessed, and pasteurized, respectively. The results from data against *S. epidermidis* were erratic, resulting in standard deviations of 6.775 mm, 12.401 mm, and 1.825 mm for manuka, unprocessed, and pasteurized, respectively.



Displays bars with median and mean ± standard deviation for each honey and minimum inhibition diameter. Colonies of bacteria were grown on petri dishes with honey-filled agar wells. After incubation of 24 hours, diameter of the ZOI was measured in millimeters.

Figure 1. Error bars of manuka, unprocessed, and pasteurized honey against *E. coli*.



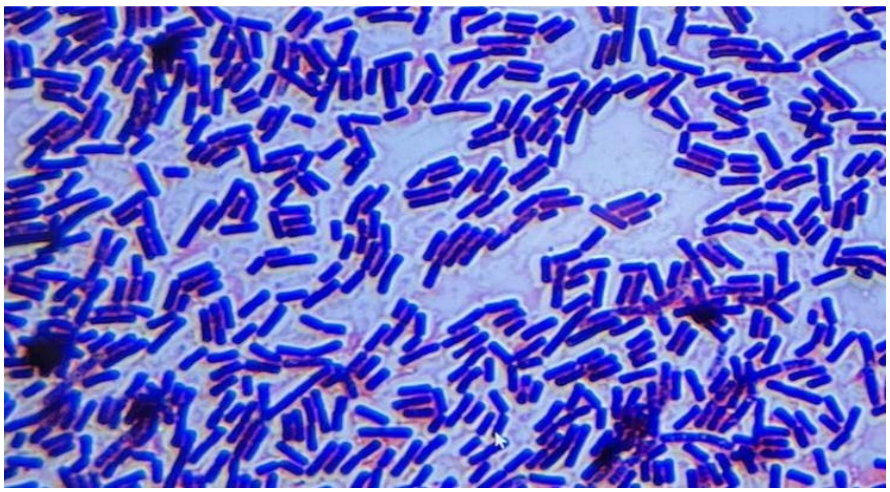
Displays bars with median and mean ± standard deviation for each honey and minimum inhibition diameter. Colonies of bacteria were grown on petri dishes with honey-filled agar wells. After incubation of 48 hours, diameter of the ZOI was measured in millimeters.

Figure 2. Error bars of manuka, unprocessed, and pasteurized honey against *S. epidermidis*.

The results of the Kruskal Wallis significance test supported the alternative hypothesis by suggesting that at least one group in each bacterial set was statistically significant ($p < 0.00001$). We subsequently performed a post hoc Mann-Whitney U test to determine which type of honey was statistically significant. Looking at the results from the *E. coli* testing, when compared to pasteurized honey, manuka honey had a p-value < 0.00001, giving it a statistically significant advantage over the pasteurized honey, and this remained true when the pasteurized honey was compared to the unprocessed also showing a p-value < 0.00001. Compared to each other, the manuka honey proved to be statistically distinct from unprocessed honey with a p-value < 0.00001. When comparing the honeys to each other with data collected from the *S. epidermidis* trials, the manuka and unprocessed honeys once again were shown to be statistically significant from the pasteurized, where $p < 0.00001$ again. However, when manuka and unprocessed honey were compared to each other, the U-value came back as 159.5; the maximum U-value accepted at $p < 0.05$ to show statistical significance was 138, and $p = 0.14007$, so the results were insignificant.

4. Discussion and Conclusions

The results of the Kruskal Wallis and Mann Whitney tests confirmed that manuka honey was the most effective honey at inhibiting *E. coli*. However, when tested against S. epidermidis, the results of manuka and unprocessed honey were unable to conclude statistical significance, displaying no clear advantages between the two. Our results displayed that the old belief that honey will clean infections is only partly true. While the results of manuka and unprocessed honey strongly suggest that they could be used as a household topical antibiotic, the saying often refers to the bear-shaped jars of honey. Based on our research, this honey proved to be ineffective at inhibiting bacteria, and there were also multiple trials where the pasteurized honey data was deemed invalid due to contamination that could have arisen from natural sources before collection or issues in quality control during the pasteurization and packing process [11]. Gram stains were the only method available to attempt to identify the contamination, and multiple stains revealed that the bacteria was a gram positive, rod shaped bacteria. To try and get an exact match, we contacted Dr. Jason Bazil at Michigan State University to see if he had any insight on how to sequence the sample. He suggested we look for a reasonably priced sequencing company, but due to a slow response from the company, and a lack of time in the lab, we were unable to conclusively determine the species. However, we suspect that the bacteria is *Clostridium botulinum*, a bacteria found in honey and the pathogen that causes botulism (see **Figure 3**) [3].

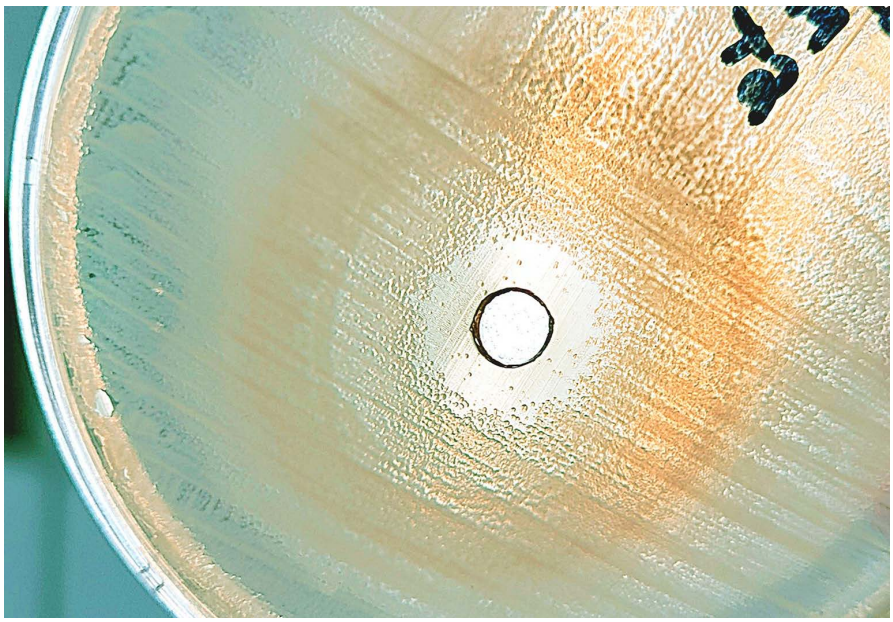


Gram stain of unknown, gram positive, rod bacteria under microscope. Gram stain was done on a contamination bacteria found in pasteurized honey, magnified at 200×.

Figure 3. Gram stain of suspected *Clostridium botulinum.*

This study was primarily limited by time and budget. Since there were only 16 weeks allotted to this research study, we did not have the time to test our initial thought. The original idea for the study was to test the honey and identify the modes of action each variety used to kill bacteria. We also had problems with materials. The unprocessed honey we used in the study came from a family farm, so

managing to obtain an exact replica of the honey would be near impossible. Three components we knew could be found in honey were hydrogen peroxide, methylglyoxal, and bee-defensin 1, but with limited technology, we had no methods to neutralize or conduct any experimentation with them [4]-[7]. Given more time and money, it is possible that this could be tested by analyzing the cause of cell death–whether it is a damaged septal ring, interrupted cell division, intracellular leakage, or interruption of peptidoglycan synthesis–after a trial with a powerful microscope, and then identifying the compound associated with that form of lysis [12]. Further research could also be conducted on an area beyond the zone of inhibition we called the "zone of facilitation" where there was an abundance of bacterial growth that outnumbered what was observed elsewhere on the plates (see **Figure 4**). We suspect this comes from a dilution of the honey to the extent that only sugars remain, providing extra food for the bacteria. We hypothesize that by analyzing the sugar content of each honey, it would be possible to compare it to the level of sugar in the zone of facilitation to determine the amount of concentration diffusion that occurred.



A petri dish with a prominent orange "zone of facilitation" surrounding the ZOI. After incubation, our petri dishes displayed a zone of inhibition, as well as a zone of facilitation (increased bacterial growth).

Figure 4. Zone of facilitation on petri dish of *E. coli*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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