2022

Variability in Antimicrobial Properties of Multifloral Honey in Southwestern Virginia

Isabella Jessee
Hollins University, jesseeil@hollins.edu

Variability in Antimicrobial Properties of Multifloral Honey

in Southwestern Virginia

Hollins University

Isabella Jessee

Supervised by Dr. Mary Jane Carmichael

Objective: The antimicrobial properties in multifloral honey have been utilized for centuries in wound healing, as well as infection treatment and prevention. The chemical properties from the nectar source, enzymes produced by the bee, as well as the digestive activity of the microorganisms in the bee gut all contribute to the antimicrobial activity of honey. Honeybee farms in four locations across southwestern Virginia (Fincastle, Covington, Troutville, and Martinsville) were visited in the fall of 2021 to collect honey (fall and spring), worker bees, and pollen pellets. Disk diffusion assays were used to
assess the antimicrobial activity of fall and spring honey against ESKAPE pathogens. Pollen analysis was completed on the spring honey and fall pollen pellets to identify the plant species on which the bees were foraging at each time of year. Lastly, the honeybee gut was dissected, and the microorganisms within the midgut and hindgut were isolated and identified using 16S rRNA gene sequencing to identify the cultivable microbial community in the bee gut.

Committee

Mary Jane Carmichael, Ph.D., Department of Biology, Thesis Advisor

Rebecca Beach, Ph.D., Department of Biology

Morgan Wilson, Ph.D., Department of Biology

VARIABILITY IN ANTIMICROBIAL PROPERTIES OF MULTIFLORAL HONEY IN SOUTHWESTERN VIRGINIA

by

Isabella Jessee
2022

Presented in partial fulfillment of the requirements for the degree of Bachelor of Science with Honors in the Department of Biology

Hollins University
Roanoke, Virginia
May 2022

Dedication

I am dedicating my senior thesis research project to my parents, Tim and Melody, as I am giving my final presentation on their 24th wedding anniversary.

Thank you for your continued love and support as I achieve my goals. I am always striving to make you proud in everything I do. Thank you for introducing me to science and medicine at a young age.

Love,

Isabella

Acknowledgements

I would like to thank the faculty and staff in the biology and chemistry departments who allowed me to borrow their equipment and gave advice in helping me carry out this project. I owe an enormous thank you to Dr. Carmichael for advising me on this thesis project and for doing so with enthusiasm and great wisdom.

Abstract

The antimicrobial properties of multifloral honey have been utilized for centuries in wound healing, as well as for infection treatment and prevention. The chemical properties from the nectar source, enzymes produced by the bee, as well as the digestive activity of the microorganisms in the bee gut all contribute to the antimicrobial activity of honey. Honeybee (*Apis mellifera*, the western or European Honeybee) farms in four locations across southwestern Virginia (Fincastle, Covington, Troutville, and Martinsville) were visited in the fall of 2021 to collect honey (fall and spring), worker bees, and pollen pellets. Disk diffusion assays were used to assess the antimicrobial activity of fall and spring honey against ESKAPE pathogens. Pollen analysis was completed on the spring honey and fall pollen pellets to identify the plant species on which the bees were foraging at each time of year. Lastly, the honeybee gut was dissected, and the microorganisms within the midgut and hindgut were isolated and identified using 16S rRNA gene sequencing to identify the cultivable microbial community in the bee gut. Martinsville spring honey demonstrated the strongest antimicrobial properties among spring and fall honey among sample site. Fall honey generally demonstrated stronger antimicrobial properties than spring honey and manuka honey (a monofloral honey from the Manuka bush in New Zealand and known to have strong antimicrobial properties). Fincastle spring honey had the most species diversity in foraged pollen, and tree pollen was unexpectedly found to be significant in bee diet. Lastly, the gut microbes identified produce antimicrobial properties, mostly antimicrobial peptides (AMPs) produced by *Bacillus* species.

Table of Contents

Introduction

Honeybees have played a key role in ecosystems worldwide for millennia. European Honeybees (*Apis mellifera*) are considered a cornerstone species because they are pollinators that ensure the plants of terrestrial ecosystems are able to produce offspring and continue providing sustenance for the honeybee population and other organisms (Engel *et al.* 2016). From working around the clock to keep the hive running, to investing energy in offspring to produce the sweet, viscous food known as honey, honeybees function at such a highly organized level that no other insect on earth is believed to compare, even though there are many eusocial insects found in nature (Engel *et al.* 2016).

Thousands of years ago, humans began utilizing honey as a food source, as well as a natural medicinal tool, because of its extensive antibacterial properties. During the Stone Age, approximately 8 millennia ago, early humans painted on cave walls pictures depicting the utilization of honey to heal the wounded and diseased, making honey the oldest healing substance known (Samarghandian *et al*. 2017). The translation below comes from the Quran and was written originally 1,400 years ago in the Middle East.

> "And thy LORD taught the bee to build its cells in hills, on trees and in men's habitations, then to eat of all the produce of the earth and find with skill the spacious paths of its LORD, there issues from within their bodies a drink of varying colors, wherein is healing for men, verily in this is a sign for those who give thought" (Al-Waili *et al*. 2014).

Aristotle also promoted the use of therapeutic honey, saying, "[honey is] good as a salve for sore eyes and wounds" (Carter *et al.* 2016). The embalming ceremonies of the Egyptians involved the use of honey to dress wounds and apply to damaged skin prior to wrapping the

body (Mijanur Rahman *et al*. 2014). In addition to wound care, Greeks treated gout, fever, and pain with honey (Nolan *et al.* 2019). The Babylonians, Chinese, Mayans, Romans, and others used honey for its medicinal and dietary benefits (Mijanur Rahman *et al*. 2014). In modern times, once antibiotics were being used in the 1960s, chemical, lab-manufactured western medicine began to soar. Natural medicinal tools, like honey, became known as "alternative," and their use declined drastically in western medicine. With the increases in antibiotic resistance, researchers are turning back to honey to combat microbial activity in wounds (Nolan *et al.* 2019, Carter *et al.* 2016). There are about 300 types of honey produced by *Apis mellifera*, all with different ranges of antimicrobial activity (Samarghandian *et al.* 2017). Aside from the antimicrobial properties, further investigation has shed a light on the many other health benefits of honey for the improvement of functionality of various body systems, including the respiratory, cardiovascular, nervous systems; honey also exhibits antidiabetic and anticancer effects (Ciancosi *et al.* 2018). To investigate the healing aspects of honey, its physical and chemical properties, as well as their derivation, need to be defined. The nectar source, as well as the honeybee's gut microbiome and enzymes its digestive system produces, lead to the curious properties of honey that medical professionals are beginning to rely on today (Fig. 1) (Nolan *et al.* 2019; Al-Waili *et al.* 2014; Mandal and Mandal 2011). While honey has numerous benefits for different systems of the human body (Ciancosi *et al.* 2018), this paper will focus on honey's antimicrobial properties.

Honeybees and Their Ecology

The Life and Behavior of a Honeybee

A honeybee hive consists of thousands of honeybees: a queen, workers (females), and drones (males). Drones make up the smallest population of the hive, as they are needed only to mate with the queen and provide genetic diversity. Drones do not do any of the work performed by worker bees to maintain the hive, and they have no stinger (Kešnerová *et al.* 2019). The worker bees only live for about a month but accomplish an unimaginable amount of work in their lifetime. The lifespan of worker bees in the winter is six times longer than that of spring or summer workers. It is thought that winter workers live much longer because they are feeding on old pollen bread, which contains a higher bacteria load and shifts the microbiome favorably (Kešnerová *et al.* 2019). Pollen bread is a combination of foraged pollen and honeybee secretions packed into cells of the comb for high protein consumption (Kešnerová *et al.* 2019). Because workers, aside from winter workers, generally live for only one month, they altruistically invest all of their time and energy into the next generation to ensure the hive's success and longevity (Wolfgang and Habersack 1998). Eusociality in honeybees has been examined in many studies. A bee stings a predator to paralyze or kill it in order to protect the colony, and as a result, the bee perishes. Workers work their whole short lives to sustain all the members of the hive (Mullen and Thompson 2015). The specific job of a worker bee changes every few days. Workers can act as nursing bees- bees that care for larvae, foragers, wax-makers, or guards at the front of the hive (Fig. 1D). Nursing bees keep the larvae and queen fed and warm (Fig. 1D). Larvae need to be kept at 95˚F and need continuous supplies of protein-dense pollen, so they consume "pollen bread," also called "bee bread" (Fig. 1D), which is a

combination of pollen packed in a cell with ripened honey and additional enzymes secreted by the workers (Wolfgang and Habersack 1998). Pollen is not only a source of protein, but also a source of fatty substances, minerals, and vitamins (Bibi *et al.* 2008). Pollen is also the only source of lipids and proteins for the honeybee (Didaras *et al.* 2020). The darker cells on a comb contain this pollen bread. All eggs are void of bacteria, and the importance of social interactions begin in the larval stage with the nursing bees passing some of their gut bacteria to the larvae to allow their bodies to begin the symbiotic relationship with their bacteria (Kwong *et al.* 2016).

Most eggs laid are also of equal rank in the social hierarchy, but those offspring fed royal jelly will grow to be queens. The queen is fed royal jelly her whole life including during development as a larva. Royal jelly consists of milky hypopharyngeal gland secretions from the workers and makes the queen much larger than the other colony members (Klose *et al.* 2017; Wolfgang and Habersack 1998). Found in the front of the head capsule, the hypopharyngeal gland is an exocrine gland with secretory cells and duct cells in sets of 12 around a long collection duct. When the job of a worker bee shifts from nursing the larvae and queen to foraging, the hypopharyngeal gland shrinks, secretes less, and begins producing proteins, including enzymes, to break down foraged carbohydrates (Klose *et al.* 2017). Foraging worker bees search for honey twelve hours a day and leave the hive in one-hour shifts. They visit hundreds of flowers a day to collect nectar and pollen. Approximately 5 million flowers' worth of nectar yields only a single pint of honey. Therefore, since foragers can travel up to a few miles from the hive, they must ensure enough flowers are present to be their long-term nectar sources (Wolfgang and Habersack 1998).

Once a population becomes too large to sustain, the queen decides it is time for a split. She signals the workers to swarm and begin searching for a new location, like a hollow tree, to construct a new hive (Wolfgang and Habersack 1998). If the swarm cannot find a home to their liking quickly, the swarm will perish (Campbell 2021). When a location is deemed fit, the queen lays approximately a dozen eggs in the original hive in an effort to replace herself. She heads to the new hive's location and takes a swarm of half of the original colony with her to start anew, leaving behind a nucleus colony ("nuc"), a colony with the bare minimum members to thrive (Wilson 2021). As the potential queens of the original hive develop into larvae, and the workers feed them all the royal jelly they can eat, the workers at the newfound hive location begin to form bee chains as a form of measuring how long their combs need to be. Once a new hive's construction process has begun, workers secrete an abdominal scent in order to deem this new home theirs, as well as aid any swarm stragglers in finding their way to their new home. A comb, made out of workers' waxy secretions, is two sheets of hundreds of hexagonal cells with a base in between the sheets. The amount of energy required to make a single ounce of wax is about a pound of honey, and from larva to adult stage, about a three-week maturation process, a worker bee consumes 142 mg of honey (Bibi *et al.* 2008; Wolfgang and Habersack 1990). This puts into perspective the number of workers with full stomachs of honey needed to construct this new home. Pollen bread, honey, and larvae are stored in these capped cells (Wilson 2021; Wolfgang and Habersack 1998). It is estimated that a colony of bees, including the larvae, consume 20 kgs of pollen every year (Seeley 1991). When workers pupate, they grow in such a way that the cell grows larger horizontally. The queens, however, grow vertically as they pupate (Wilson 2021). Back at the original hive with the dozen potential queens pupating, one queen

hatches first. She will begin stinging the other still-pupating queens to death in their sensitive abdomen through their chrysalis, ensuring her victory as queen. A week after a virgin queen has crowned herself, she takes her mating flights a few days consecutively to mate with about ten drones per day. The drones have much larger eyes than the workers and the queen to allow them to spot the virgin queen on her mating flight. Once a drone has successfully mated with the queen, he dies and plummets to the ground, the queen holding the remainder of his abdomen to signal to the workers upon her return to the hive that she was successful. Over the course of a few days of mating flights, the queen collects and stores enough sperm to create eggs for the remainder of her lifetime, which is one to four years. A queen lays about 1,500 eggs daily (Fig. 1D), except in the winter, and about 200,000 eggs per year (Wolfgang and Habersack 1998). This polyandrous mating yields genetic heterogeneity among the members of the hive (Wolfgang and Habersack 1998).

Honeybee workers do not mate and reproduce. Surrendering the chance to pass on their own genes and raise their own young, workers do not reproduce and leave the queen with the responsibility of creating offspring (Pirk *et al.* 2002). Fertilized eggs yield workers, while unfertilized eggs yield drones (Wilson 2021). Workers cannot mate, so they resort to asexual reproduction in which they can only produce haploid drones (Pirk *et al.* 2002).

Honeybees are considered eusocial insects because colonies involve generational overlap, reproductive division of labor, and cooperative care for brood (Queller and Strassman 2014). Eusocial insects have evolved to be highly efficient in their division of labor and benefit from group living (Keller and Chapuisat 2014). What is now referred to as kin selection was an idea of W. D. Hamilton in the 1960s, which quantified the concept of gene sharing, or

relatedness (Queller and Strassman 2014). Kin selection is selection on genes causing behavior in one individual through the effects on fitness of other individuals who are genetically related (Queller and Strassman 2014). Kin selection is related to inclusive fitness, the sum of an individual's fitness effects on self and others multiplied by the individual's relatedness to each party (Queller and Strassman 2014). Hamilton's rule states that inclusive fitness is positive only when behavior evolves (Queller and Strassman 2014). Kinship and inclusive fitness directly apply to eusocial insects of the order Hymenoptera, including bees, ants, and wasps (Queller and Strassman 2014). Instead of a parent caring for its offspring as seen in direct fitness, indirect fitness involves alloparenting in which family members, aside from parents, care for offspring. Honeybees exhibit indirect fitness because the honeybee sisters care for their siblings rather than reproducing themselves (Seehuus *et al.* 2006).

Honeybees exhibit strange genetic characteristics due to their haplodiploidy, which is a sex determination system involving female diploids randomly passing half of their genome to offspring and male haploids passing their full genome only to daughters (Keller and Chapuisat 2014; Queller and Strassman 2014). This leads to Hamilton's haplodiploidy hypothesis that unusually high relatedness in sisters was critical in evolution of eusociality (Queller and Strassman 2014). The honeybee queen stores drone sperm from her mating flights in which drones from other hives mate with her, supplying a key source of genetic diversity (Queller and Strassman 2014). The eggs she does not fertilize are fatherless haploid males, or drones, whereas the eggs she does fertilize are diploid females (Queller and Strassman 2014). Especially when sister bees share the same drone father, their degree of genetic relatedness is unusually high (Queller and Strassman 2014).

Polyphenism is excellently demonstrated by the honeybee (Evans and Wheeler 1999). Polyphenism is the expression of different morphologies with physiological and behavioral accompaniments (Evans and Wheeler 1999). The morphological phenotypes of the honeybee contribute to the division of labor (Evans and Wheeler 1999). The following are examples of physiological differences based on labor. Workers have larger hypopharyngeal glands for honey-making, but when the worker bee switches jobs and becomes a forager, the hypopharyngeal gland shrinks since it is not needed for secretions (Klose *et al.* 2017; Pernal and Currie 2000). Lastly, the queen is large and fed solely royal jelly, and her reproductive system develops much faster than other females (Evans and Wheeler 1999). Different morphologies exist even though all females within a hive are genetically identical diploid sisters (Evans and Wheeler 1999).

Foragers' Selection of Nectar Sources

A foraging worker bee does not simply spot a flower and extract some of its nectar. The forager works as part of a collective determining which nectar sources should be used to produce their honey to feed the colony (Wolfgang and Habersack 1998). When a forager finds a new food source, she performs a "waggle dance" in which she moves her abdomen to direct the others to her newfound food source. The speed, direction, and number of patterns in the abdomen movement acts as directions for the others to memorize. Foragers also use the sun as a reference point in explaining to each other if the nectar is right, left, or ahead (Wolfgang and Habersack 1998).

Honeybees must rely on different plants depending on the time of year. When a specific plant is prosperous during one season, the foragers shift their diet to include the nectar from

that particular plant. This the primary reason honey is darker during certain season; the nectar source varies based on available vegetation. Colors of honey range from amber, to dark amber, to yellow, to reddish amber to virtually black. The combination of active compounds in different nectar sources contributes to the color, taste, and smell of honey (Nolan 2019). Climate and colony size (and therefore the queen's ability to lay eggs) also affect the characteristics and properties of the honey yield. The darker the honey, the richer in flavor and the more antimicrobial it will be (White and Doner 1980). Honey is marketed as multifloral and unifloral, also called varietal. For honey to be deemed unifloral or varietal, the majority of the nectar used to create it must come from one particular species of plant, like orange blossom honey or avocado honey, whereas multifloral honey is produced by bees using various nectar sources, not one much more than another (Campbell 2021; White and Doner 1980). Pollen grains in honey can even be used to identify the plant species from which the nectar originated (Bibi *et al.* 2008).

According to a Virginia beekeeper, some nectar sources in southwestern Virginia, depending on season, are as follows: red maple, black locust, blackberries/dewberries, and non-native bush honeysuckle in the spring; tulip poplar, clover, and wildflower/dearth in early summer; and asters, goldenrod, and wingstem in the fall. From June through August, honeybees do not have nearly as many available nectar sources due to heat and drought, which causes a dead spot in the bloom sequence. This limits them extremely, causing starvation. In southwestern Virginia, the plants in the spring provide nectar that yields very light amber honey, and the plants in the fall and winter provide nectar that yields dark honey (Campbell 2021).

Seeley and colleagues conducted a study with over 4,000 Italian honeybees (*Apis mellifera ligustica*) in Ithaca, New York, to evaluate the behavior associated with nectar source profitability (Seeley *et al.* 1991). Profitability includes sugar content, proximity to the hive, and quantity and condition of the flowers. Over two 8-hour periods, researchers recorded how many workers approached one of two feeders, a feeder with low profitability nectar (low sugar), and a feeder with high profitability nectar (high sugar) (Table 1). The researchers studied the bees' behavior as well. When first assessing the two feeders, the same number of bees approached and attempted to extract nectar from the feeders. However, as time went on, the workers began to increase foraging only at the high profitability nectar feeder, deserting the low profitability feeder. To make worthwhile use of the colony's energies, foragers must determine profitability of each nectar source they find and share this information with the rest of the colony. This points to the concept of the "supraorganism," the one large organism (the colony) composed of many smaller organisms, the bees. Honeybees work as a collective unit to maximize use of energy, offspring yield, and honey yield. Each forager consumes 0.5 mg of honey per kilometer she expects to travel (Bibi *et al.* 2008). Minimizing the cost of this energy to obtain the nectar is of utmost importance. Seeley describes the selection of nectar by the supraorganism as a naturally selective process with a better outcome (a larger amount of a high-quality honey) when more foragers collect nectar from the most profitable source as opposed to a less than satisfactory outcome (a smaller amount of low-quality honey) when foragers collect form the less profitable source. Seeley and his colleagues concluded there must be a component of the bee's nervous system allowing the worker bees to measure profitability of nectar sources while foraging (Seeley *et al.* 1991).

Honey-Making Process

While humans have harvested honey since ancient times, honeybee colonies have used it to nourish their workers to allow them to continue working and performing their daily activities, all crucial to the maintenance of the hive. A colony of honeybees consumes about 60 kgs of honey annually (Seeley 1991). The process by which honey is made involves the following steps. First, the foragers collect nectar, a watery, sweet liquid from various floral sources (Wolfgang and Habersack 1998; White and Doner 1980). Nectar fills the crop, or honey stomach, and is the honeybees' main source of carbohydrates (Didaras *et al.* 2020). Workers store pollen in "pollen baskets," sacs on the back of the legs (Seeley 1991; Wolfgang and Habersack 1998). Worker bees from a given colony collect approximately 10-26 kg of pollen per year (Didaras *et al.* 2020). When the foragers return to the hive with full stomachs of nectar and pollen baskets on their legs, they pass the pollen to the workers who then add phytocidal acid to prevent germination and bacterial growth, and they add hypopharyngeal gland enzymes to prevent fermentation or anaerobic metabolism from occurring (Bibi *et al.* 2008). Then, they bring the nectar up from their stomachs into their mouths, and workers extract it with their tongue out of the forager's mouth, add their own enzyme master mix, and regurgitate it into an empty cell. The workers fan it dry with their wings to reduce the water content to about 17%, which takes about three days (Olaitan *et al.* 2007; White and Doner 1980). Because nectar has a high moisture, it easily spoils; therefore, without dehydrating the nectar, the honey would lose its viscosity and spoil (Campbell 2021; White and Doner 1980). Workers then add their enzymes to the nectar and allow it to ripen until it thickens into honey. Once ripe, workers excrete wax and cap the honey for storage. Without capping the honey, it would absorb water from the air and spoil because

of ripened honey's hygroscopicity (Campbell 2021). From this point on, unless tampered with, the honey will never spoil (Wolfgang and Habersack 1998).

Honeybee Microbiome

The antimicrobial properties of honey derive from both the nectar source as well as the honeybee itself and its enzymes, some of which are produced by the microbiome. Although the honeybee gut microbiome is much more simplistic, it parallels the human gut microbiome because it is dominated my facultative anaerobes, which make ATP by aerobic respiration in the presence of oxygen, but switch to fermentation in the absence of oxygen, and microaerophiles, which require lower concentrations of oxygen to grow. Nine core bacterial clusters inhabit the gut microbiome of the honeybee, all of which are easily passed from bee to bee by social contact and have adapted alongside their hosts for millions of years. Other microbes, such as *Frischella perrera* of the *Proteobacteria*, are present in much lower concentrations and serve little known functional purposes (Kwong and Moran *et al.* 2016). Of the nine core clusters, there are two gram-negative proteobacteria, *Snodgrassella alvi* and *Gilliamella apicola*, two gram-positive fermentative Firmicute species, *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, and the remaining five are *Actinobacteria* of the species *Bifidobacterium asteroides* and are present in lower populations.

Much like the human gut, each region of the honeybee's abdomen (hindgut-ileum, distal hindgut-rectum, and midgut) contains different microorganisms aiding the bee in performing its daily functions (Kwong and Moran *et al.* 2016). The midgut, in which food is digested and absorbed, does not provide a conducive environment for many microorganisms because the lining is shed frequently, and the environment contains enzymes aiding in digestion and

nutrient absorption (Ellis 2015). The ileum and rectum form the hindgut and are crucial in water and salt absorption prior to excretion (Ellis 2015). The ileum is a tube with six folds teaming with *S. alvi* and *G. apicola* (Kwong and Moran *et al.* 2016). The rectum contains fecal matter prior to defecation. In the rectum, *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5 are the dominant species (Kwong and Moran *et al.* 2016). The queen's gut is filled with the *Acetobacteraceae*, *P. apium* and Alpha2, likely due to her difference in diet. *P. apium* is the only known bacteria to be able to flourish in royal jelly (Kwong and Moran *et al.* 2016).

The primary benefit of the symbiotic relationship for the bees is that the bacteria work to digest the plant carbohydrates the bee is incapable of digesting. This symbiosis works to sustain the bee's own body as well as add to the enzyme secretions injected into nectar to ripen it and form sugar-concentrated, antimicrobial honey. Zheng and identified the microbes involved in polysaccharide digestion (Zheng *et al.* 2019). *B. asteroides* and the Proteobacterium, *G. apicola*, are the primary degraders of hemicellulose and pectin and both have wide-ranging strain diversity. Genes in *B. asteroides* encode for many different carbohydrate-active enzymes, CAZymes, that degrade polysaccharides, same as *Bacteroides* in the human gut (Zheng *et al.* 2019). *B. asteroides* and *G. apicola* break the bonds in polysaccharides to release sugars, short-chain fatty acids, that the host can absorb (Zheng *et al.* 2019). Some of these CAZymes are glycoside hydrolase, polysaccharide lyase, carbohydrate esterase, glycosyl transferase, and carbohydrate-binding module (Zheng *et al.* 2019). The honeybee gut microbiota also produce amino acids: *G. apicola*, *S. alvi*, and *B. asteroides* are able to produce most of the 20 core amino acids. *G. apicola and S. alvi* also recycle nitrogenous waste, like uric acid and ammonia (Zheng *et al.* 2019). The gut microbiome also differs amongst honeybees based on their jobs. In a study

by Kešnerová, winter bees and nursing bees showed the heaviest bacterial loads, while winter bees showed the least diversity of gut microbes. *Enterobacteriaceae*, which ferment sugars, were commonly found among nursing bees. Because a worker's job changes every few days, this study suggests that the microbiome readily shifts according to the bee's new job (Kešnerová *et al.* 2019).

In his research, Engel and colleagues sequenced the metagenome of the gut microbes and realized through comparative analysis of gene contents that highly functional, distinct metabolic niches are filled by the core microorganism in the honeybee's gut (Engel *et al.* 2012). Microbes and their hosts evolved alongside one another in a polysaccharide-rich world where plants represent a chief energy source. Honeybees and their microbes live in a codependent relationship to utilize the energy the earth offers (Zheng *et al.* 2019). While relatively little is known about the bee gut microbiome and the interaction between it and its host, it is an active area of research (Kwong and Moran *et al.* 2016).

Chemical Properties of Honey

Phytochemicals in Honey

Multifloral honey is among the most common types of honey and is a likely honey seen in the grocery store (Campbell 2021). There are 180 compounds in honey, from water to sugars to many phytochemicals to free amino acids, proteins, enzymes, vitamins, and minerals (Ciancosi *et al.* 2018). Phytochemicals, so called plant components with discreet bio-activities…. or non-peroxide antimicrobial factors, are secondary metabolites produced by bacteria that are responsible for honey's flavor, color, and antimicrobial properties (Dillon and German 2002; Manyi-Loh *et al.* 2011). Because phytochemicals determine a large aspect of the nectar's

contribution to the antimicrobial properties of honey, plants from different locations with varying climates and soils affects the antimicrobial power of a honey type (Table 1). Phytochemicals include three subdivisions: carbohydrates, phenolic compounds, and volatile organic compounds (VOCs) (Fig. 2)(Manyi-Loh *et al.* 2011).

Carbohydrates

Carbohydrates are the primary component of honey, comprising 95% of honey's dry mass. Dextrose (glucose) and levulose (fructose), both monosaccharides, are the two dominant sugars in honey. The following ten disaccharides have also been found in honey: sucrose, maltose, isomaltose, maltulose, nigerose, turanose, kojibiose, laminaribiose, a, B-trehalose, and gentiobiose (White and Doner 1980). In addition, the following ten trisaccharides have been found in honey: melezitose, 3-aisomaltosylglucose, maltotriose, l-kestose, panose, isomaltotriose, erlose, theanderose, centose, and isopanose (White and Doner 1980). The only two polysaccharides found in honey are isomaltotetraose and isomaltopentaose (White and Doner 1980). Aside from dextrose and levulose, the sugars beyond the complexity level of monosaccharides are present in minute amounts; however, all sugars must be converted to monosaccharides by the bee's enzymes. None of the above sugars, with the exception of sucrose, are present in nectar; they arise as a result of the enzymatic breakdown of the complex sugars into monosaccharides. Therefore, the higher the sugar content, the greater the enzymatic activity because more enzymes need to be produced to metabolize these sugars (Olaitan *et al.* 2007; White and Doner 1980). Here, profitability of nectar source in relation to sugar contents contributes to the formation of antimicrobial-rich honey. According to Masoura, honey with a sugar content of 80% or greater is considered bactericidal because lower

concentrations of 40% or less have been found to break down saccharides to form monosaccharides and further lactate (Masoura *et al.* 2020). This is why sugars counteract the effect of acid/oxidative stress. However, it has been found that sugars at a concentration up to 50% decreased cell size dramatically due to plasmolysis and osmotic stress, which cause the inner and outer membrane of the bacterial cell to separate and the cell to collapse; however, cell growth is not inhibited. This suggests that sugars may contribute to the antibacterial effect by altering the physiology of cells (Table 1). Sugars, however, moderate the toxicity caused by the gluconic acid and H_2O_2 (Masoura *et al.* 2020).

Phenolics

Honey contains much more than its easily detectable sugar content. Phenolic compounds (polyphenols) are abundant in pollen (Didaras *et al.* 2020). Polyphenols are deemed either flavonoids (including flavanols, chalcones, isoflavovones, flavonols, flavones, flavanones, and anthocyanidin) or non-flavonoids (phenolic acids) (Ciancosi *et al.* 2018). Non-flavanoids consist of one benzene ring whereas flavonoids are water-soluble and consist of two benzene rings with a linear chain of three carbon atoms, but this structure generally shifts to form three rings with 15 carbons (Ciancosi *et al.* 2018; Li and Duan 2018). Some flavonoids honey commonly contains are catechin, pigenin, pinocembrin, genistein, chrysin, pinobanksin, quercetin, luteolin, galangin, and kaemferol (Ciancosi *et al.* 2018). Some phenolic acids honey contains are ferulic, gallic, syringic, p-Coumaric, vallinic, caffeic,4-(Diethylamino) benzoic, chlorogenic, and ellagic acids (Ciancosi *et al.* 2018). The darker the honey, the more phenolic compounds, and therefore, the more antimicrobial activity (Estivinho *et al.* 2008).

Volatile Organic Compounds

Manyi-Loh considers VOCs fingerprints of honey used to determine if the honey is unifloral or multifloral as well as its geographical location (Manyi-Loh *et al.* 2011). VOCs are subdivided into aldehydes, ketones, acids, alcohols, hydrocarbons, norisoprenoids, terpenes, and benzenes (da Silva *et al.* 2016; Manyi-Loh *et al.* 2011). More than 600 VOCs have been identified in low concentration in common honey (Hawkins 2015). An important VOC in Manuka honey is methylglyoxal, which is a 1,2-dicarbonyl compound responsible for this particular type of honey being antimicrobial (Manyi-Loh *et al.* 2011; Mavric *et al.* 2008; Nolan *et al.* 2019)(Table 1). Methylglyoxal is an aldehyde that arises non-enzymatically when methylglyoxal synthase converts dihydroxyacetone-phosphate in the Manuka tree (*Leptospermum scoparium*) into methylglyoxal (Nolan *et al.* 2019). Manuka honey is viewed as one of, if not the most, antimicrobial honey on the market, and because of this, it is very expensive to purchase (Nolan *et al.* 2019). According to Carter, methylglyoxal operates against pathogens in a non-specific manor by reacting with the pathogen's macromolecules like DNA and RNA (Carter *et al.* 2016). For this reason, it was thought that methylglyoxal might be harmful to eukaryotic cells, but it is not (Carter *et al.* 2016). However, according to Rabie and colleagues, methylglyoxal has the capacity to alter fimbriae and flagella of bacterial cells, limiting their motility and therefore decreasing growth (Rabie *et al.* 2016).

Acids

Acids are not phytochemicals; rather, they result from the enzymatic breakdown of sugar into monosaccharides. Glucose oxidase produces gluconic acid upon the metabolism of dextrose. Gluconic acid, the predominant acid in honey, is a driving force in the low pH of

honey, which also contributes to its antimicrobial effects (Table 1). The pH of honey ranges from an acidic 3.2 to 4.5, which can deter the growth of certain groups of microorganisms (da Silva *et al* 2016) (Table 1). Sugar and gluconic acid have a direct positive relationship, so that when the sugar concentration of honey increases, so does the concentration of gluconic acid. Additional acids in honey include formic, acetic, butyric, lactic, oxalic, succinic, tartaric, maleic, pyruvic, pyroglutamic, a-ketoglutaric, glycollic, citric, malic, 2- or 3- phosphoglyceric acid, a- or B-glycerophosphate, and glucose 6-phosphate (White and Doner 1980). When humans harvest honey and process it to stabilize it and sell it, heating is involved to rid the honey of any harmful microorganisms. However, heating honey denatures the enzymes, leading to a decreased antimicrobial effect (White and Doner 1980).

Bee-defensin and H_2O_2

Bee-defensin and hydrogen peroxide do not belong in any of the above chemical categories, yet they are both crucial to the antimicrobial activities in honey (Table 1). Bee-defensin is an antimicrobial peptide secreted by the hypopharyngeal glands of the honeybee and found in the bee's hemolymph (blood). Bee-defensin acts by producing proteins that create a pore on a bacterial cell's surface and cause apoptosis by inhibiting transcription and translation of proteins. According to Nolan and colleagues, bee-defensin is most effective against gram-positive bacteria like *Staphylococcus aureus* (Nolan *et al.* 2019). However, Masoura and colleagues state that at a concentration of 0.5 µg/ml or greater, bee-defensin combats both gram-positive and gram-negative bacteria (Masoura *et al.* 2020). The quantity of bee-defensin depends on the activity of the bee's hypopharyngeal glands. Both methylglyoxal and bee-defensin inhibit the formation of biofilms (Nolan *et al.* 2019).

When a worker collects nectar, she adds three enzymes: invertase, diastase, and glucose oxidase (Nolan *et al.* 2019). The nectar contains plant sucrose. Invertase, also called sucrase, breaks down sucrose into dextrose and levulose while diastase hydrolyzes starch to produce dextrin and β-amylases which pave the way for maltose formation (Ciancosi *et al.* 2018; White and Doner 1980). In short, invertase and diastase convert sucrose from the nectar into fructose and glucose, allowing glucose oxidase to oxidize glucose once oxygen is added. The byproducts of this are D-glucono-δ-lactone and hydrogen peroxide (Nolan *et al.* 2019). Hydrogen peroxide is a weak acid, but it has been called the most known inhibine by Mavric because it inhibits the growth of microbes (Campbell 2021; Mavric 2008). Hydrogen peroxide is referred to as an oxidative biocide because it oxidizes chemicals in honey, inhibiting bacterial growth and irreversibly damaging bacterial DNA by producing hydroxyl radicals. In a study conducted by Masoura and colleagues, the synergy of hydrogen peroxide and gluconic acid led to rapid depolarization of *Escherichia coli*'s cell membrane, followed by apoptosis and overall growth inhibition (Masoura *et al.* 2020). Typically, hydrogen peroxide concentration in honey varies depending on glucose oxidase added by the worker bee and the pollen-derived catalase; these two components depend on bee health and diversity in the bee's diet, meaning the pollen and nectar (Nolan *et al.* 2019). Low concentrations of hydrogen peroxide only caused transient depolarization of the membrane, and the bacterial cells did not suffer. The concentration of hydrogen peroxide in honey is antimicrobial even though it is 900 times smaller than the concentration humans use to cleanse wounds. Polyphenol compounds in honey can directly produce hydrogen peroxide or reduce Fe (III) to Fe (II), which excites hydrogen peroxide production (Masoura *et al.* 2020). Also, upon dilution, the antimicrobial activity in honey

increases because glucose oxidase can bind glucose much more readily, leading to continuous hydrogen peroxide production (Nolan *et al.* 2019).

Antimicrobial Activity of Honey Against Pathogens

The Variety of Microbes Honey Combats

In 1892, the first observation of the antimicrobial properties of honey was made using the following gram-positive and gram-negative organisms: *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *S. aureus*, *Bacillus subtilis*, and *Listeria monocytogens* (Nolan *et al.* 2019). Olaitan and colleagues found that *E. coli, Vibrio cholrae, Yersinia enterocolitica, Plesiomonas shigelloides, Aeromonas hydrophila, Salmonella typhi, Shigella boydi,* and *Clostridium jejuni* populations could be inhibited at or above a honey concentration of 50% (Olaitan *et al.* 2007) (Table 2). Honey research has only recently begun to skyrocket because of the abundance of antibiotic-resistant pathogens. Multiple drug resistant pathogens have finally been defeated by honey or by the synergy of drugs and honey. For example, *S. aureus* and *MRSA* were both eradicated by oxacillin-honey. Any attempts at recreating a pathogenic strain resistant to honey have failed (Carter *et al.* 2016). Honey has been found to inhibit planktonic cells, yeasts, viruses, and bacteria. Over 60 bacterial species have been shown to be sensitive to honey, which changes shape of and shrinks the cell (Table 1) (Carter *et al.* 2016; Olaitan *et al.* 2007). Some yeast can live in honey diluted with water and cause spoiling of the honey through fermentation. *Candida albicans*, *Clostridum oedemantiens*, *Aspergillus spp.*, and *Penicillium spp.* can be killed by honey, but in the microsporidian *Nosema apis*, only decreased spore viability has been documented (Carter *et al.* 2016; Olaitan *et al.* 2007).

P. aeruginosa is a common agent in infected wounds, but honey has completely eradicated the infection in studies, allowing skin grafting to occur (Olaitan *et al.* 2007). Particular genes involving virulence, quorum sensing, and formation of biofilms are down-regulated upon administration of honey. Honey causes down-regulation of flagellation proteins in *P. aeruginosa*. Flagellation normally allows *P. aeruginosa* to travel and cause an invasive infection (Carter *et al.* 2016). Prior to treatment, Olaitan recommends a swab of the infected wound to allow pathologists to identify the pathogens and test their susceptibility to honey (Olaitan *et al.* 2007).

Manuka Honey vs. Tualang Honey as Antibacterial Agents

Manuka honey and Tualang honey have been revered as the most potent of honeys, with regard to their strong antimicrobial properties. Manuka honey is made from the nectar of the Manuka Tree (*Leptospermum scoparium*) in New Zealand and Australia. In the 1980s at Waikato University in New Zealand, professor Molan was the first person to question and study Manuka honey's potential health benefits while testing it against many bacterial species. Even he did not realize that low concentrations of Manuka honey still act as powerful antimicrobial agents (Carter *et al.* 2016). An additional key component of any honey in healing wounds is that its viscosity and hygroscopicity keep the wound moist, while also killing pathogens (Campbell 2021).

Manuka honey is different from common multifloral honey because it is a non-peroxide honey, meaning hydrogen peroxide is not the primary source of its antimicrobial activity. In 2008, it was discovered that methylglyoxal, however, is the source of Manuka honey's antimicrobial activity (Carter *et al.* 2016). The pH of Manuka honey ranges from 3.2 to 4.21, and

it is light brown in color (Ahmed and Othman 2013). Methylglyoxal is present in multifloral honey but in much smaller concentrations. In a study conducted by Mavric and colleagues, it was discovered that methylglyoxal concentrations were 100 times higher in Manuka honey than multifloral honey (Mavric *et al.* 2008). Because of this, Manuka honey was officially registered with a medical governing body in New Zealand as a medicinal product (Carter *et al.* 2016). Manuka honey has been studied as a possible treatment for the flu as well as chicken pox and shingles, which are caused by the varicella-zoster virus (Carter *et al.* 2016). MRSA has been shown to cease growth with the synergistic use of Manuka honey and the antibiotics oxacillin, tetracycline, imipenem and mupirocin (Carter *et al.* 2016).

In other cases, doctors used Manuka honey as a last resort once antibiotics did not eradicate infection, and those patients successfully healed. Veterinarians have begun using Manuka honey on animal wounds and on dressings post-surgery as well (Carter *et al.* 2016). Cooper and fellow researchers conducted a study on 58 strains of coagulase-positive *S. aureus*, the most pathogenic strain of this organism. Some of these samples from infected surface lacerations were treated with Manuka honey while others were treated with pasture honey, commonly multifloral honey. Minimum inhibitory concentrations (MICs) ranged from 2% to 3% for Manuka honey treatment and 3% to 4% for pasture honey treatment; therefore, Manuka honey did not show preferential healing compared to the multifloral honey. The MICs revealed both honeys' capability of preventing growth of *S. aureus*, even upon dilution by bodily fluids up to 14 times past the point at which osmolarity halts inhibition (Cooper *et al*. 1999). A recently studied multispecies biofilm revealed the only known pathogen Manuka honey cannot kill or inhibit is *Enterococcus faecalis* (Carter *et al.* 2016).

Rock bees (*Apis dorsata*) produce unifloral, deep brown Tualang honey from the nectar of the Tualang tree (*Kompassia excelsa*) in the northwestern area of the peninsula of Malaysia. The Tualang tree grows in the rainforest and can reach 250 feet with honeycombs 6 feet long and over 30,000 honeybees. The pH of this honey is 3.55 to 4.21, very similar to the pH of Manuka honey. While Manuka honey's antibacterial strength lies in its methylglyoxal concentration, Tualang honey's antibacterial strength is its increased concentration of phenolic acids and flavonoids. Tualang honey is slightly more bactericidal than Manuka honey, especially against gram-negative bacteria (Table 2). In fact, medical professionals who have used Tualang honey to dress infected wounds have found it to be very effective at inhibiting the growth of *P. aeruginosa, Acinetobacter baumannii,* and *K. pneumoniae*, all gram-negative species. In third-degree burns, hydrofibre silver gel is usually used with wound dressings; however, 32.26% of wounds dressed with Tualang honey decreased significantly in size because the honey triggers fibroblast and epithelial cell renewal (Ahmed and Othman 2013).

Rationale for Project and Goals

According to the Virginia State Beekeepers' Association, there are approximately 20 beekeeper organizations in southwest Virginia. The present study was designed to assay the antimicrobial properties of honey from southwest Virginia. The chemical properties from the nectar source, the enzymes produced by the bee itself, and the processing of the nectar source by the bee gut microbiome all contribute to the antimicrobial activity of honey. Because of this, a three-part study (Fig. 3) was designed to determine the antimicrobial properties of multifloral honey in southwestern Virginia. First, spring and fall honey from four sites were collected in 2021 and assayed for antimicrobial properties against the ESKAPE pathogens using a disc

diffusion assay. ESKAPE pathogens (*Escherichia coli, Staphylococcus aureus, Klebsiella Pneumoniae, Acinetobacter Baumannii, Pseudomonas aeruginosa, Enterococcus faecium*) are common opportunistic human pathogens and have been used in many disk diffusion assay studies (Antonova et al. 2019; Hijazi et al. 2018; Fleeman et al. 2015). In order to determine the foraging sources of the bees, honey and pollen pellets were processed and examined by microscopy to identify the pollen varieties found in each sample. Finally, the bee gut microbiome was characterized from each site using culture-dependent assays.

Materials and Methods

Site Description

In fall 2021, samples of honey were collected from different regions of Southwestern Virginia (Fig. 4). The Fincastle site (Fig. 5A) was visited in the late afternoon. The Fincastle beekeeper, Mr. Williams, reported that he has had his most stable hives for about four years. He evaluates hive health weekly and reported that he was treating for varroa mites (*Varroa destructor*) with 99.6% oxalic acid at the time of sample collection. He had treated for varroa three times total since 2018. Varroa mites feed on the bees, eventually killing them; these mites destroy hives if not treated (Locke 2017). He did not introduce plants to any area specifically to provide foraging sources. Mr. Williams collects spring honey from his hives in July for sale and personal use. He does not supplement with pollen patties or sucrose.

The Covington site (Fig. 5B) was visited in late afternoon. The Covington beekeeper, Mr. Wright, reported that he has had his most stable hives for about thirteen years. He evaluates hive health weekly and has never needed to treat his hives for varroa mites. He does not supplement with pollen patties of sucrose and has not introduced plants to any area specifically

to provide foraging sources. Mr. Wright collects spring honey from his hives toward the end of June or beginning of July for sale and personal use.

The Trouville site (Fig. 5C) was visited in the afternoon. The Trouville beekeeper, Mr. Wolfe, reported that he has had his most stable hives for about forty years, since the 1980s. He evaluates hive health every three weeks (the worker bee gestation period). After July 10th when spring honey is drained off for sale and personal use, Mr. Wolfe performs three-week cycles of varroa mite treatment with 99.6% oxalic acid. He treats for three weeks then allows the hives three weeks to adjust then treats again. He does not supplement with pollen paddies of sucrose. He has introduced fields of wildflowers to his property specifically to provide foraging sources. He performs prescribed burns every three years as well.

The Martinsville site (Fig. 5D) was visited in the morning. The Martinsville beekeeper, Mr. Whitlock, reported that he has had his most stable hives for approximately three years. He evaluates hive health every two weeks (the worker bee gestation period). Annually, Mr. Whitlock treats for varroa mites the entire month of August with 100% oxalic acid. His hives also had hive beetles (*Aethina tumida*) for which he does not treat; he simply allows the bees to drive the beetles to the top of the hive. Hive beetles have been referred to as minor pests, not nearly as serious as varroa mites (Neumann 2016). He harvests spring honey for sale and personal use in July. He was the only beekeeper to supplement with pollen patties, as well as 2:1 sucrose syrup in water (v/v). He has not introduced plants to the area specifically to provide foraging sources. He performs prescribed burns every three years as well.

Sample Collection

Honeybee farmers from each location provided 8-12 oz samples of honey obtained in July, representing spring honey samples since the honey was cumulatively produced over the entire spring season. When beekeepers harvest honey in the beginning of summer, they use that honey for sale and personal use since it is believed to have more floral diversity and better taste (personal communication with beekeepers). For the fall honey samples, honey was freshly collected in either September or October by scraping the honeycomb into a large plastic bowl and allowing the honey to drain off the comb through a sieve (Fig. 6).

In order to collect the honeybees, the beekeeper removed a frame with many worker bees on it, and approximately 30-40 bees were brushed into plastic vials (with a leather gloved hand). The vials were quickly capped with a sponge, and the vials were placed in a cooler and buried with ice up to the sponge top in order to sedate the bees and keep them docile during transportation back to the laboratory. Then, each bee was placed in its own capped vial and placed in a freezer at -15˚C until needed for gut dissection. The bees remained refrigerated for three months until dissections were performed in January 2022.

In addition to the collection of fall and spring honey at each site, pollen pellets were collected for species identification. While honey contains pollen that can be analyzed, using pollen pellets allowed for a direct analysis of the plants bees in particular areas are foraging on in a more concentrated sample. Pollen pellet samples were collected by using a pollen trap (shown attached to a hive in Fig. 5B). The pollen trap was placed at the entrance of the hive, secured with lab tape, and left for half an hour or so or until the necessary amount of pollen pellets were collected. As the bees fly through the trap, the pollen pellets get scraped off of their hind legs, forelegs, and middle legs and deposited in the collection tray. Once five to ten

pollen pellets were collected, the pollen trap was removed, and the pellets were placed in a single collection tube labeled with the specific farm from which the pollen came. The tubes were placed in a cooler with ice, along with the bees, to be transported back to the laboratory. The tubes were placed in the freezer at -15˚C to prevent growth of any fungi that might have been associated with the sample.

Antimicrobial Assays

Assays to determine what honey concentration to use with ESKAPE pathogens.

Samples of Manuka honey were diluted to different honey concentrations to determine which single concentration was best to test for the antimicrobial assay. Manuka honey was used for these assays because it is known to be one of, if not the strongest, antimicrobial honeys on the market due to its methylglyoxal content, a volatile organic compound (1,2-dicarbonyl) (Nolan *et al.* 2019). Methylglyoxal is an aldehyde that arises non-enzymatically when methylglyoxal synthase converts dihydroxyacetone-phosphate in the Manuka tree into methylglyoxal (Nolan *et al.* 2019). One honey sample was diluted to 30% (30 mL honey: 70 mL deionized water), one to 50% (50 mL honey: 50 mL deionized water), and one to 70% (70 mL honey: 30 mL deionized water). A full concentration (100%) sample was also used for the assays. The dilutions were refrigerated until needed. To create each dilution, the desired amount of honey and water were poured into a 50 mL falcon tube and was taped onto a nutator overnight to mix thoroughly. When ready to be used, the honey dilutions were removed from the refrigerator and heated by placing the 50 mL falcon tube and placed in a 40˚C water bath for approximately 15 minutes or until the honey became less viscous and could be filtered via syringe filtration to eliminate pathogens (Hamden 2010). This temperature was

not high enough to denature any bioactive enzymes that may have antimicrobial properties in the honey (Subramanian *et al.* 2007). Once removed from the water, the dilutions were left to return to room temperature prior to dipping the disks.

Staphylococcus aureus and *E. coli* were the two pathogens used to test the concentration of the honey to be used in the antimicrobial assays (Al-Waili *et al.* 2011). *S. aureus* was chosen to represent the gram positive ESKAPE pathogens, and *E. coli* was chosen to represent the gram negative ESKAPE pathogens. Mueller-Hinton agar was poured into the petri dish to a depth of 4 mm in 100 mL (or 150 mL) petri dishes. Cultures were incubated overnight and then diluted to match a 0.5 McFarland turbidity standard. Each pathogen was transferred to a plate, pipetting 100 µL of each pathogen onto its own plate and using a spreader for even distribution. The disks (n=9) that had been soaking in a particular honey for two minutes were gently placed onto the plate to ensure full contact with the pathogen. Each plate contained three honey-soaked disks, and a plate for each pathogen was created with three control disks soaked in sterile, deionized water for two minutes. Three replicates per culture per honey type were plated in addition to a control for each pathogen. The plates were inverted and incubated at 40˚C for 16-18 hours. When the plates were removed from the incubator, zones of inhibition were measured in millimeters using a ruler, and organized in a chart according to honey dilution the disks had soaked in as well as the pathogen by which the disks were surrounded. Entire zones of inhibition, including the disk, the clear zone around the disk if applicable, and the diffuse halo (i.e. the cloudy, visibly distinct area of reduced bacterial growth compared to the control), were measured and recorded (Fig. 7). It is possible that the diffuse halo was produced by a chemical in the honey interacting with the media to produce

the color change, as we did not confirm that it consisted of bacterial growth. To test this in future studies, disks soaked in the honey could be plated with no bacteria spread on the plate. If a diffuse halo is produced, this would mean there is likely a chemical present in the honey causing the growth media to alter in appearance. SigmaPlot version 14.5 (Systat, Palo Alto, CA) was used to test for significant differences in assay response among spring and fall honeys from each sample location, as well as Manuka honey (positive control), deionized water (negative control), and tetracycline (positive control). No statistical difference was found between the 30% and 50% dilutions or between the 70% and 100% dilutions; however, there was a significant difference (P= 0.001) between the 30% and 50% dilutions and the 70% and 100% dilutions. Because of this, a 70% dilution was used for subsequent assays to reduce viscosity for filter sterilization.

Antimicrobial properties of summer and fall honey against ESKAPE pathogens

Summer and fall honey from each site were tested for antimicrobial activity using the same disk diffusion assay as described in the previous section. In these assays, the honey was tested for its efficacy against all six ESKAPE pathogens: *E. faecium* (Presque Isle Cultures® 524™), *S. aureus* (Presque Isle Cultures® 4651™), *Klebsiella pneumoniae* (Presque Isle Cultures® 344™), *A. baumannii* Bouvet and Grimont (American Type Culture Collection® 19606™), *P. aeruginosa* (Presque Isle Cultures® 99™), and *E. coli* (Presque Isle Cultures® 336™) as the representative *Enterobacter* (De Oliveira et al. 2020). Twenty-four hours in advance, broth cultures of each of the six ESKAPE pathogens were inoculated, incubated overnight, and then diluted to match a 0.5 McFarland turbidity standard. One hundred µL of each pathogen was transferred to a plate and spread for even distribution. The disks that had been soaking in a particular honey for two

minutes were gently placed onto the plate to ensure full contact with the pathogen. Each plate contained three honey-soaked disks, and a plate for each pathogen was created with three control disks soaked in deionized water for two minutes. Three replicates per culture per honey type were plated in addition to a control for each pathogen. Seventy precent Manuka honey plates were used as a positive control; the negative control was plates with disks that were soaked in sterile, deionized water. The plates were inverted and incubated at 40˚C for 16-18 hours. When the plates were removed from the incubator, zones of inhibition were measured as above, and organized in a chart according to honey type the disks had soaked in as well as the pathogen by which the disks were surrounded. Lastly, the pH of each 70% dilution of honey was measured and recorded using EMD colorpHast pH strips (pH 2.0-9.0) (Gibbstown, New Jersey) because honey's low pH plays a role in defense against pathogens (da Silva *et al* 2016).

Pollen Analysis

The spring honey and fall pollen pellet samples were collected and then shipped to Global Geolab Limited (Alberta, Canada) laboratory for processing following their standard protocols for pollen pellets and honey, respectively, and then sent directly to Dr. Sophie Warny at Louisiana State University for palynology analysis and identification via microscopy. Palynological analysis was conducted on using an Olympus BX41 microscope. Microphotographs were taken with an Olympus QColor5 mounted digital camera. First, the samples were scanned using a 40x objective making initial identifications of each pollen type and taking photographic images of unknown pollen types. In making quantitative counts, each pollen type was identified to the family, genus, or in some cases species level. Second, a quantitative pollen count for each sample was conducted to determine the pollen types

present and the frequency of each taxon using both 60x and 100x oil immersion objectives. Statistically valid quantitative pollen count of a minimum of 300 pollen grains per sample were used.

Honeybee Gut Dissection and Culture-Dependent Identification of the Bee Gut Community

Honeybees (n = 8 per location) were dissected, and their gastrointestinal microflora was cultivated using the following procedure. To dissect the honeybee, the bases of the wings were pinned to the paraffin wax in a glass petri dish. The paraffin wax filled the petri dish halfway. The petri dish was set on a small ice pack that had been wrapped in one layer of paper towel to preserve the microbial communities in the gut. The wings and legs were first removed with forceps. Forceps, previously sterilized with 70% ethanol, were used to gently pinch the tip of the abdomen, where the stinger is located, until the rectum and ileum came out, followed by the midgut and crop. If needed, an additional pin was used to pin the abdominal exoskeleton. The digestive tract was laid out in a straight line on the wax in the petri dish, and the midgut and hindgut were cut and separated from one another. Each of the three gut sections was placed into its own separate falcon tubes with sterile broth media. The four media used were brain heart infusion (BHI), tryptic soy agar (TSA), Colombia agar with 5% defibrinated sheep's blood, and sugar water [50% sucrose in deionized water (v/v)] (Romero *et al*. 2019). The liquid broth of each media containing sections of the bee gut were vortexed for a couple minutes. Then, 300 µL were dispensed onto plates with plated media of each of the three media types and spread to cover the entire plate. The plates were incubated for 24 hours at 40˚C. Since the cultures grew very well in three of the media (TSA, Colombia agar with 5% defibrinated sheep's blood, and BHI), serial dilutions were performed to allow distinct colonies to grow instead of

lawns when plated. Dilutions of 10^{-6} and 10^{-7} were used to plate. When morphologically distinct colonies could be visualized, a sterile loop was used to transfer the growing colonies to fresh plates of the three media types to streak for isolation. A gram stain was then performed on each isolate.

Identification of isolates by the 16S rRNA gene sequence

Isolates were grouped by morphotype, and a representative isolate for each morphotype was chosen for 16S rRNA gene sequencing. A fresh broth culture of each culture was inoculated and grown in the incubator overnight. For each isolate, genomic DNA extraction and preparation for sequencing were conducted following a modified bead beating protocol with the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH). The concentration of extracted DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The isolates were prepared for PCR amplification in duplicate using PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA) and custom sequencing primers (Integrated DNA Technologies, Coralville, IA), the forward primer 27F 5'-AGAGTTTGATCMTGGCTCAG-3' (Lane 1991) and the reverse primer 1492 rM 5'-RGYTACCTTGTTACGACTT-3' (Emerson and Moyer 2002). Five ng μL^{-1} of DNA was used for each PCR reaction. The samples were placed in the thermocycler for PCR amplification using the following protocol: an initial denaturation of 95˚C for 10 min, followed by 30 cycles of 94˚C for 1 min, 55˚C for 90 sec, 72˚C for 3 min, and a final extension of 72˚C for 7 min following Carmichael *et al.* (2013). Gel electrophoresis was used to confirm amplification of the 16S rRNA gene sequencing using 2% ultrapure agarose with SYBR Safe DNA Stain in 1X TAE running buffer. Following confirmation of amplification, the 1500kb long 16S rRNA gene, a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) was used to purify

the PCR products for Sanger sequencing. Bi-directional sequencing of each isolate was performed at Eurofins Scientific (location). Sequencher (GeneCodes, Ann Arbor, MI) was used to build and edit contiguous sequences from the forward and reverse sequences of each isolate. The edited sequences were identified using NCBI BLAST. *Statistical Analyses*

All statistical analyses were conducted using SigmaPlot version 14.5 (Systat, Palo Alto, CA). Analysis of variance (ANOVA) was used to test for significant differences ($p < 0.05$) among honey dilutions in order to determine which concentration of honey to use for experimental assays. When assumptions for normality were not met, a non-parametric equivalent was used. When significant differences were found, appropriate pairwise comparisons were completed. A similar procedure was used to 1) test for significant differences among deionized water, Tetracycline, and fall, spring, and Manuka honey at each site and 2) test for differences in antimicrobial properties across sites both seasonally and over the course of the growing season.

Results

Antimicrobial Assays

pH

Among all spring and fall honey, Troutville spring honey had the lowest pH (3.8), followed by Fincastle fall and Covington spring honey (4.0). Covington fall, Fincastle spring, Martinsville spring, and Martinsville fall all had a pH of 4.3. Troutville fall honey acidity was 4.5.

Positive and Negative Controls

Disks soaked in deionized water (the negative control), all showed no zones of inhibition (hereafter, ZOI); all honey and Tetracycline (positive control) produced ZOI. A majority of Tetracycline disks showed significantly larger mean ZOI than manuka honey and spring and fall sample site honey ($P<0.05$) (Figs. 8 and 9). However, in the Martinsville honey test group, Tetracycline mean ZOI was significantly lower than all honey samples in *E. coli* and *A. baumannii* plates (Fig. 8D).

Comparison of Manuka Honey to Fall and Spring Honey at Each Site

Fincastle

For *E. coli*, the mean ZOI of manuka honey (22.67 ± 2.40 mm) was significantly larger ($P<0.001$) than the mean ZOI for spring honey (13.00 ± 2.05 mm). The mean ZOI of manuka honey was significantly larger ($P<0.001$) than fall honey mean ZOI (14.50 ± 1.00 mm) (Fig. 8A). In *K. pneumoniae*, the mean ZOI of fall honey (20.39 ± 1.69 mm) was significantly larger ($P=0.020$) than the mean ZOI manuka honey (17.39 ± 2.71) (Fig. 8A). In *S. aureus*, the mean ZOI of spring honey (20.22 ± 3.77 mm) was significantly larger ($P=0.001$) than the mean ZOI for manuka honey (15.83 ± 1.54 mm) (Fig. 8A). In *A. baumannii*, the mean ZOI of fall honey (14.78 ± 2.68 mm) was significantly larger ($P=0.013$) than the mean ZOI for fall honey (11.89 ± 0.74 mm). The mean ZOI for spring honey (14.06 ± 1.29 mm) was also significantly larger ($P=0.015$) than

the mean ZOI for manuka honey (Fig. 8A). All other comparisons between fall, spring, and manuka honey were not significantly different (P>0.05).

Covington

For *E. coli*, the mean ZOI for fall honey (23.61 ± 3.86 mm) was significantly larger (P=0.004) than the mean ZOI for manuka honey (14.22 ± 1.18 mm). In *S. aureus*, all pairwise comparisons were significant (P<0.05). The mean ZOI for fall honey (25.00 ± 1.32 mm) was significantly larger (P<0.001) than the mean ZOI for manuka honey (15.50 ± 3.22 mm). In addition, the mean ZOI for spring honey (18.17 ± 2.74 mm) was significantly larger (P=0.041) than the mean ZOI for manuka honey (Fig. 8B). All other comparisons between fall, spring, and manuka honey were not significantly different (P>0.05).

Troutville

In *E. faecium,* the mean ZOI for fall honey (24.00 ± 3.12 mm) was significantly larger (P=0.034) than the mean ZOI for manuka honey (18.22 ± 5.84 mm) (Fig. 8C). In *S. aureus*, the mean ZOI for manuka (24.22 ± 4.58 mm) was significantly larger (P=0.013) than the spring honey mean ZOI (19.33 ± 2.87) (Fig. 8C). In *A. baumannii*, all pairwise comparisons were significant (P<0.05). The mean ZOI for manuka honey was the largest (19.61 ± 3.13 mm), followed by fall mean ZOI (15.67 ± 1.75 mm), then spring mean ZOI (13.17 ± 1.54 mm) (Fig. 8C). In *K. pneumoniae*, the mean ZOI of manuka honey (20.44 ± 2.24 mm) was significantly larger (P=0.021) than the mean ZOI for spring honey (16.22 ± 3.54 mm) (Fig. 8C). All other comparisons between fall, spring, and manuka honey were not significantly different (P>0.05).

Martinsville

In *E. faecium*, the mean ZOI for spring honey (10.72 ± 1.25 mm) was significantly larger (P=0.03) than the mean ZOI for manuka honey (8.94 ±1.45 mm) (Fig. 8D). All other comparisons between fall, spring, and manuka honey were not significantly different (P>0.05).

Comparison of Fall and Spring Honey at Each Site

Fincastle

In *S. aureus*, the mean ZOI for spring honey (20.22 ± 3.77 mm) was significantly larger (P=0.017) than the mean ZOI for fall honey (16.56 ± 0.73 mm). In *K. pneumoniae*, the mean ZOI for fall honey (20.39 ± 1.69 mm) was significantly larger (P<0.001) than the mean ZOI for spring honey (15.33 ± 2.29 mm) (Fig. 8A). All other comparisons between spring and fall honey were not significantly different (P>0.05).

Covington

In *S. aureus*, the mean ZOI for fall honey (25.00 ± 1.32 mm) was significantly larger (P<0.001) than the mean ZOI for spring honey (18.17 ± 2.74 mm). In *E. faecium*, the mean ZOI for fall honey (19.89 ± 8.68 mm) was significantly larger (P=0.004) than the mean ZOI for spring honey (17.56 ± 6.14 mm) (Fig. 8C). In *E. coli*, the mean ZOI for fall honey (23.61 ± 3.86 mm) was significantly larger (P<0.001) than the mean ZOI for spring honey (13.44 ± 1.51 mm) (Fig. 8B). All other comparisons between spring and fall honey were not significantly different (P>0.05).

Troutville

In *A. baumannii*, the mean ZOI for fall honey (15.67 ± 1.75 mm) was significantly larger (P=0.022) than the mean ZOI for spring honey (13.17 ± 1.54 mm). In *E. coli*, the mean ZOI for fall honey (20.00 ± 0.97 mm) was significantly larger (P=0.009) than the mean ZOI for spring honey

(17.33 ± 1.28 mm) (Fig. 8C). All other comparisons between spring and fall honey were not significantly different (P>0.05).

Martinsville

In *S. aureus*, the mean ZOI for fall honey (27.33 ± 3.39 mm) was significantly larger (P=0.015) than the mean ZOI for spring honey (21.56 ± 2.24 mm). In *E. faecium*, the mean ZOI for spring honey (10.72 ± 1.25 mm) was significantly larger (P=0.030) than the mean ZOI for fall honey (10.22 ± 1.00 mm) (Fig. 8D). All other comparisons between spring and fall honey were not significantly different (P>0.05). Among sample sites, significant differences occurred more frequently in Martinsville honey (Figs. 8 and 9).

Pollen Analysis

Dr. Warny analyzed a total of 2,539 pollen grains from the four sample sites (Figs. 10 and 11). Overall, spring honey was more diverse in pollen composition than fall honey. In particular, Fincastle spring honey had the most diversity among all of the samples with 18 species identified. The dominant species were clover (33%), Russian olive (9%), and goldenrod (7%) (Figs. 10 and 11). Martinsville spring honey was the second most diverse with 15 species identified, the dominant species being clover (39%), locust tree (7%), and fabaceae from the pea family (9%). Following Martinsville spring honey was Covington spring honey with 13 species identified, the dominant species being clover (24%), basswood tree (39%), and the genus *Prunus* (peach, plum, or cherry) (9%). Finally, Troutville spring honey had 11 species identified, the dominant species being clover (42%), mustards (12%), and dogwood tree (14%). Among the fall pollen pellets, one species was recovered from Covington fall pellet: golden rod (100%). Two species were identified in the Martinsville fall pellet: goldenrod (59%) and grass

(41%), and three species were identified in fall pellets from Fincastle and Troutville. The Fincastle species recovered were goldenrod (72%), and crepe myrtle (26%), and Asteraceae (likely dandelion) (2%). The Troutville species recovered were goldenrod (82%), elm (16%), and Asteraceae (likely sunflower) (2%).

Honeybee Gut Dissection and Culture-Dependent Identification of the Bee Gut Community

Forty-two different morphotypes were isolated from the honeybee gut (Figs. 12 and 13). One appeared to be fungal, so it was disregarded. A representative culture for each morphotype was maintained for DNA sequencing. Thirty six of the 42 isolates had high-quality data that allowed for successful contiguous sequence (forward and reverse sequence combined) construction in Sequencher (Fig. 14). Ten different microorganisms total (across all four locations, across all media types, and across midgut and hindgut segmentations) were identified in NCBI BLAST (Fig. 15). Six identified species were from the *Bacillus* genus (*B. megaterium, B. thuringiensis, B. mycoides, B. anthracis, B. cereus*, and *B. weidmannii*). The remaining microbes identified were *P. vagans, P. agglomerans, P. alvei*, and *S. marcescens*.

According to results from the entire bee guts dissected in location, disregarding midgut or hindgut location, in Fincastle, *B. mycoides* made up the largest portion of the bee gut species identified (40%), followed by *S. marcescens* (30%), *P. vagans* (10%), *B. weidmannii* (10%), and *B. cereus* (10%) (Fig. 16A). In Troutville, *B. mycoides* made up the largest portion of the bee gut species identified (66.6%), followed by *B. thuringiensis* (11.1%), *B. megaterium* (11.1%), and *P. agglomerans* (11.1%) (Fig. 16B). In Covington, *B. mycoides* again made up the largest portion of the microbes identified (75%), followed by the only other microbe identified in Covington bees, *S. marcescens* (25%) (Fig. 13C). In Martinsville, *B. mycoides* made up 33.3% of identified gut

bacteria, followed by *B. megaterium* (22.2%), *B. thuringiensis* (22.2%), *P. alvei* (11.1%), and *B. anthracis* (11.1%) (Fig. 16D).

Looking at species identified in bees' midgut and hindgut at a given location, of the Fincastle midgut community three species were identified: 40% *S. marcescens*, 40% *B. mycoides*, and 20% *B. weidmannii* (Fig. 17A). In the hindgut of the bees dissected from Fincastle, four species were identified: *B. mycoides* (40%), *S. marcescens* (20%), *B. cereus* (20%), and *P. vagans* (20%) (Fig. 17B). Of the Covington bee midgut community, there were two species identified: *B. mycoides* (66.7%) and *S. marcescens* (33.6%) (Fig. 17C). In the hindgut community, the only species recovered and identified was *B. mycoides* (100%) (Fig. 17D). In the Troutville honeybee midgut, only two species were identified: *B. mycoides* (75%) and *B. megaterium* (25%) (Fig. 17E). In the Troutville hindgut community, three species were identified: *B. mycoides* (60%), *P. agglomerans* (20%), and *B. thuringiensis* (20%) (Fig. 17F). In the Martinsville midgut community, three microbes were identified: *B. mycoides* (50%), *B. megaterium* (25%), and *P. alvei* (25%) (Fig. 12G). From the Martinsville honeybee hindgut, four species were identified: *B. thuringiensis* (40%), *B. mycoides* (20%), *B. megaterium* (20%), and *B. anthracis* (20%) (Fig. 17H).

Discussion

In order to determine antimicrobial strength of honey across the four sample sites in southwest Virginia, antimicrobial assays were performed with ESKAPE pathogens to measure and analyze ZOI, the larger ZOI demonstrating stronger antimicrobial properties of a given honey. Pollen from the sample site spring honey and fall pollen pellets were analyzed to determine foraging diversity because the phytochemicals in plants have the capacity to impart

antimicrobial properties in honey. Lastly, honeybee gut dissection and microbe identification were performed to identify potential microbial products contributing to honey's antimicrobial properties.

Antimicrobial Assays

Naturally occurring antimicrobial agents, chemicals that kill or inhibit growth of microorganisms, can be -cidal (kill microbes), -lytic (microbial cell lysing), or -static (inhibit but do not kill microbes) (Madigan *et al.* 2014). Bactericidal agents kill the cell by tightly binding to cellular targets, which causes a linear decrease in the viable cell count and only flattens total cell count (Madigan *et al.* 2014). Bacteriolytic agents lyse a bacterial cell, therefore killing it, which causes a linear decrease in the total cell count and viable cell count (Madigan *et al.* 2014). Bacteriostatic agents inhibit crucial biochemical processes in a way that, if the agent is removed, the bacterial cell can continue growing (Madigan *et al.* 2014). The linear increase of viable cell count and total cell count flattens upon the addition of the bacteriostatic agent then increases linearly again once the agent it removed (Madigan *et al.* 2014). Antimicrobial agents also have a wide variety of functional targets in the cell, for example cell wall structure, protein synthesis, and membrane transport processes (Madigan *et al.* 2014).

Because of the floral, and therefore foraging, diversity in the spring, fall honey was expected to be less antimicrobial than spring honey from the sample sites. However, fall honey from each sample site tended to be stronger than spring honey in microbial growth inhibition. Although some pathogens responded better to spring honey over fall, overall, fall honey was more potent. Specifically, Martinsville fall honey was demonstrated to have the strongest antimicrobial properties among sample site honey. The antimicrobial agents cannot be

hypothesized; however, the difference in antimicrobial properties of the honey samples was demonstrated.

pH

Due to honey's acid content, primarily gluconic acid, the lower the pH, the stronger the honey's inhibition of microbial growth (da Silva *et al* 2016). The pH of honey commonly ranges from 3.2 to 4.5, and the honey from spring and fall from each sample location lie within this expected range (da Silva *et al* 2016). Martinsville seemed to produce the most antimicrobial honey among sites and seasons even though its pH (4.3 in spring honey and fall honey) was in the middle of the pH values of the sample honey, which contradicts the findings in da Silva *et al.* 2016. This suggests that compounds other than gluconic acid may be giving Martinsville honey its antimicrobial strength. Although Troutville spring honey was the most acidic, it had the least antimicrobial effect on the pathogens. Although not known, specific compounds are likely present that are giving this honey its antimicrobial properties. Martinsville, although a moderate 70% dilution pH among the other sample sites, was the darkest honey followed by Covington then Fincastle and Troutville being the lightest of the fall honey (Fig. 6).

pH testing was the last of the methods to be conducted, and by the time it was performed, most of the honey, all except Troutville, Martinsville, and Covington fall honey, crystallized (i.e. became semi-solid, with large, visible sugar crystals). Crystallization is favored when water content is elevated; however, if water content is too low, the honey becomes supersaturated with glucose, which is less soluble than fructose (Conforti *et al.* 2006). This is why bees stop dehydrating their honey once it reaches about 17% water concentration (Olaitan *et al.* 2007; White and Doner 1980). If pH had been tested with the pure honey samples immediately upon

collection, and if honey was stored at room temperature instead of being refrigerated, results may have varied, and crystallization would likely have not occurred since dilution and cooler temperatures promote crystallization (Conforti *et al.* 2006).

Positive and Negative Controls

In order to test the strength of the honey samples, deionized water was used to act as the negative control, while manuka honey and tetracycline were used as the positive controls. Disks soaked in deionized water on the ESKAPE pathogen plates did not produce ZOI. Manuka honey was used because it is a known, strong antimicrobial. In order to test the honeys against a medical grade antimicrobial, the broad-spectrum antibiotic, tetracycline, was used. Tetracycline is bacteriostatic, meaning it halts the growth of additional bacteria in a given area (Grossman 2016). It binds the ribosomal complex, which prevents the association of aminoacyl-tRNA with the bacterial ribosome, and therefore inhibits bacterial protein synthesis. (Roberts 2002). Change in pH drives the tetracycline to move across the membrane through porin channels and into the periplasmic space where it binds reversibly to the prokaryotic 30S ribosomal subunit, halting protein synthesis. Because of its mechanism of action, Tetracycline is considered broad-spectrum because it is effective against both gram-negative and gram-positive bacteria as well as intracellular chlamydiae, mycoplasmas, and rickettsiae. In some cases, it is also effective against eukaryotic pathogens (Roberts 2002).

Comparison of Manuka Honey to Fall and Spring Honey at each Site

While significant differences were found in response of certain ESKAPE pathogens to honey from particular sample sites, overall, Martinsville spring and fall honey was the most effective against *S. aureus*, having the largest mean ZOI recorded (27.33 ± 3.39 mm) across all

honey and sample sites. Although spring honey was expected to be more effective as an antimicrobial, and it was in some cases, fall honey across sites was more effective against ESKAPE pathogens in mean ZOI calculated. Various phytochemicals were expected to be present in spring honey due to the diversity of foraged pollen (Nolan *et al.* 2019). Although that might have been the case in the spring sample site honey, something caused fall honey to be more potent than spring. Manuka honey's methylglyoxal content being 100 times higher than that in average honey made it a standard for comparison (Mavric *et al.* 2008). Because fall honey produced larger mean ZOIs in many cases than manuka honey, a particular potent phytochemical, or combination of phytochemicals were likely present.

Pollen Analysis

According to Di Pasquale, diversity of pollen in a honeybee's diet is important in bee health; however, quality of pollen is much more important than diversity (Di Pasquale 2016). Depending on the bee's job and hierarchy, it will allocate crude protein from pollen in a way to better itself physiologically (Pernal and Currie 2000). For example, workers use the protein to grow their ovaries and hypopharyngeal glands (Pernal and Currie 2000). Poor quality pollen, meaning pollen that has lost its protein-based nutritional value due to dehydration, negatively affects the hypopharyngeal glands, brood development, and worker longevity (Di Pasquale 2016). Habitat loss also leads to nutritional stress (Di Pasquale 2016). Alaux and colleagues found that polyfloral diets boosted the bees' immunocompetence, specifically by increasing glucose oxidase activity (Alaux *et al.* 2010). Glucose oxidase produces gluconic acid, a driving force in the acidity of honey (da Silva *et al* 2016).

When beekeepers were surveyed regarding known forage in the area, the reported forage differed from the recovered pollen (Fig. 10). The shaded boxed in Fig. 10 represent pollen species that were reported as well as recovered. Some reported species were not recovered; however, some recovered species had not been reported. It was unexpected that trees would be just as prominent in honeybee diet. Walnut, Russian olive, pine, prickly ash, elm, and crepe myrtle were among the tree pollen sources in the honeybee diet (Fig. 10). Pollen was recovered that the beekeepers were unaware was in the area. Beekeepers speculated that the *Fabaceae* identified in the spring honey samples from Troutville, Fincastle, and Martinsville were likely black-eyed peas (*Vigna unguiculata*), which are commonly planted as a cover crop in the fall in the region. Goldenrod provided the majority of the bees' fall diet across sample sites, consistent with beekeeper reports (Fig. 10).

Due to the fields of wildflower planted in the foraging vicinity, Troutville spring honey was expected to be the most diverse in pollen species, therefore having an increased number of plant phytochemicals causing it to have more antimicrobial strength than the rest of the honey samples (Dillon and German 2002). However, pollen collected from the spring honey revealed the smallest number of species identified (11) and that the bees were not primarily foraging on the nearby wildflowers; rather, they were foraging mainly on holly trees, dogwood trees, mustard, and Russian olive. Among spring honey samples, Fincastle had the most species diversity (18), followed by Martinsville (16), Covington (12), and Troutville (11). Among fall honey samples, Fincastle and Troutville had the most species diversity (3) followed by Martinsville (2), and Covington (1). Based on the results, plant diversity being directly related to antimicrobial strength was not demonstrated. However, Martinsville and Fincastle spring honey

could indicate that it is not necessarily the pollen species diversity that is of importance in the relation to honey's antimicrobial properties; rather, it may be the chemical composition of pollen from a particular species or group of species that influences the antimicrobial properties of honey. For example, Starks and colleagues isolated the antimicrobial compounds in goldenrod *(Solidago sp.*), which was prominent in the fall samples. The compounds identified, two of which were clerodane diterpenes are solidagoic acids, are known to have moderate antimicrobial activity against *S. aureus* (Starks *et al.* 2010). Additionally, Elm (*Ulmus spp.*) trees possess a gene encoding for antimicrobial peptide (AMP) production (Newhouse 2005). Therefore, some of the plants represented in the pollen samples are known to produce compounds with antimicrobial activity. It is important to note that the pollen identified in the spring honey is representative of the entire foraging season; however, the pollen pellets were a single sample collected once during the season. Therefore, it is unknown if the fall pollen pellets were indeed representative of the pollen on which bees were foraging during the entire fall season at each sample site.

Honeybee Gut Dissection and Culture-Dependent Identification of the Bee Gut Community

Overall, ten microbial species were isolated from the honeybee gut, two of which were specific to the midgut (*B. weidmannii* and *P. alvei*) and five of which were specific to the hindgut (*B. thuringiensis*, *B. anthracis*, *P. vagans*, *B. cereus*, and *P. agglomerans*). *B. mycoides* was the dominant species identified in both the midgut and hindgut of bees from each sample location (Fig. 16 and Fig. 17). The second most prominent microbe found was *S. marcescens*, found in the Fincastle and Covington midgut and the Fincastle hindgut (Fig. 16 and Fig. 17). The

third most prominent species was *B. megaterium*, found in the Troutville and Martinsville midgut and the Martinsville hindgut (Fig. 16 and Fig. 17).

B. mycoides was isolated from the midgut and hindgut from all four sample sites using BHI and columbia agar supplemented with sheep's blood. *B. mycoides* is Gram-positive, rod-shaped, and spore-forming anaerobe, which has been isolated from soil and coastal sediment (NCBI BLAST). *B. mycoides* is known to produce the volatile compounds phenylacetic acid and methylphenyl acetate, which have antifungal and phytotoxic properties (Wu *et al.* 2020).

S. marcescens was isolated from both the Fincastle midgut (on BHI) and hindgut (on TSA) as well as in the Covington midgut (on BHI and TSA). *S. marcescens* is a rod-shaped, Gram-negative, facultative anaerobe in the family *Yersiniaceae*. It is known to be an opportunistic pathogen, causing infections in wounds as well as the respiratory and urinary tract (Hejazi and Falkiner 1997). *S. marcescens* has been found in a wide variety of environments, but because it prefers damp conditions, as such it is commonly observed in bathrooms in toilets and on shower walls as a pink-orange biofilm. *S. marcescens* can perform casein hydrolysis, allowing it to produce extracellular metalloproteinases, important in cell-to-extracellular matrix interactions. According to Duanis-Assaf, κ-casein may have anti-biofilm activity by attaching to adhesion-like proteins, prohibiting bacterial adhesion to surfaces (Duanis-Assaf 2020). Metalloproteinases inhibit *E. faecalis* biofilms formation as well (Tay 2015). The locations from which the top 5 most closely matched BLAST hits were identified, using the microbe's accession number, were mixed greens, rhizosphere soil, urine in canines, human blood, and human sputem.

B. thuringiensis was isolated only from the hindguts from Martinsville and Troutville on TSA. *B. thuringiensis* is a Gram-positive, sporulating bacterium mostly found in soil but also occurs has been found in the gut of many insects. When certain *B. thuringiensis* strains sporulate, they produce insecticidal crystal proteins, also called delta endotoxins or Bt toxins, as in *B. thuringiensis* toxins (Kumar *et al.* 1996). The Bt gene is divided into cry I, II, III, and IV classes; cry codes a parasporal inclusion protein making it a biological pesticide, the most commonly used biological pesticide all over the world (Kumar *et al.* 1996; Yilmaz *et al.* 2005). *B. thuringiensis* is closely related to *B. anthracis* and *B. cereus*, and like *B. cereus*, *B. thuringiensis* has been found to be more effective at treating *S. aureus* infection than some antibiotics (Yilmaz *et al.* 2005). According to Yilmaz and colleagues, when tested against antibiotic disks, some of which were Erythromycin, Vancomycin, Cephazolin, and Azithromycin, *B. thuringiensis* was effective at inhibiting *S. aureus* growth (Yilmaz *et al.* 2005). Antibiotics produced by *Bacilli* are effective against both Gram-positive and Gram-negative bacteria, but are more so effective against Gram-positive (Yilmaz *et al.* 2005). The locations from which the top 5 most closely matched BLAST hits were identified, using the microbe's accession number, were mosquito larva breeding sites, soybean nodules, soil, the shrimp gastrointestinal tract, and swamp forest soil.

B. megaterium, also called *Priestia megaterium*, was isolated from the Martinsville hindgut (on sucrose agar) and the midgut from Troutville and Martinsville (on TSA). It is a rod-shaped, Gram-positive bacterium that is primarily aerobic and spore-forming. *B. megaterium* is also among the largest known bacteria (Bunk *et al.* 2010). It produced penicillin amylase, which is used to make synthetic penicillin. *B. megaterium* has been identified in honey and bee pollen

(Mohammad *et al.* 2020). Emimycin, which inhibits bacterial replication, and oxetanocin, and antiviral antibiotic, are two of the antibiotics *B. megaterium* produces, in addition to some fungicidal toxins (Andrei and Snoeck 2021; Morita 1999; Vary 1994). The locations from which the top 5 most closely matched BLAST hits were identified, using the microbe's accession number, were soil rice straw, cryoconite, saffron rhizosphere, soil, and wheat.

B. anthracis was solely isolated from the Martinsville hindgut and was grown on TSA. *B. anthracis* is also the causative agent of anthrax, a lethal disease in livestock which can be zoonotically transferred to humans (Spencer 2003). *B. anthracis* endospores are extremely resilient, hence their past use as a bioweapon. *B. anthracis* is a Gram-positive and rod-shaped bacterium and the only obligate pathogen in the *Bacillus* genus. PurE (N5-carboxy-amino-imidazole ribonucleotide mutase) is an enzyme produced by *B. anthracis* that is believed to be a potential target in antibiotic development (Kim *et al.* 4014). Little research has been done since Kim's study to discover the mechanism of action of PurE. The location from which the top most closely matched BLAST hit was identified, using the microbe's accession number, was the rhizosphere.

P. alvei was solely isolated from the Martinsville midgut on TSA. It is a Gram-positive, rod-shaped, motile anaerobe. *P. alvei* is a secondary invader during outbreaks of European foulbrood, a severe bacterial disease that affects brood, or larvae, caused by the Gram-positive bacterium *Melissocccus plutonius (*Forsgren 2010; Djukic *et al.* 2012). Djukic and his colleagues sequenced the genome of a strain of *P. alvei* called DSM 29, and they identified putative genes encoding an antimicrobial peptide (AMP) (Djukic *et al.* 2012). Since 2012, further research has demonstrated that *P. alvei* NP75 (a different strain) produces two antimicrobial peptides,

paenibacillin N and P, both of which have effectiveness against clinical pathogens, paenibacillin N against *E. coli* and paenibacillin P against *Bacillus sphaericus* (Jagadeesan *et al*. 2020). *Bacillus*-derived AMPs are broad-spectrum (Sumi *et al.* 2014). Although their mechanism of action is unclear, AMPs are thought to kill bacteria by either by forming channels in or disrupting the bacterial cell wall (Sumi *et al.* 2014). The locations from which the top most closely matched BLAST hits were honey, bee pollen, pond water, fish feces, and cow feces.

P. vagans was solely isolated from the Fincastle hindgut and was grown on BHI. It is a Gram-negative, rod-shaped aerobe. According to Kamber, *P. vagans* strain C9-1 produces at least two antibiotics, one being herbicolin I. While the mechanism of action is unknown, it has been examined and demonstrated as antibiotic, specifically in pears and apples infected with *Erwinia amylovora*, a Gram-negative organism that is the causative agent of fire blight disease (Kamber *et al.* 2012). The locations from which the top most closely matched BLAST hits were *Megymenum gracilicorne* (saw toothed stinkbug) gut, infant blood, wounds, and pine.

P. agglomerans, closely related to *P. vagans*, was solely isolated from the Troutville hindgut on BHI. It is a Gram-negative, rod-shaped aerobe known to produce antibiotics, including herbicolin, pantocins, phenazine (Rezonnico 2017). According to Rezonnico (2017), *P. agglomerans* strains are the most promising biocontrol agents from many plant diseases. A lipopolysaccharide (IP-PA1) produced by *P. agglomerans*, has been shown to prime macrophage activation in healing and protect against infection, allergies, and cancer (Nakata *et al*. 2011). The locations from which the top most closely matched BLAST hits were cherries, perithecium (the fruiting body of an ascomycete phylum fungus), and soil.

B. cereus was solely isolated from the Fincastle hindgut on TSA. *B. cereus* is a Gram-positive, rod-shaped, motile, facultatively anaerobic that has the capacity to sporulate. As mentioned in the *B. thuringiensis* section, *B. cereus*, specifically strain M15, inhibits both Gram-positive and Gram-negative bacteria and is more effective than some of the test antibiotics in (Yilmaz *et al.* 2005). A study showed that *B. cereus* M15 was effective at inhibiting growth of other *B. cereus* strains as well as *Pseudomonas fluorescens, Micrococcus flavus, and B. thuringiensis* (Yilmaz *et al.* 2005). Some of the polypeptide antibiotics produced by *Bacillus* that work to inhibit bacterial growth of various species are bacitracin, gramycidin S, polymyxin, tyrotricidin (Yilmaz *et al.* 2005). The locations from which the top 5 most closely matched BLAST hits were outbreaks of infection in hospitals, the rhizosphere, spiders, a mosquito larva breeding site, and a soybean nodule.

B. weidmannii was solely isolated from the Fincastle midgut and was grown on TSA. It is Gram-positive, rod-shaped, aerobic, and spore-forming. According to Muriuki, *B. weidmannii* is associated with dairy spoilage (Muriuki 2020). There is no known antimicrobial compound produced by *B. weidmannii* that has been shown. The locations from which the top most closely matched BLAST hits were farm water, fish gills, raw milk, and primarily soil.

Although the honeybee's nine core gut bacterial clusters discussed in Kwong and Moran's literature review (*Snodgrassella alvi, Gilliamella apicola*, *Lactobacillus* Firm-4*, Lactobacillus* Firm-5, and five *Actinobacteria* of the species *Bifidobacterium asteroides*) were not identified, ten gut bacteria were isolated from the midgut, hindgut, or both and identified (2016). The 10 microorganisms identified in this study were: six Gram-positive species from the *Bacillus* genus (*B. megaterium, B. thuringiensis, B. mycoides, B. anthracis, B. cereus*, and *B.*

weidmannii), two Gram-negative species from the *Pantoea* genus (*P. vagans* and *P. agglomerans*), Gram-positive *P. alvei*, and Gram-negative *S. marcescens*.

Overall, 90% of the bacteria identified in the southwest Virginia honeybee gut are known to produce compounds (like acids, enzymes, or AMPs), in particular strains, that could contribute to the antimicrobial activity of honey. When the bee ingests and regurgitates its nectar, adding its secreted enzymes, and likely some of its gut microbes, the honey produced by the bees at the sample sites may contain some of the products of the gut microbes found. It is unknown what these antimicrobial compounds are and in what concentration they are present.

Conclusion

This study provided evidence of antimicrobial properties in multifloral spring and fall honey from southwest Virginia. Honey is a powerful antimicrobial agent when it comes to fighting infections (Ciancosi *et al.* 2018). While manuka honey is known to have strong antimicrobial properties due to its methylglyoxal content, fall sample site honey from southwest Virginia rivaled manuka honey and were more effective at inhibiting bacterial growth compared to the Manuka honey used. While the Fincastle sample site in the spring possessed the highest pollen species diversity, and theoretically a higher diversity of phytochemicals, its antimicrobial strength did not quite match that of Martinsville (Dillon and German 2002; Manyi-Loh *et al.* 2011). On the other hand, Martinsville honey was a moderate pH compared to Troutville spring honey, which was the most acidic, but the least antimicrobial. Several plants that are represented in the pollen analysis are known to have antimicrobial properties. Similarly, particular strains of the gut microbes identified from the honeybee's hindgut and midgut are known to produce acids, enzymes, and other bioactive agents that potentially

contributed to the antimicrobial properties of the sample site honey. While only particular strains of each of the ten identified microorganisms in the gut community possess genes encoding for production of antimicrobial agents, those strains could have been the ones present in the bee guts samples. For future studies, fresh dissection would be preferable in order to avoid potential bias in species that could be cultivated since the bees were frozen. Freezing the bees possibly allowed sporulating microorganisms, like *Bacillus*, to predominate because microbes tend to sporulate when environmental conditions, like change in temperature and nutrient availability, are not ideal for growing.

Overall, honey in southwest Virginia indeed possessed antimicrobial properties whose sources can be hypothesized, but future studies should aim to isolate and identify the major contributors, as well as their concentrations, among antimicrobial compounds in the honey samples collected. In addition, it would be preferable to visit sample sites at multiple times in each season to attempt to capture the full diversity in gut organisms and foraging preferences. Sampling over the course of a year might capture the diversity of the honeybee's diet and therefore the phytochemicals contributing to honey's antimicrobial properties.

Figures and Tables

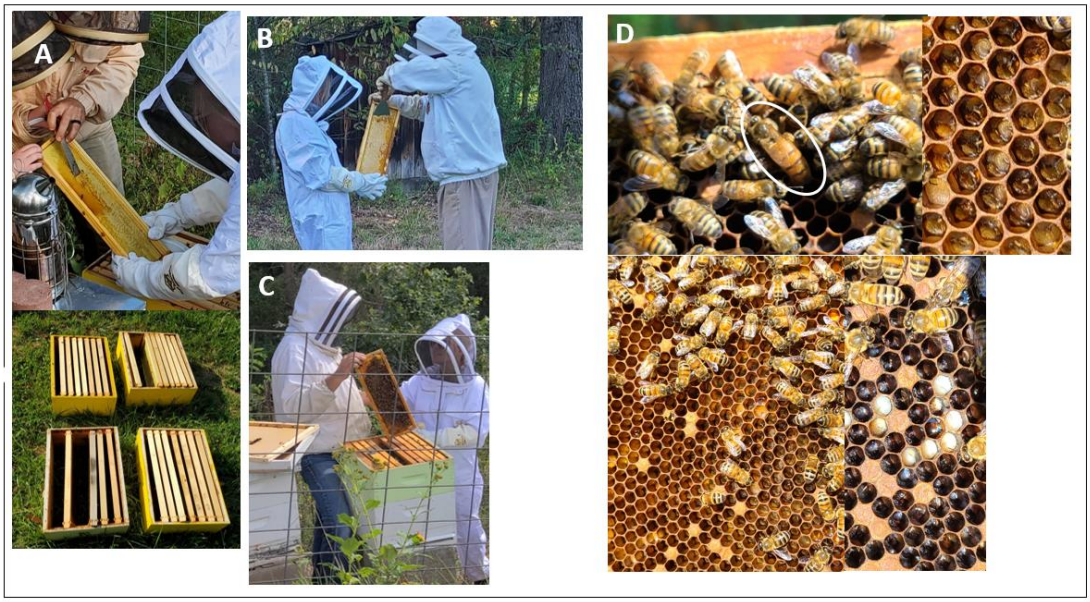

Fig. 1. Inside and outside the hive from frame to comb: (A) Fincastle, (B) Covington, (C) Troutville, (D) Martinsville.

Honey's Antimicrobial Properties

Direct Contributors	Indirect Contributors
High osmotic pressure- increase extracellular water potential	Polyphenolic compounds- alters DNA in damaging way
Low pH- alters bacterial enzymes	Hydrogen peroxide- oxidation causes hydroxyl radicals to change DNA
High sugar content- reduces bacterial growth rate	Methylglyoxal- alters fimbriae and flagella
	Bee-defensin 1- disrupts membrane permeability

Table 1. Contributors to honey's antimicrobial properties. Direct contributors affect cellular mechanisms, while indirect contributors have various effects on the bacterial cell. (inspired by Nolan *et al.* 2019)

Gram-Positive Species and Strains	Gram-Negative Species and Strains
Actinomyces pyogenes	*Acinetobacter baumannii*
Actinomyces viscosus	*Acinetobacter calcoaceticus*
Bacillus cereus	*Alcaligenes faecalis*
Bacillus stearothermophilus	*Burkholderia ambifaria*
Bacillus subtilis	*Burkholderia anthina*
Bifidobacteria	*Burkholderia cenocepacia*
Candida albicans	*Burkholderia cepacia* complex
Candida dubliniensis	*Burkholderia cepacia* group K
Candida glabrata	*Burkholderia cepacian*
Clostridium difficile	*Burkholderia multivorans*
Coagulase negative *Staphylococci*	*Burkholderia pyrrocinia*
Coagulase-negative *Staphylococcus aureus (CONS)*	*Burkholderia stabilis*
Enterococcus	*Burkholderia vietnamensis*
Enterococcus faecalis	*Campylobacter spp.*
Haemolytic streptococci	*Citrobacter freundii*
Lactobacilli	*Enterobacter aerogenes*
Listeria monocytogenes	*Enterobacter agglomerans*
Methicillin-resistant -Staphylococcus aureus (MRSA)	*Enterobacter cloacae*
Mycobacterium tuberculosis	*Escherichia coli*
Nocardia asteroids	Extended-spectrum beta lactamase producing *Enterobacter clocae*
Rhodotorula spp.	Extended-spectrum beta lactamase producing *Enterobacter sp*
Staphylococcus aureus	Extended-spectrum beta lactamase producing *Escherichia coli*
Staphylococcus epidermidis	*Helicobacter pylori*
Staphylococcus equi subsp. Zooepidemicus	*Klebsiella pneumonia*
Staphylococcus sciuri	*Morganella morganii*
Streptococcus agalactiae	*Porphyromonas gingivalis*
Streptococcus dysgalactiae	*Proteus mirabilis*
Streptococcus mutans	*Proteus vulgaris*
Streptococcus pyogenes	*Pseudomonas aeruginosa*
Streptococcus sobrinus	*Salmonella enterica* Serovar *typhi*
Streptococcus uberis	*Salmonella enteritidis*
Vancomycin-resistant Enterococcus	*Salmonella Mississippi*
Methicillin-resistant -Staphylococcus epidermis (MRSE)	*Salmonella typhi*
	Salmonella typhimurium
	Serratia marcescens
	Shigella flexneri
	Shigella sonnei
	Stenotrophomonas maltophilia
	Vibrio cholerae
	Yersinia enterocolitica

Table 2. Microorganisms sensitive to honey. Honey, whether Manuka, Tualang, or simply raw, multifloral honey, has proven to be effective at killing and preventing further growth of the above microorganismal species and strains. The ESKAPE pathogens are highlighted. (Ahmed and Othman 2013) (Al-Waili *et al.* 2011) (Carter *et al.* 2016) (Ciancosi *et al.* 2018) (Cooper *et al.* 1999) (Olaitan *et al.* 2007)

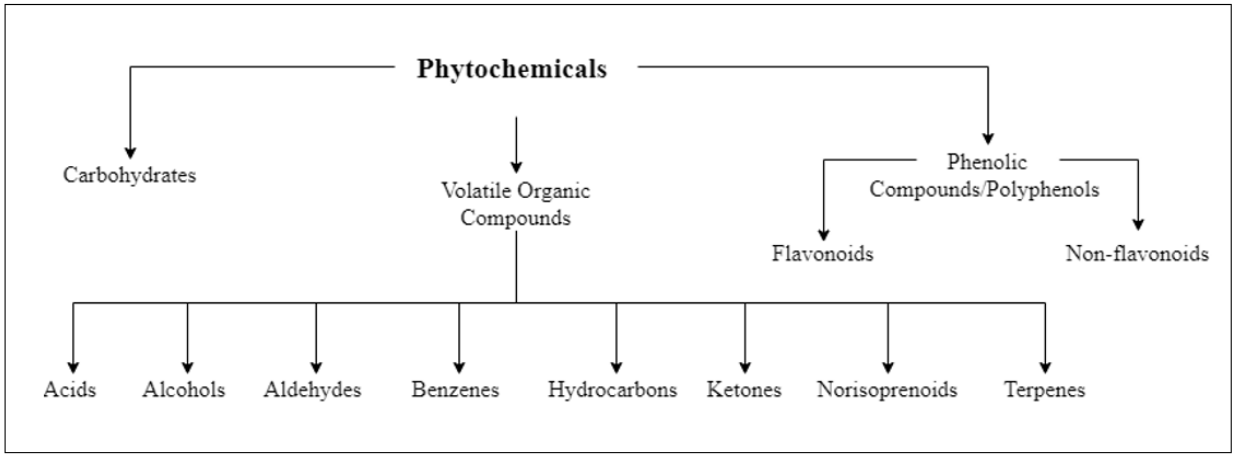

Fig. 2. Divisions of phytochemicals. The three types of phytochemicals are carbs, VOCs, and phenolic compounds. The latter two branch further into their own subdivisions. All honey contains methylglyoxal, an aldehyde; however, Manuka honey contains the highest concentration of methylglyoxal, which is the primary reason for Manuka honey's its antimicrobial properties. Bee-defensin, hydrogen peroxide, and pH also are responsible for honey's antimicrobial properties.
(Patra 2012)

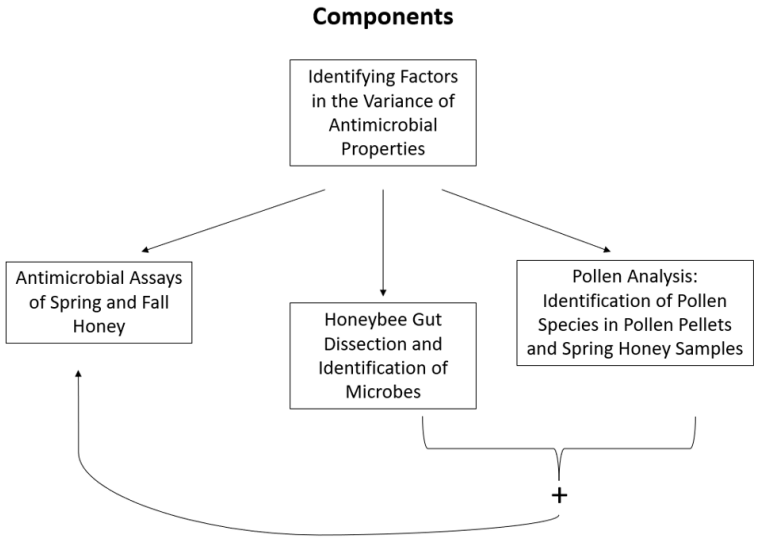

Fig. 3. Experimental design. The antimicrobial properties of honey are a combination of what the bees are foraging on, as well as how the bee gut community processes the diet. Therefore, the three components of this research project included antimicrobial assays, honeybee gut dissection, and pollen analysis.

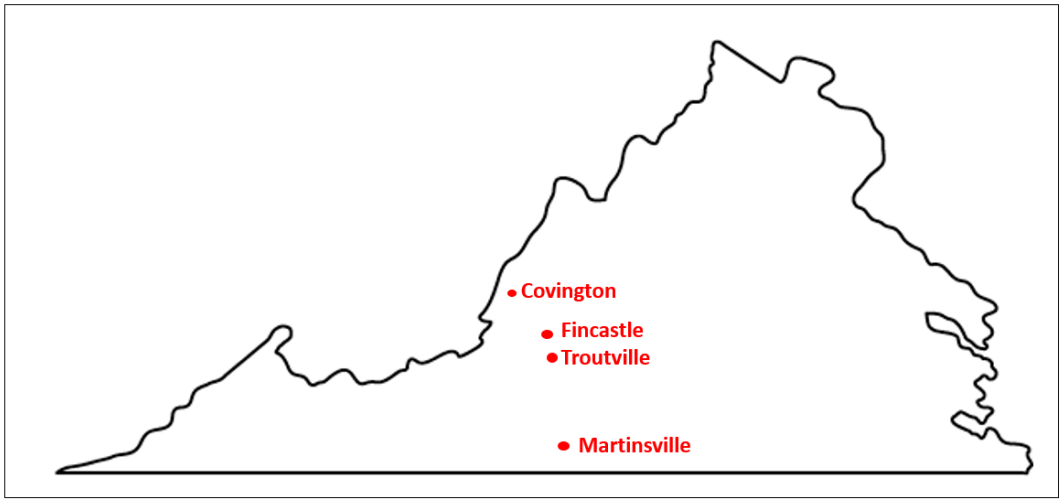

Fig. 4. Sample sites. Honey, honeybees, and pollen pellets were collected from four regions across Southwestern Virginia: Covington, Fincastle, Troutville, and Martinsville. (Virginia map from the National Speleological Society)

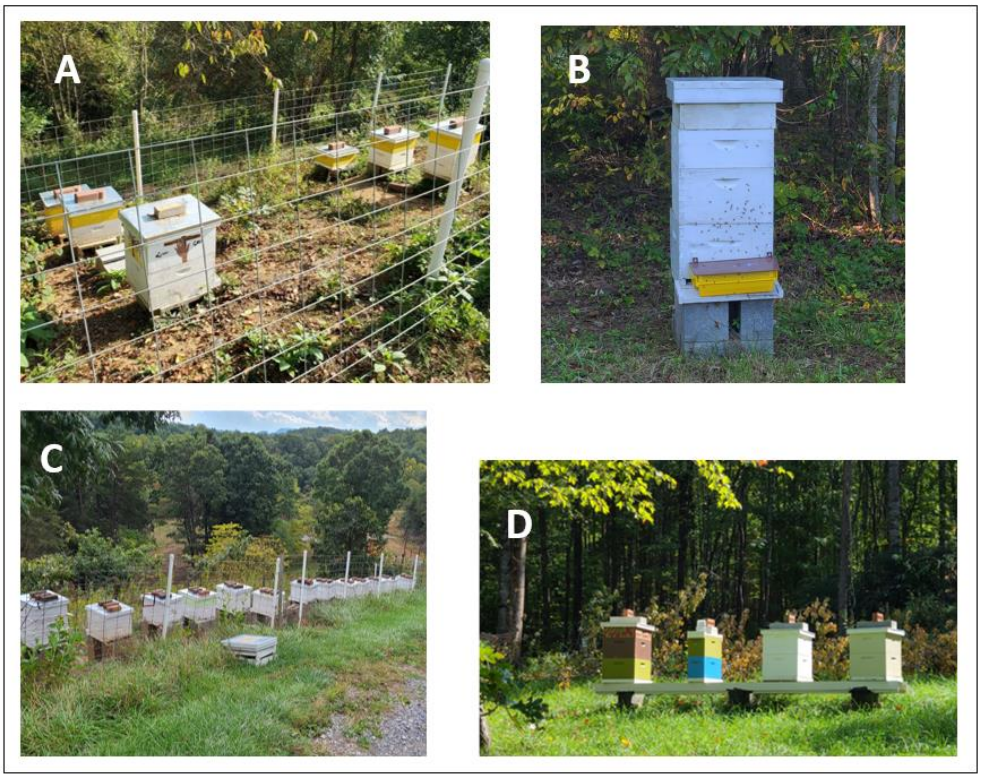

Fig. 5. Hives at sample sites: (A) Fincastle, (B) Covington, (C) Troutville, (D) Martinsville.

Fig. 6. Lightest to darkest honey. Left to right: Troutville, Fincastle, Covington, Martinsville fall honey. Darker honey tends to have a better taste and stronger antimicrobial properties (White and Doner 1980).

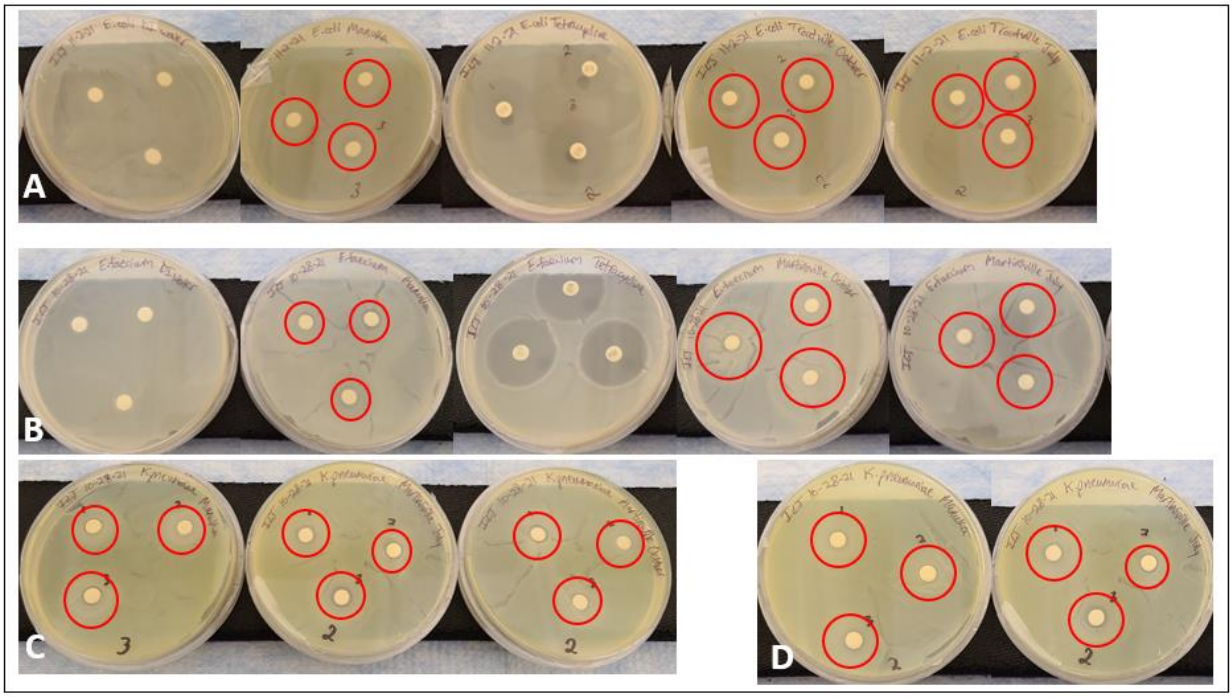

Fig. 7. Antimicrobial assays. (A)Troutville gram negative *E. coli*, (B) Martinsville gram positive *E. faecium*, (C) Martinsville Manuka, July, and October honey *K. pneumoniae*, (D) Martinsville Manuka larger ZOI but only diffuse halo (L) and July honey smaller ZOI but clear (R).

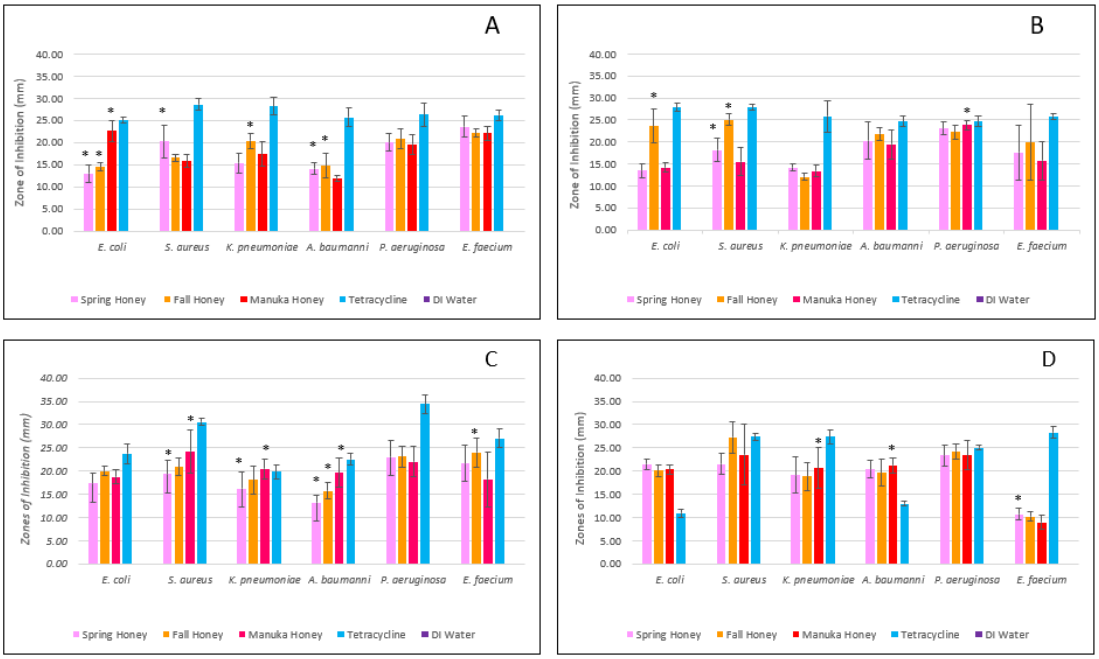

Fig. 8. Zones of inhibition for ESKAPE pathogens. Disks soaked in either spring honey, fall honey, Manuka honey, Tetracycline, or deionized water produced zones of inhibition (ZOI) when placed on plates with a lawn of each ESKAPE pathogen. Mean ZOIs were plotted for each ESKAPE pathogen across the four sample locations: (A) Fincastle, (B) Covington, (C) Troutville, (D) Martinsville. Values given as mean ± standard deviation. Asterisks indicate either the spring or fall honey is significantly different from manuka honey.

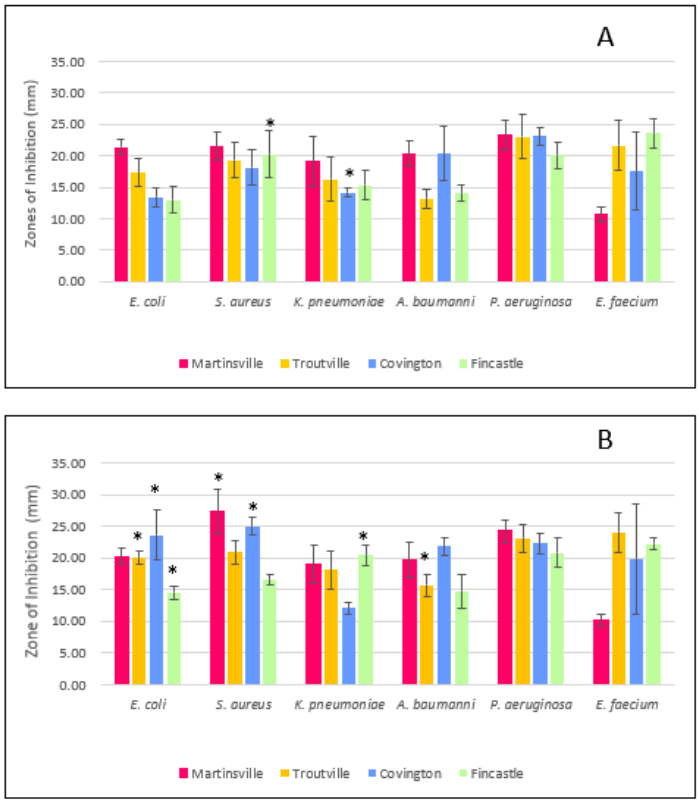

Fig. 9. Zones of inhibition for ESKAPE pathogens from spring (A) and fall (B) honey by sample location. Values given as mean ± standard deviation. Asterisks indicate a significant difference between spring and fall honey for the specific pathogen at the given site. The asterisk is placed above the mean ZOI that is significantly higher than its counterpart in the opposite season.

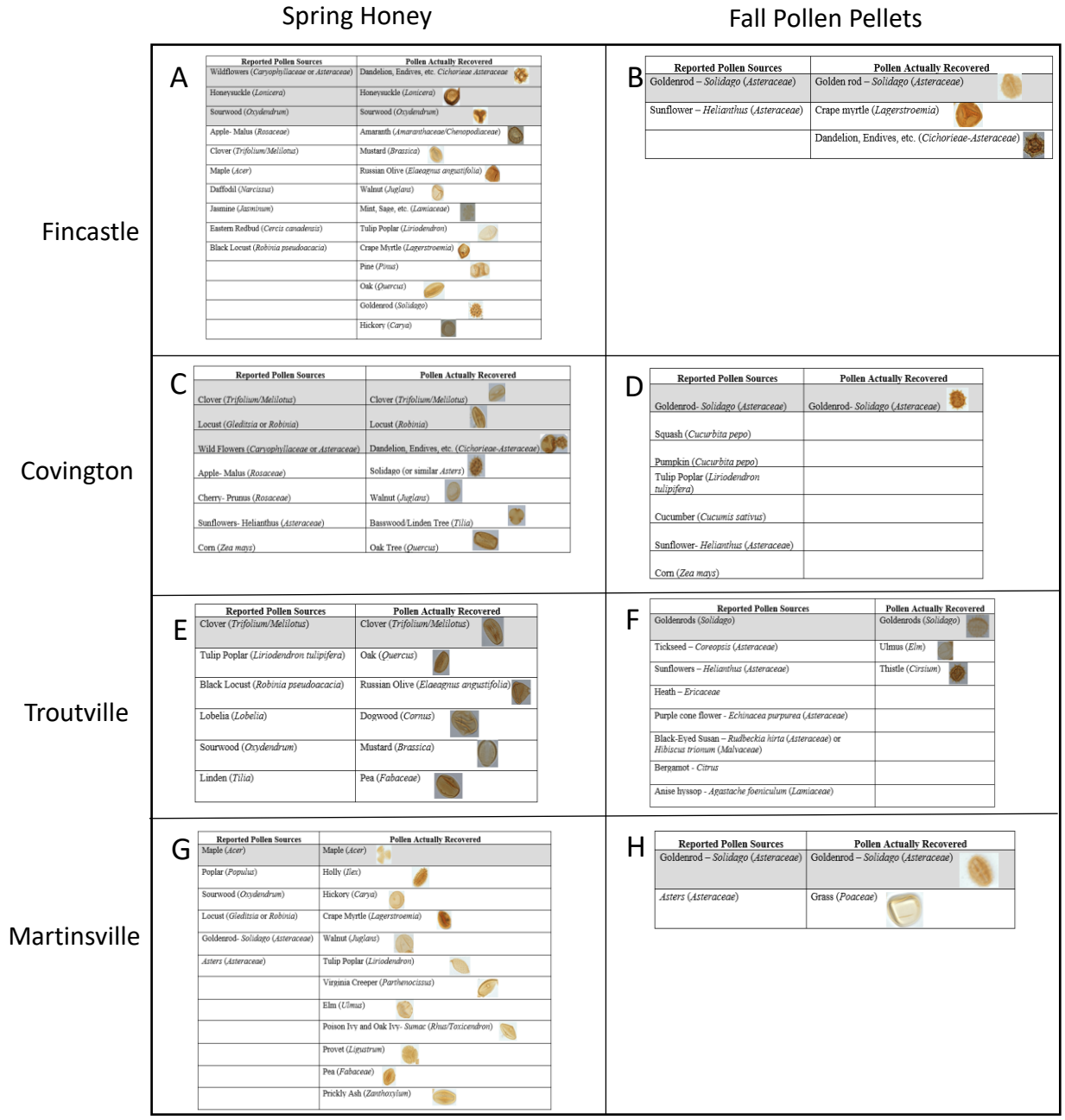

Fig. 10. Reported and recovered pollen from spring honey and fall pollen pellets from sites. Fincastle pollen from spring honey (A) and pollen from fall pollen pellets (B). Covington pollen from spring honey (C) and pollen from fall pollen pellets (D). Troutville pollen from spring honey (E) and pollen from fall pollen pellets (F). Martinsville pollen from spring honey (G) and pollen from fall pollen pellets (H). The shaded sections of the table indicate reported

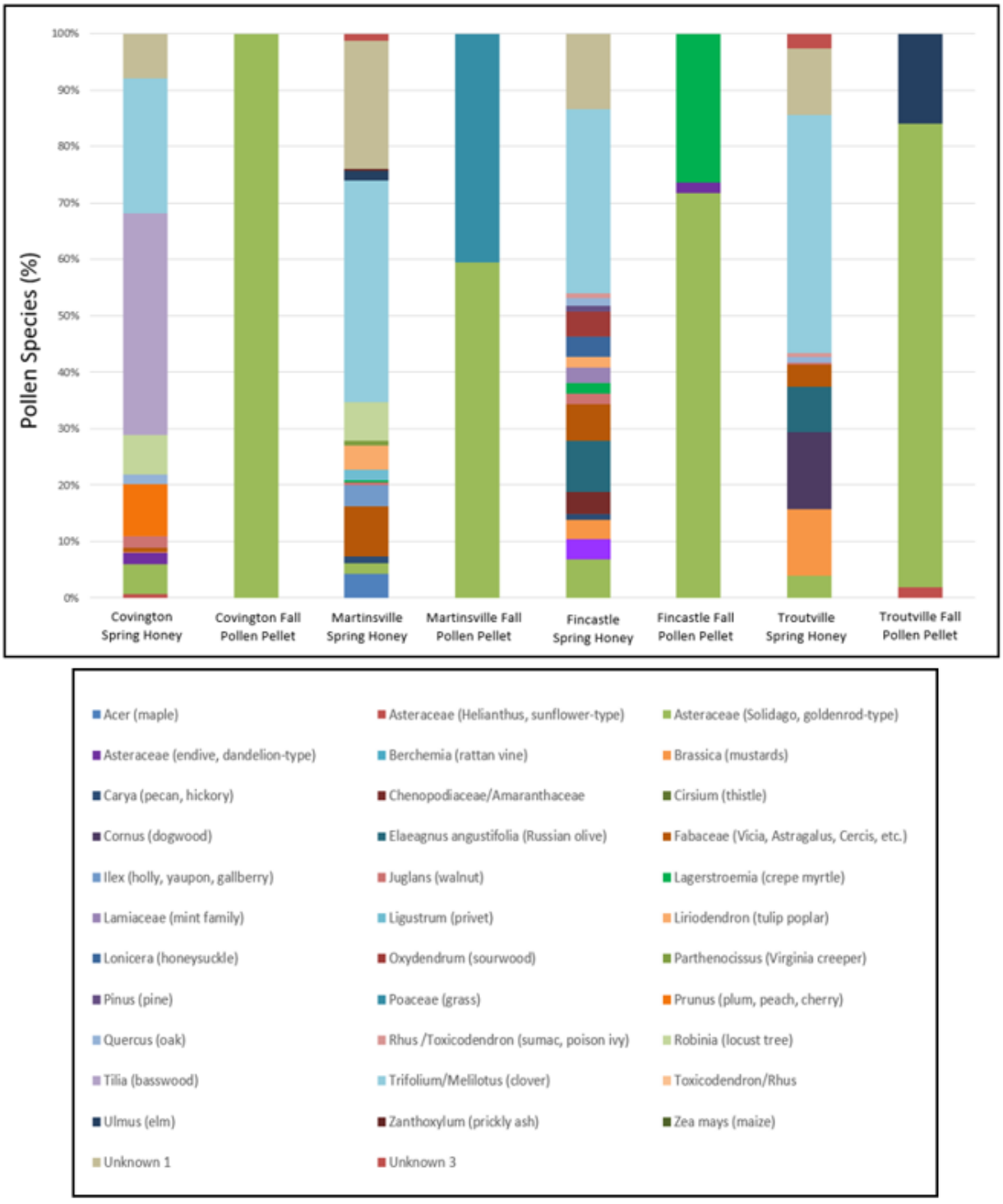

Fig. 11. Pollen recovered from each sample site in spring honey and fall pollen pellets. This figure shows the percentages of the pollen species recovered from each location's spring honey as well as each location's fall pollen pellet samples. Species diversity is the highest in Fincastle spring honey, with 18 species recovered.

Fig. 12. Gut dissection.

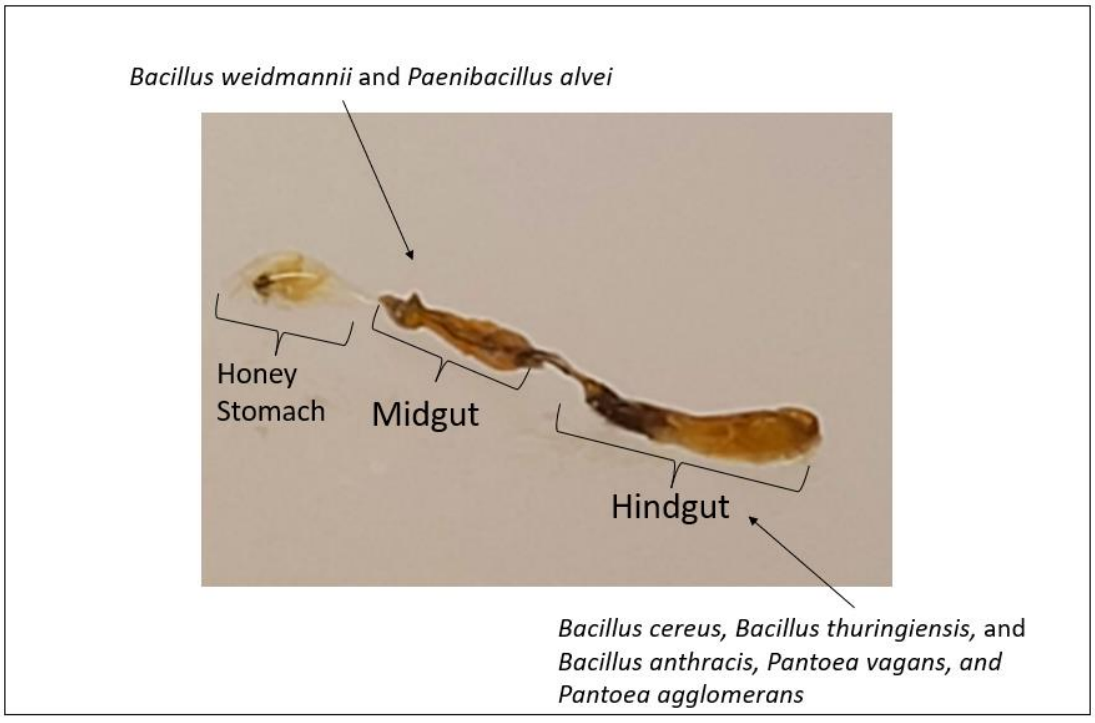

Fig. 13. Labeled dissected gut. Species found strictly in the midgut and hindgut are labeled.

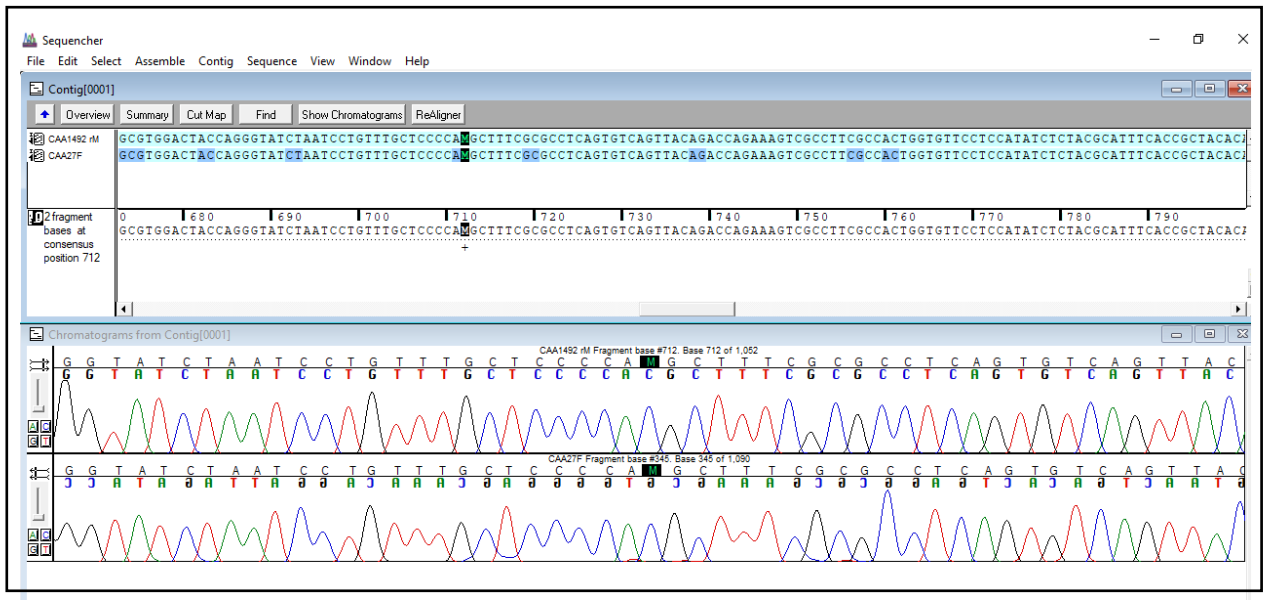

Fig. 14. Building a contiguous sequence. Once forward and reverse reads of the 16S rRNA gene were received, the sequences were edited, choosing nucleotide base pairs based on the chromatogram left undecided by Eurofins Scientific, and removing the beginnings and ends of the sequences that did not have clean peaks. Once a contiguous sequence was built and edited, the sequence was pasted into NCBI BLAST for microbe identification.

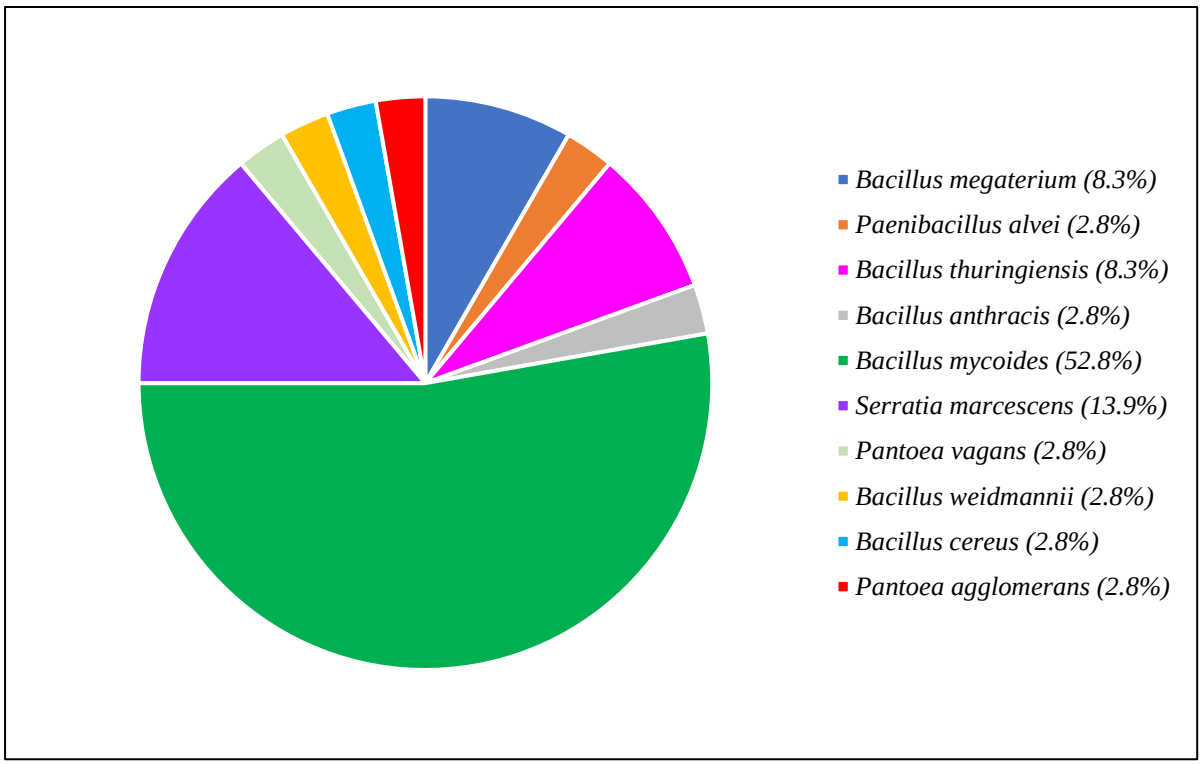

Fig. 15. Cultivable gut microorganisms in the southwest Virginia bee gut. Percentages were calculated based on all species identified across sample sites.

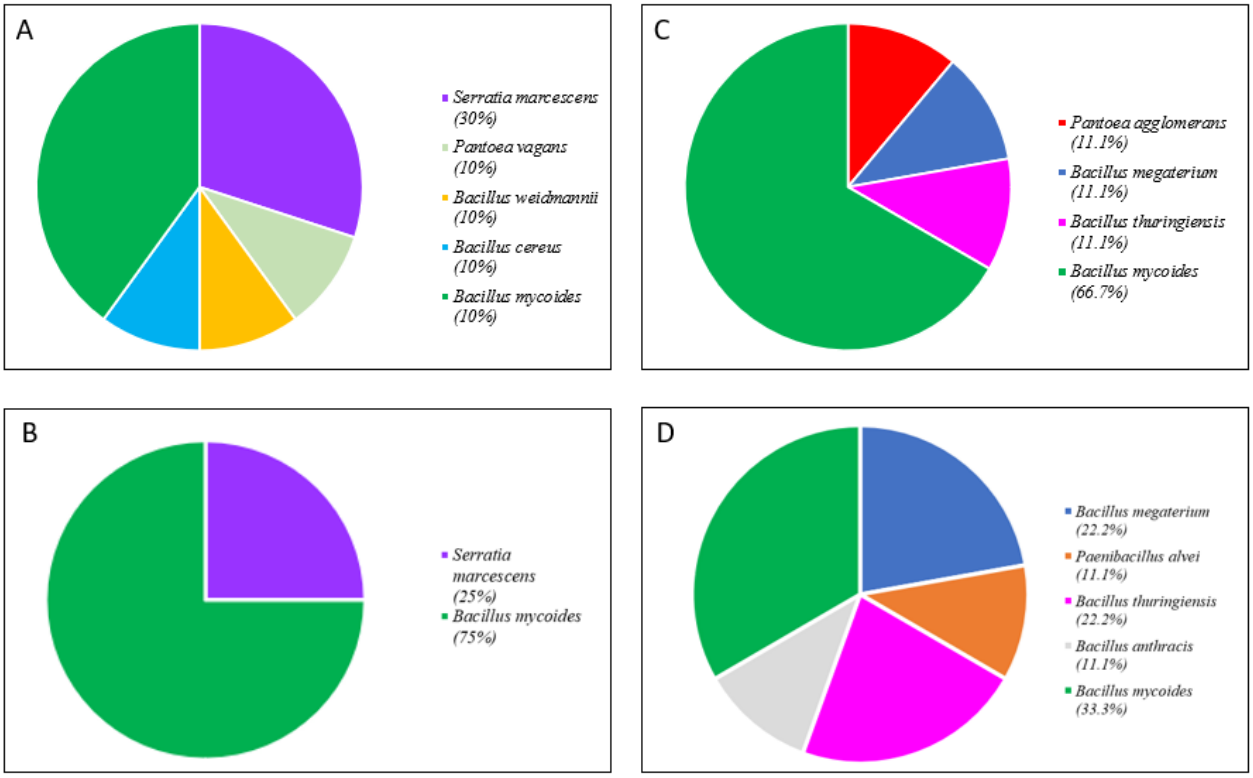

Fig. 16. Cultivable communities from the whole bee gut by sample location: (A) Fincastle, (B) Covington, (C) Troutville, (D) Martinsville. Percentages were calculated based on the species that were identified at each sample site in the whole gut.

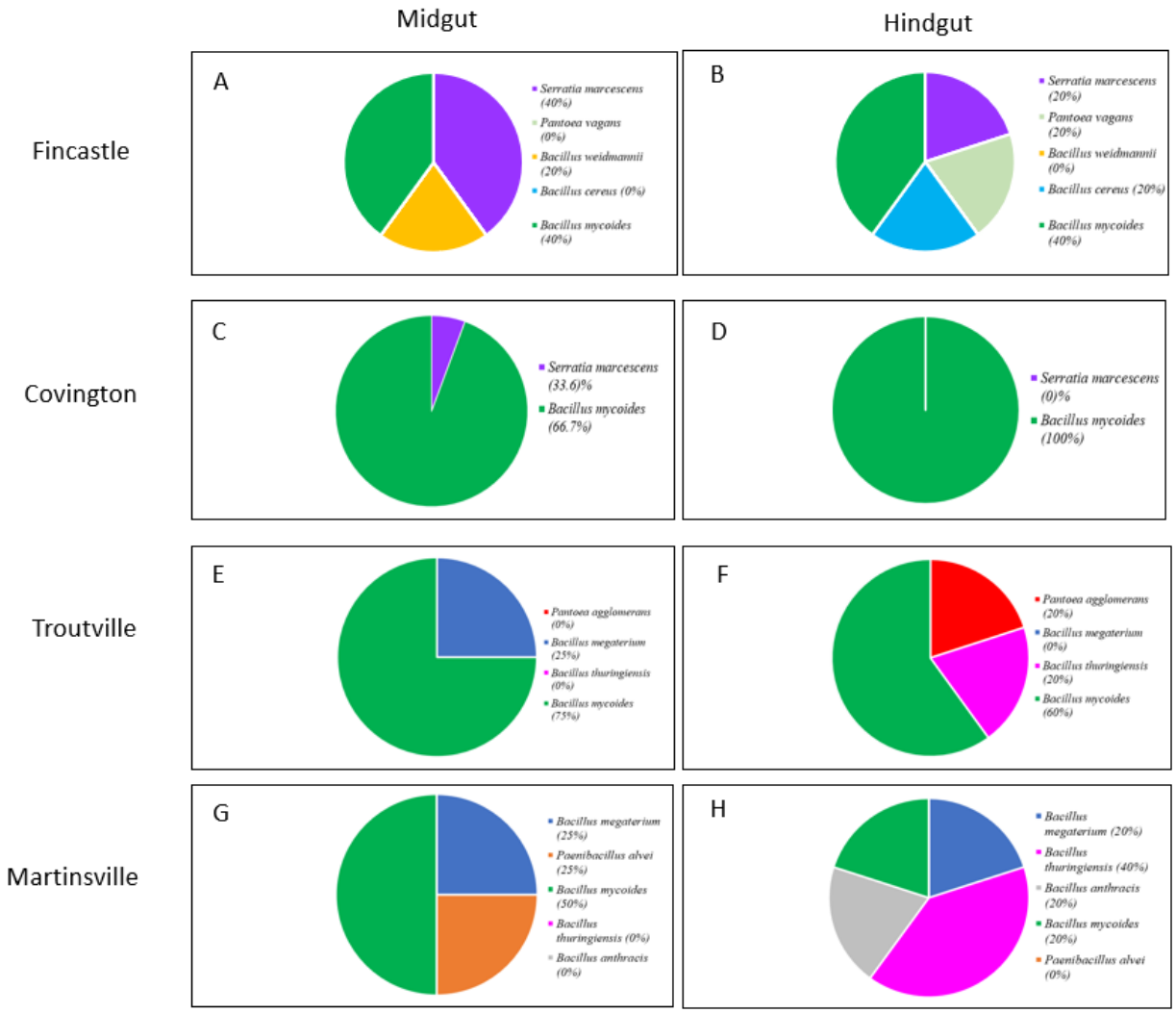

Fig. 17. Cultivable communities from the bee midgut and hindgut by sample location. Percentages were calculated based on the species that were identified at each sample site in each gut region.

Literature Cited

Ahmed, S. and Othman, N. H. (2013). Review of the Medicinal Effects of Tualang Honey and a Comparison with Manuka Honey. *The Malaysian journal of medical sciences:* vol. 20,3.

Alaux, C., Ducloz, F., Crauser, D., and Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. *Biology letters*, *6*(4), 562-565.

Al-Waili, N. S., Al-Waili, F. S., Akmal, M., Ali, A., Salom, K. Y., and Al Ghamdi, A. A. (2014). Effects of natural honey on polymicrobial culture of various human pathogens. *Archives of medical science: AMS*, *10*(2), 246.

Andrei, G. and Snoeck, R. (2021). Advances and Perspectives in the Management of Varicella-Zoster Virus Infections. *Molecules*, *26*(4), 1132. MDPI AG. Retrieved from http://dx.doi.org/10.3390/molecules26041132

Antonova, N. P., Vasina, D. V., Lendel, A. M., Usachev, E. V., Makarov, V. V., Gintsburg, A. L., and Gushchin, V. A. (2019). Broad bactericidal activity of the Myoviridae bacteriophage lysins LysAm24, LysECD7, and LysSi3 against Gram-negative ESKAPE pathogens. *Viruses*, *11*(3), 284.

Bibi, S., Husain, S. Z., and Malik, R. N. (2008). Pollen analysis and heavy metals detection in honey samples from seven selected countries. *Pak J Bot*, 40(2), 507-516.

Bunk, B., Schulz, A., Stammen, S., Münch, R., Warren, M. J., Jahn, D., and Biedendieck, R. (2010). A short story about a big magic bug. *Bioengineered bugs*, *1*(2), 85-91.

Campbell, Chad. (2021). Personal Interview. 8 Jan.

Carter, D. A., Blair, S. E., Cokcetin, N. N., Bouzo, D., Brooks, P., Schothauer, R., and Harry, E. J. (2016). Therapeutic manuka honey: no longer so alternative. *Frontiers in microbiology*, *7*, 569.

Cianciosi, D., Forbes-Hernández, T. Y., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P. P., and Battino, M. (2018). Phenolic compounds in honey and their associated health benefits: A review. *Molecules*, *23*(9), 2322.

Conforti, P. A., Lupano, C. E., Malacalza, N. H., Arias, V., and Castells, C. B. (2006). Crystallization of honey at− 20 C. *International Journal of Food Properties*, *9*(1), 99-107.

Cooper, R. A., Molan, P. C., and Harding, K. G. (1999). Antibacterial activity of honey against strains of Staphylococcus aureus from infected wounds. *Journal of the royal society of medicine*, *92*(6), 283-285.

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., and Fett, R. (2016). Honey: chemical composition, stability and authenticity. *Food chemistry*, *196*, 309-323.

Didaras, N. A., Karatasou, K., Dimitriou, T. G., Amoutzias, G. D., and Mossialos, D. (2020). Antimicrobial activity of bee-collected pollen and beebread: State of the art and future perspectives. *Antibiotics*, *9*(11), 811.

Di Pasquale, G., Alaux, C., Le Conte, Y., Odoux, J. F., Pioz, M., Vaissière, B. E., and Decourtye, A. (2016). Variations in the availability of pollen resources affect honey bee health. *PloS one*, *11*(9), e0162818.

Djukic, M., Becker, D., Poehlein, A., Voget, S., and Daniel, R. (2012). Genome sequence of *Paenibacillus alvei* DSM 29, a secondary invader during European foulbrood outbreaks.

Duanis-Assaf, D., Kenan, E., Sionov, R., Steinberg, D., and Shemesh, M. (2020). Proteolytic activity of *Bacillus subtilis* upon κ-Casein undermines its "caries-safe" effect. *Microorganisms*, *8*(2), 221.

Ellis, Jamie. (2015). Field Guide to Beekeeping. *American Bee Journal.* https://americanbeejournal.com/the-internal-anatomy-of-the-honey-bee/

Engel, P., Martinson, V. G., and Moran, N. A. (2012). Functional diversity within the simple gut microbiota of the honey bee. *Proceedings of the National Academy of Sciences*, *109*(27), 11002-11007.

Engel, P., Kwong, W. K., McFrederick, Q., Anderson, K. E., Barribeau, S. M., Chandler, J. A., and Dainat, B. (2016). The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. *MBio*, *7*(2), e02164-15.

Evans, J. D., and Wheeler, D. E. (1999). Differential gene expression between developing queens and workers in the honey bee, Apis mellifera. *Proceedings of the National Academy of Sciences*, *96*(10), 5575-5580.

Fleeman, R., LaVoi, T. M., Santos, R. G., Morales, A., Nefzi, A., Welmaker, G. S., and Shaw, L. N. (2015). Combinatorial libraries as a tool for the discovery of novel, broad-spectrum antibacterial agents targeting the ESKAPE pathogens. *Journal of medicinal chemistry*, *58*(8), 3340-3355.

Forsgren, E. (2010). European foulbrood in honey bees. *Journal of invertebrate pathology*, *103 Suppl 1*, S5–S9. https://doi.org/10.1016/j.jip.2009.06.016

Hawkins, J. (2015). Investigating antibacterial plant-derived compounds from natural honey. *PLOS*. https://orca.cardiff.ac.uk/77005/

Hijazi, S., Visaggio, D., Pirolo, M., Frangipani, E., Bernstein, L., and Visca, P. (2018). Antimicrobial activity of gallium compounds on ESKAPE pathogens. *Frontiers in cellular and infection microbiology*, 316.

Jagadeesan, Y., Athinarayanan, S., Ayub, S. B. M., and Balaiah, A. (2020). Assessment of Synthesis Machinery of Two Antimicrobial Peptides from *Paenibacillus alvei* NP75. *Probiotics and Antimicrobial Proteins*, *12*(1), 39-47.

Kamber, T., Lansdell, T. A., Stockwell, V. O., Ishimaru, C. A., Smits, T. H., and Duffy, B. (2012). Characterization of the biosynthetic operon for the antibacterial peptide herbicolin in *Pantoea vagans* biocontrol strain C9-1 and incidence in *Pantoea* species. *Applied and environmental microbiology*, *78*(12), 4412-4419.

Keller, L. and Chapuisat, M. (2014). *The Princeton Guide to Evolution*. Princeton University Press. (pp. 697–702).

Kešnerová, L., Emery, O., Troilo, M., Liberti, J., Erkosar, B., and Engel, P. (2020). Gut microbiota structure differs between honeybees in winter and summer. *The ISME journal*, *14*(3), 801-814.

Kim, A., Wolf, N. M., Zhu, T., Johnson, M. E., Deng, J., Cook, J. L., and Fung, L. W. M. (2015). Identification of *Bacillus anthracis* PurE inhibitors with antimicrobial activity. *Bioorganic & medicinal chemistry*, *23*(7), 1492-1499.

Klose, S. P., Rolke, D., and Baumann, O. (2017). Morphogenesis of honeybee hypopharyngeal gland during pupal development. *Frontiers in zoology*, *14*(1), 1-14.

Kumar, P. A., Sharma, R. P., and Malik, V. S. (1996). The insecticidal proteins of *Bacillus thuringiensis*. *Advances in applied microbiology*, *42*, 1-43.

Kwong, W. K. and Moran, N. A. (2016). Gut Microbial Communities of Social Bees. *Nature Reviews Microbiology* vol. 14,6.

Locke, B. (2016). Natural Varroa mite-surviving *Apis mellifera* honeybee populations. *Apidologie*, *47*(3), 467-482Madigan, M. T., Martinko, J. M., and Parker, J. (2017). Brock biology of microorganisms (Vol. 11, p. 136). Upper Saddle River, NJ: *Pearson Prentice Hall.* 167-169.

Mandal, M. D. and Mandal S. (2011). Honey: Its Medicinal Property and Antibacterial Activity. *Asian Pacific Journal of Tropical Biomedicine* vol. 1,2.

Manyi-Loh, C. E., Ndip, R. N., and Clarke, A. M. (2011). Volatile compounds in honey: a review on their involvement in aroma, botanical origin determination and potential biomedical activities. *International Journal of Molecular Sciences*, *12*(12), 9514-9532.

Masoura, M., Passaretti, P., Overton, T. W., Lund, P. A., and Gkatzionis, K. (2020). Use of a model to understand the synergies underlying the antibacterial mechanism of H2O2-producing honeys. *Scientific reports*, *10*(1), 1-14.

Mavric, E., Wittmann, S., Barth, G., and Henle, T. (2008). Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Molecular nutrition & food research*, *52*(4), 483-489.

Mijanur Rahman, M., Gan, S. H., and Khalil, M. (2014). Neurological effects of honey: current and future prospects. *Evidence-based complementary and alternative medicine*.

Mohammad, S. M., Mahmud-Ab-Rashid, N. K., and Zawawi, N. (2020). Probiotic properties of bacteria isolated from bee bread of stingless bee *Heterotrigona itama*. *Journal of Apicultural Research*, *60*(1), 172-187.

Morita, M., Tomita, K., Ishizawa, M., Takagi, K., Kawamura, F., Takahashi, H., and Morino, T. (1999). Cloning of oxetanocin A biosynthetic and resistance genes that reside on a plasmid of *Bacillus megaterium* strain NK84-0128. *Bioscience, biotechnology, and biochemistry*, *63*(3), 563-566.

Mullen, E. K. and Thompson, G. J. (2015). Advances in Insect Physiology: Genomics, Physiology and Behaviour of Social Insects. *Elsevier,* vol. 48. p. 325-354.

Muriuki, S. W., Neondo, J. O., and Budambula N. L. M. (2020). Detection and Profiling of Antibiotic Resistance among Culturable Bacterial Isolates in Vended Food and Soil Samples, *International Journal of Microbiology*, https://doi.org/10.1155/2020/6572693

Nakata, K., Inagawa, H., and Soma, G. I. (2011). Lipopolysaccharide IP-PA1 from *Pantoea agglomerans* prevents suppression of macrophage function in stress-induced diseases. *Anticancer research*, *31*(7), 2437-2440.

Neumann, P., Pettis, J. S., and Schäfer, M. O. (2016). *Quo vadis Aethina tumida*? Biology and control of small hive beetles. *Apidologie*, *47*(3), 427-466.

Newhouse, A. E. (2005). Transformation of American elm with a gene encoding a synthetic antimicrobial peptide for resistance to Dutch-elm disease. *ProQuest Dissertations and Theses Global*. https://hollins.idm.oclc.org/login?url=https://www.proquest.com/dissertations-theses/transformation-american-elm-with-gene-encoding/docview/305392666/se-2?accountid=27472

Nolan, V. C., Harrison, J., and Cox, J. A. (2019). Dissecting the antimicrobial composition of honey. *Antibiotics*, *8*(4), 251.

Olaitan, P. B., Adeleke, O. E., and Iyabo, O. O. (2007). Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *African health sciences*, *7*(3).

Patra, A. K. (2012). An Overview of Antimicrobial Properties of Different Classes of Phytochemicals. *Dietary Phytochemicals and Microbes*, 1–32. https://doi.org/10.1007/978-94-007-3926-0_1

Pernal, S. F., and Currie, R. W. (2000). Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera L*.). *Apidologie, 31*, 387-409.

Pirk, C. W., Neumann, P., and Hepburn, H. R. (2002). Egg laying and egg removal by workers are positively correlated in queenright Cape honeybee colonies *(Apis mellifera capensis*). *Apidologie*, *33*(2), 203-211.

Queller, D. C. and Strassman, J. E. (2014). *The Princeton Guide to Evolution*. Princeton University Press. (pp. 215–219).

Rabie, E., Serem, J. C., Oberholzer, H. M., Gaspar, A. R. M., and Bester, M. J. (2016). How methylglyoxal kills bacteria: An ultrastructural study. *Ultrastructural pathology*, *40*(2), 107-111.

Rezzonico, F., Smits, T. H., Montesinos, E., Frey, J. E., and Duffy, B. (2009). Genotypic comparison of *Pantoea agglomerans* plant and clinical strains. *BMC microbiology*, *9*(1), 1-18.

Samarghandian, S., Farkhondeh, T., and Samini, F. (2017). Honey and health: A review of recent clinical research. *Pharmacognosy research*, *9*(2), 121.

Seeley, T. D., Camazine, S., and Sneyd, J. (1991). Collective decision-making in honey bees: how colonies choose among nectar sources. *Behavioral Ecology and Sociobiology*, *28*(4), 277-290.

Seehuus, S. C., Norberg, K., Gimsa, U., Krekling, T., and Amdam, G. V. (2006). Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proceedings of the National Academy of Sciences*, *103*(4), 962-967.

Spencer, R. C. (2003). *Bacillus anthracis*. *Journal of Clinical Pathology*. 56 (3): 182–187. doi:10.1136/jcp.56.3.182. PMC 1769905. PMID 12610093

Starks, C. M., Williams, R. B., Goering, M. G., O'Neil-Johnson, M., Norman, V. L., Hu, J. F., and Eldridge, G. R. (2010). Antibacterial clerodane diterpenes from Goldenrod (*Solidago virgaurea*). *Phytochemistry*, *71*(1), 104-109.

Subramanian, R., Umesh Hebbar, H., and Rastogi, N. K. (2007). Processing of honey: a review. *International Journal of Food Properties*, *10*(1), 127-143.

Sumi, C. D., Yang, B. W., Yeo, I. C., and Hahm, Y. T. (2015). Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. *Canadian journal of microbiology*, *61*(2), 93-103.

Tay, C. X., Quah, S. Y., Lui, J. N., Yu, V. S. H., and Tan, K. S. (2015). Matrix metalloproteinase inhibitor as an antimicrobial agent to eradicate *Enterococcus faecalis* biofilm. *Journal of Endodontics*, *41*(6), 858-863.

Vary, P. S. (1994). Prime time for *Bacillus megaterium*. *Microbiology (Reading, England)*, *140 (Pt 5)*, 1001–1013. https://doi.org/10.1099/13500872-140-5-1001

White, J. W. Jr. and Doner, L. W. (1980). Honey Composition and Properties. *Beekeeping in the United States Agriculture Handbook Number 335*.

Wilson, James. (2021). Virginia Beekeepers' Association Meeting.

Wolfgang, T. and Habersack, H. (1998). *Tales from the Hive*. NOVA Online. https://www.youtube.com/watch?v=I6C9th9rO0U.

Wu, J. J., Huang, J. W., and Deng, W. L. (2020). Phenylacetic Acid and Methylphenyl Acetate from the Biocontrol Bacterium *Bacillus mycoides* BM02 Suppress Spore Germination in *Fusarium oxysporum* f. sp*. lycopersici*. *Frontiers in microbiology*, 3022.

Yilmaz, M., Soran, H., and Beyatli, Y. (2006). Antimicrobial activities of some. *Bacillus spp*.

Zheng, H., Perreau, J., Powell, J. E., Han, B., Zhang, Z., Kwong, W. K., and Moran, N. A. (2019). Division of labor in honey bee gut microbiota for plant polysaccharide digestion. *Proceedings of the National Academy of Sciences*, *116*(51), 25909-25916.