Journal of
Emerging Investigators

Fall and Spring Honeys are Equally Effective at Reducing Growth Numbers of *E. coli, S. aureus, P. aeruginosa*, and *S. epidermidis*

Liam Gilligan[1], Veronica Arseneault[1], Yaa Konama Pokuaa[1], Kathleen Mungai[1], Stephanie Wallace[1], Katelyn St. Louis[1], Lauren O'Keefe[1], Keroline Santos[1], Jahnvi Patel[1], Kruti Patel[1], Pooja Patel[1], Kimberly A. Gonzalez[2]

[1]Lowell High School, Lowell, MA

[2]Middlesex Community College, Bedford, MA

Summary

An anticipated lack of effective antibiotics available for treating bacterial diseases has led scientists toward researching alternative therapeutics, such as honey. The impact honey has on bacterial growth can be credited to its catalase activity, acidic pH, as well as the sugar and hydrogen peroxide content, but these attributes may vary depending on whether the honey was produced in the spring or the fall season. In this study, locally produced fall and spring honeys were tested to determine whether there was a significant difference in their abilities to limit or prevent bacterial growth of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Staphylococcus epidermidis*. ANOVA analyses showed that the growth of all organisms was significantly decreased as the concentration of honey was increased, yet comparisons show there was no statistically significant difference between fall or spring honey in terms of their effectiveness in limiting bacterial growth.

Received: December 5, 2016; **Accepted:** August 3, 2017 ; **Published:** October 17, 2017

Introduction

The increase of antibiotic-resistant microbes has led to a re-evaluation of the therapeutic use of ancient medicinal remedies such as plant roots, bark, and honey as treatments for bacterial infection (1). Molecules found within these substances are thought to have a broad spectrum of antimicrobial activity against a variety of antibiotic resistant bacteria. Honey has been one of the most widely investigated compounds as an antimicrobial agent due to its ability to increase the body's healing processes (2). Studies have shown that honey speeds up wound healing, as well as decreases the duration of infectious bacterial diarrheal diseases. Some of the properties that make honey an effective antimicrobial agent are its high osmolarity, its acidic pH range, its catalase activity, and its hydrogen peroxide content (5). Catalase content in honey is derived from flower pollen, whereas hydrogen peroxide presence is due to the activity of the enzyme glucose oxidase (3). The presence of catalase and the production of hydrogen peroxide are dependent on weather, season, type of bee pollinating the flower, and flower type. Furthermore, the levels of both catalase and hydrogen peroxide are dependent on one another; low levels of catalase in honey translate into higher levels of hydrogen peroxide, and vice versa. Since honeys can vary in their antimicrobial compounds, this leads to the question of whether there are antimicrobial differences between fall and spring honeys. In terms of overall appearance, fall honeys are darker in color than spring honeys. This can be attributed to differences in the vegetation used by bees for honey production, producing differences in polyphenol, flavonoid, and carotenoid content within the honey (4). Antimicrobial activity of both flavonoids and polyphenols can be credited to their antioxidative properties (7). Polyphenols, including flavonoids, are phenolic compounds that contain one carbonyl group as well as varying levels of hydroxylation (16). Studies investigating the antimicrobial activity of polyphenols have produced mixed results as to whether or not hydroxylation is responsible for the antimicrobial activity of polyphenols and flavonoids, but it is thought that the more lipophilic (less hydroxylated) the phenolic compound is, the more likely it is to target the bacterial membrane resulting in destruction of its structure. Furthermore, polyphenols also exhibit antimicrobial activity due to inhibition of hydrolytic enzymes, which prevent antimicrobial adhesion to cells (6). Studies using phenols extracted from berries have been shown to prevent attachment of organisms such as *Escherichia coli, Helicobacter pylori,* and *Streptococcus mutans* to human epithelial tissue (14). Polyphenols extracted from cranberry juice have been shown to inhibit biofilm formation in *Porphyromonas gingivalis* and *Fusobacterium nucleatum* via interference with Arg-gingipain and Lys-gingipain extracellular cysteine proteases (17). Studies of how polyphenols work in honey have produced mixed results (8).

In this study, we aimed to explore whether there were antimicrobial differences between locally produced (Shaw Farm, Dracut, MA) fall and spring honeys. These honeys are collected from beehives located within 150 acres of land, which contain a variety of wild flowers and vegetation depending on the season (15). Beekeepers collect, store, and distribute the honey as a raw product, meaning it is not processed or heated prior to distribution. We tested the fall and spring honeys against the growth of *Escherichia coli, Staphylococcus aureus, Pseudomonas*

aeruginosa, and *Staphylococcus epidermidis* on nutrient agar and honey-nutrient agar containing 5%-20% honey concentration. These organisms were chosen so that a range of pathogens versus commensals, as well as a range of susceptibility patterns could be examined. The results showed that increased honey concentrations did exhibit antimicrobial activity against all of the tested organisms; however, there was no statistical difference between the fall versus spring honey in terms of antimicrobial activity.

Results

In order to test whether fall and spring honeys have antimicrobial differences against *E. coli, S. aureus, P. aeruginosa,* and *S. epidermidis*, these organisms were grown on nutrient agar and honey-nutrient agar plates containing different concentrations of fall or spring honey. Provided in **Table 1** are the descriptive statistics for *E. coli, S. aureus, P. aeruginosa*, and *S. epidermidis* after being spot-titered onto the different agars. As shown in **Figure 1**, the mean, along with the standard error, and sample variance decreases as the concentration of honey increases, showing little to no growth at a concentration of 20% fall or spring honey. The data in **Table 1** suggests that there is large sample variance. Levene's test for equality of variance was performed to determine whether sample variances were equivalent at α = 0.05. For *E. coli,* Levene's produced p = 0.0002, indicating that the variance was significantly different. Levene's test produced p = 0.0002 for *S. aureus*, p = 0.007 for *P. aeruginosa*, and p = 0.0002 for *S. epidermidis*. Since the variances were different for all tests, a Kruskal-Wallis analysis of variance (ANOVA) test was used to determine whether there was a statistically significant decrease in bacterial growth with increasing honey concentration. For *E. coli*, the Kruskal-Wallis ANOVA produced p= 0.0047, with α = 0.05. Since p < 0.05, the data support a significant decrease in the growth of *E. coli* on increased concentrations of honey agar. Kruskal Wallis ANOVA testing produced p = 0.007 for *S. aureus*, p = 0.02 for *P. aeruginosa*, and p = 0.03 for *S. epidermidis*, respectively. **Figure 1** shows the decrease in E. coli growth for each at increasing concentrations of honey (top), as well as the data quantification with the standard error (bottom). Similar growth patterns were observed for all of the tested organisms (**Figures 2-4**).

Organism	Nutrient agar	5% Honey nutrient agar Spring	5% Honey nutrient agar Fall	10% Honey nutrient agar Spring	10% Honey nutrient agar Fall	20% Honey nutrient agar Spring	20% Honey nutrient agar Fall
E. coli mean CFU/mL	2.19E+09	1.67E+09	1.14E+09	1.18E+08	1.69E+08	8.67E+04	0.00E+00
Standard error	2.06E+09	1.52E+09	9.35E+08	1.06E+08	1.35E+08	8.67E+04	0.00E+00
Sample variance	1.27E+19	6.89E+18	2.62E+18	3.38E+16	5.50E+16	2.25E+10	0.00E+00
S. aureus mean CFU/mL	1.40E+09	1.46E+09	1.77E+09	1.52E+06	1.63E+06	0.00E+00	0.00E+00
Standard error	1.35E+09	1.42E+09	1.71E+09	1.29E+06	1.59E+06	0.00E+00	0.00E+00
Sample variance	5.47E+18	6.07E+18	8.81E+18	5.02E+12	7.54E+12	0.00E+00	0.00E+00
P. aeruginosa mean CFU/mL	6.87E+08	9.07E+08	7.27E+08	2.90E+08	7.50E+07	2.35E+05	3.33E-01
Standard error	9.87E+07	3.45E+08	4.05E+08	7.94E+07	3.52E+07	2.13E+05	3.33E-01
Sample variance	2.92E+16	3.56E+17	4.92E+17	1.89E+16	3.72E+15	1.36E+11	3.33E-01
S. epidermidis mean CFU/mL	8.00E+07	1.49E+08	7.57E+07	1.40E+08	4.90E+07	0.00E+00	0.00E+00
Standard error	5.20E+06	9.58E+07	2.52E+07	8.53E+07	1.15E+07	0.00E+00	0.00E+00
Sample variance	8.10E+13	2.75E+16	1.90E+15	2.18E+16	3.97E+14	0.00E+00	0.00E+00

Table 1. Descriptive statistics for the organisms tested. The data show the mean CFUs/mL, standard error (CFU/mL), and sample variance (CFU/mL)^2 for *E. coli, S. aureus, P. aeruginosa*, and *S. epidermidis*.

To determine if there was an antimicrobial difference between fall versus spring honey, a one-tailed unequal variance t-test was performed comparing the growth of each organism on 5% fall honey nutrient agar versus 5% spring honey nutrient, 10% fall versus spring honey agar, and 20% fall versus spring honey agar. The results of the one-tailed unequal variance t-tests for all organisms tested produced p > 0.05, with α = 0.05,

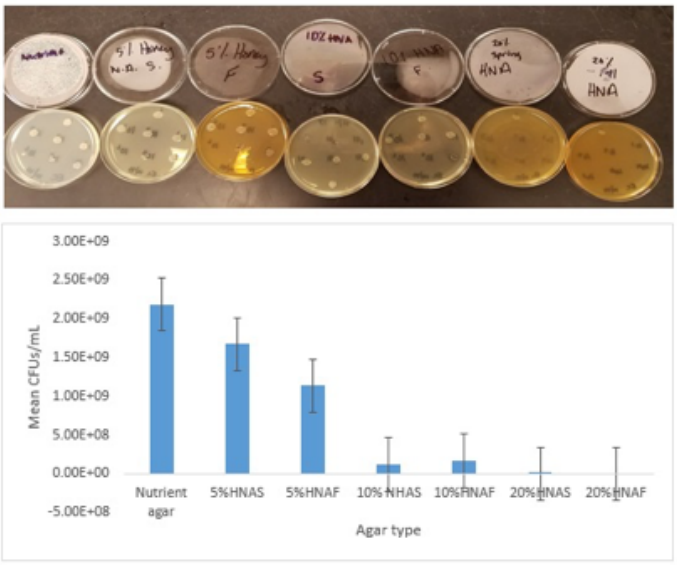

Figure 1: *E. coli* growth on different honey agar concentrations. The top of the image shows *E. coli* growth on nutrient agar (far left) and the honey agars in increasing concentration from left to right (5%-20%). The lighter-color plates contain spring honey whereas the darker-color plates contain fall honey. In the bottom image, error bars indicate standard error of the mean. "HNAS" indicates Honey-nutrient agar spring; "HNAF" indicated Honey-nutrient agar fall.

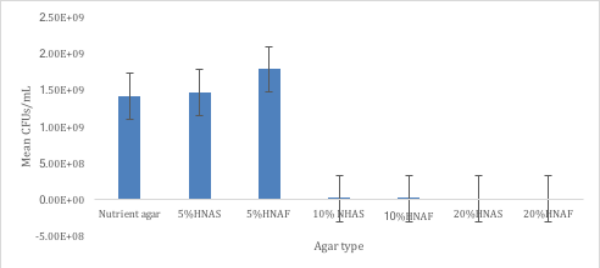

Figure 2: *S. aureus* growth on different honey agar concentrations.

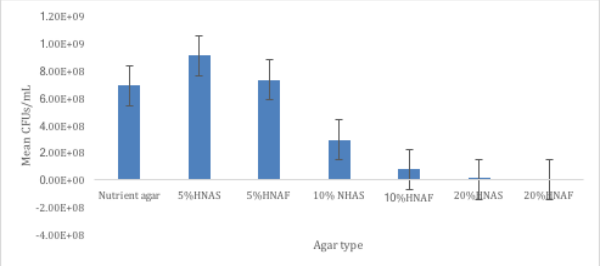

Figure 3: *P. aeruginosa* growth on different honey agar concentrations.

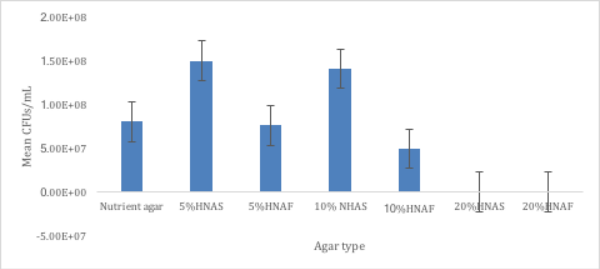

Figure 4: *S. epidermidis* growth on different honey agar concentrations.

meaning there was no statistically significant difference in antimicrobial activity between fall versus spring honeys at the concentrations tested (**Table 2**). **Figures 5 and 6** show the reduction in mean CFUs/mL with increasing concentration, from 5-20% spring and fall honey, respectively.

	5% Honey-agar one-tailed unequal variance T-test	10% Honey-agar one-tailed unequal variance T-test	20% Honey-agar one-tailed unequal variance T-test
E. coli	p = 0.39	p = 0.39	p = 0.21
S. epidermidis	p = 0.26	p = 0.20	No growth
S. aureus	p = 0.45	p = 0.48	No growth
P. aeruginosa	p = 0.13	p = 0.05	p = 0.19

Table 2. One-Tailed Unequal Variance t-test.

Discussion

These results suggest that increased honey concentrations decrease bacterial growth, which supports previous findings from other studies (5, 9); however, the data also shows no statistically significant difference in antimicrobial activity between fall and spring honeys. The similar outcomes for fall and spring

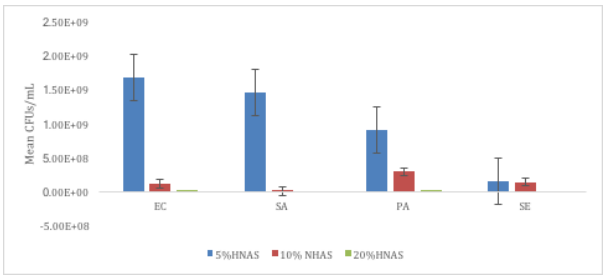

Figure 5: Mean CFUs/mL of bacterial growth on spring honey-nutrient agars, along with the standard error.

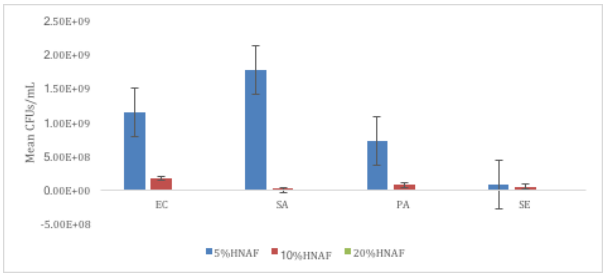

Figure 6: Mean CFUs/mL of bacterial growth on fall honey-nutrient agars, along with the standard error.

honey may be due to a lack of seasonal influence on the product. A study by Requier *et al*. showed that bees undergo bimodal seasonal honey production and that their diet consists of pollens and nectars harvested from local flowers, trees, and weeds (10). The diversity of vegetation used in honey production by the bees may explain why there is little antimicrobial difference between fall and spring honey, perhaps because higher levels of nectar from weeds and trees were used during honey production, with less reliance on local, seasonal flowers that might influence the antimicrobial characteristics of the honey. This is an area of future exploration that warrants investigation.

Upon further examination of the data, it does not appear that honey is an effective antimicrobial agent until it reaches a concentration of at least 20%. While growth did appear to decrease as honey concentration was increased, it was not until 20% honey that no growth was observed. This indicates that at this concentration the sugars, hydrogen peroxide, and catalase are at high enough levels to be effective at inhibiting bacterial growth. Sugars create a hypertonic environment for bacteria, which can be detrimental to their growth (11), whereas hydrogen peroxide and catalase induce oxidative stress to bacteria, as previously indicated (7). Future experiments will examine whether it is the high sugar concentration, catalase activity, or a combination of both that is responsible for the inhibition of bacterial growth. This can be done by comparing honey agar to sugar agar, and by using filtration to purify the honey so as to eliminate heating the honey, which may disrupt the catalase activity.

While this study shows that there is no statistically significant antimicrobial difference between fall and spring honeys, there are limitations to this work. One

limitation was the very small sample size of the study. Only three trials were performed for each organism at each honey concentration. While unlikely, a larger sample size may provide different results. Another limitation is the sample variance present within the data, which can be attributed to the fact that the McFarland standard used to determine the growth numbers was based on visual observation and is therefore inherently subjective (12). Furthermore, since bacteria have different growth rates, the bacterial numbers in each thioglycolate broth may be different depending on the organism. Both of these limitations could be corrected for by using optical density to determine cell numbers in the inoculum post incubation (13).

An interesting observation that warrants future investigation was noticed while examining the growth of *E. coli* and *P. aeruginosa* on the 20% honey agars when compared to *S. aureus* and *S. epidermidis*. Both *E. coli* and *P. aeruginosa* exhibited small amounts of growth at this concentration, whereas the *Staphylococci* species did not grow at all. While this may be due to possible breakthrough growth of both *E. coli* and *P. aeruginosa* as a result of over-inoculation of the initial growth medium, it would be worth investigating whether cellular differences accounted for this observation.

Despite the limitations of this study, it can be deduced that increasing honey concentration effectively decreases bacterial growth, and that there is no remarkable difference between fall versus spring honeys. Public health officials and the general public can use this information as possible alternative treatments for bacterial infections. With the growing antibiotic resistance, alternative medicinal therapies may provide additional tools to be used as treatment options when antibiotics fail.

Methods

Thirty-one (31) grams of nutrient agar (Frey Scientific) was added to 1000 mL of distilled water. Fifty (50), one hundred (100), or two hundred (200) grams of fall or spring honey (Shaw Farm, Dracut, MA) was then added to the agar suspension, increasing the volume of the flask over 1,000 mL, but ensuring honey concentrations of 5%, 10%, and 20%, respectively. Next, the agar with honey was heated until the solution was translucent. The solution was then autoclaved for 15 minutes at 121°C and then poured into sterile Petri dishes (Frey Scientific). The agar was allowed to cool and was then stored in refrigeration (4°C) until use.

When testing began, thioglycolate broths were inoculated with either *E. coli, S. aureus, P. aeruginosa*, or *S. epidermidis* to a 0.5 McFarland standard and then incubated at 35°C for 24 hours. After 24 hours of incubation, a 1:10 serial dilution was made from each thioglycolate broth to a 106 dilution factor. This was accomplished by placing 0.5mL of each inoculated thioglycolate broth into 4.5 mL of sterile blood bank saline. Saline was chosen because it was readily available and the salt concentration was not thought to adversely affect the growth of the organisms. From each dilution tube, a spot-titer was performed onto either nutrient agar, fall honey nutrient agar, or spring honey nutrient agar, in order to estimate CFUs/mL. For the spot-titer, 10µL of the inoculated thioglycolate tube was pipetted onto the honey agar plates, with concentrations of honey at 5%, 10%, or 20%. These plates were then incubated for 24 hours at 35°C, as this was the temperature measured on the incubators used. After 24 hours, colony counts were performed on the plate that contained 25-250 CFUs, as this is considered a countable range. The CFU number was then divided by 0.01 and multiplied by the dilution by the dilution factor to estimate CFUs/mL. This process was repeated for a total of three trials for each organism and agar type.

References

1. "Medicinal Plants." Medicinal Plants, www.piam.com/mms_garden/plants.html.
2. Lulat, A. and Zulma, A. "Honey-a Remedy Rediscovered." *J R Soc Med*, 82.7 (1989) 384–85.
3. Roderick, WJ. "The Contribution of Catalase and Other Natural Products to the Antibacterial Activity of Honey: A Review." *Food Chemistry* 21.2 (2000) 235–39.
4. Zhoa, T. "What Causes Honey to Have Different Colors?" 14 June 2013, www.quora.com/What-causes-honey-to-have-different-colors.
5. Mandal, MD, and Mandal, S. "Honey: Its Medicinal Property and Antibacterial Activity." *Asian Pac J Trop Biomed* 1.2 (2011) 154–60.
6. Baba, SA, and Malik, SA. "Determination of Total Phenolic and Flavonoid Content, Antimicrobial and Antioxidant Activity of a Root Extract of Arisaema Jacquemontii Blume." *Journal of Taibah University for Science* 9.4 (2015) 449–454.
7. Cartea, ME *et al.* "Phenolic Compounds in Brassica Vegetables." *Molecules* 16.1 (2011) 251–80.
8. Giorgi, A *et al.* "The Relationships between Phenolic Content, Pollen Diversity, Physicochemical Information and Radical Scavenging Activity in Honey." *Molecules* 16.12 (2011) 336–47.
9. Lusby, PE. *et al.* "Bactericidal Activity of Different Honeys against Pathogenic Bacteria." *Archives of Medical Research* 36.5 (2005) 464–67.
10. Requier, F *et al.* "Honey Bee Diet in Intensive Farmland Habitats Reveals an Unexpectedly High Flower Richness and a Major Role of Weeds." *Ecological Applications*, 25.4 (2015) 881–90.
11. Adeleke, O *et al.* "Honey: A Reservoir for Microorganism and an Inhibitory Agent for Microbes." *African Health Sciences* 7.3 (2007) 159–65.
12. Sutton, S. "Measurement of Cell Concentration in Suspension by Optical Density." PMF Newsletter, Aug. 2006, www.microbiol.org/resources/monographswhite-papers/measurement-of-cell-concentration-in-suspension-by-optical-density/.
13. Myers, JA *et al.* "Improving Accuracy of Cell and Chromophore Concentration Measurements Using Optical Density". *BMC Biophysics* 6.1 (2013) 4.
14. Toivanen, M, Huttunen, S, Lapinjoki, S, Tikkanen-

Kaukanen, C. “Inhibition of adhesion of Neisseria meningitidis to human epithelial cells by berry juice polyphenolic fractions”. *Phytotherapy Research* 25.6 (2010) 828-32.
15. Shaw Farm Employee. Personal Interview. 3 July 2017.
16. Cowan, MM. “Plant Products as Antimicrobial Agents”. *Clinical Microbiology Reviews* 12.4 (1999)
17. Yamanaka A, Kouchi T, Kasai K, Kato T, Ishihara K, Okuda K. “Inhibitory Effect of Cranberry Polyphenol on Biofilm Formation and Cysteine Proteases of Porphyromonas gingivalis”. *Journal of Periodontal Research* 42.6 (2007) 589–92.

Acknowledgments

We’d like to thank Shaw Farm in Dracut, MA for providing information about their honey products.