Honey Bees (*Apis mellifera*) and Agricultural Chemicals: Global to Micro Perspectives

By

Ann Bernert

An Undergraduate Thesis Submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in BioResource Research,
Sustainable Ecosystems & Biotechnology

and

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APPROVED:

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Ramesh Sagili, Dept. of Horticulture Date

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Ken Johnson, Dept. of Botany and Plant Pathology Date

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Katharine G. Field, BRR Director Date

I understand that my project will become part of the permanent collection of the Oregon State University Library, and will become part of the Scholars Archive collection for BioResource Research. My signature below authorizes release of my project and thesis to any reader upon request.

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Ann Bernert Date

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Chapter 1

Global perspectives in honey bee health

Global perspectives in honey bee health

Mounting evidence reflects that our natural world is changing drastically. Global climate change, an unprecedented human population, and the sixth mass extinction event on this planet are all factors of this transformation. According to the Food and Agriculture Organization of the United Nations, the predicted population in 2050 is expected to be 9.2 billion humans in the year 2050 meaning the agriculture industry must begin to produce more to meet the growing demand for food, biofuels, and fiber. However, agriculture is at competition with urban growth as well as protected natural areas for land, water, and energy. Our planet has a finite reserve of all of these (Dirzo & Raven 2003; Tilman et al. 2011). In order to sustain the growing human population, the sustainability and efficiency of agricultural production should be a priority (Godfray 2010). As a stable food source is important to establishing developed countries, sustainable and reliable agriculture will be critical for ending poverty and world hunger. Maize, rice, soybean and wheat currently provide the majority of the world's calories yet, yields of these crops have stagnated in the past years (Ray et al. 2012). This stagnation provides incentive to explore the yield improvement in other crops in order to meet raising food demands (Figure 1).

Figure 1 A small scale farm in Monteverde, Costa Rica makes use of sustainable farming practices and natural pollinators. Pictured here are rows of maize intercropped with yucca. This farm also produces organic coffee and sugar cane. Pressure to increase yield may drastically alter current farming practices currently employed at this location.

One way to improve the efficiency of agricultural production in a number of other crops is through effective pollination. There is an estimation by the Food and Agriculture Organization of the United Nations that of the 100 crops providing the vast majority of the world's food, 71 benefit from bee pollination. Also, pollination dependent fruits are also some of the most nutritious, typically containing a high number of vitamins and antioxidants. These crops also tend to be more economically valuable, thus growing pollination dependent crops can lead to a higher farming income than non-pollination dependent crops. Some of the crops that benefit significantly from insect pollination include almonds, avocados, cherries, blueberries, cucumbers, oranges, and pumpkins.

In the United States, the value of pollination has been estimated at $15.12 billion USD and is on an upward trend (Calderone 2012). Exact estimates are hard to determine on an international scale but are no doubt substantial (Hein 2009). A number of developed countries have an estimate of the economic value and the impact of pollinators. The United Kingdom Department for Environment, Food & Rural Affairs considers the magnitude of economic value of hundreds of millions £ and that 80% of plants in Europe are pollinated by insects. Chile is another country in which that value of pollination is recognized and a number of measures are employed to help support beekeepers in the region (Figure 2). Part of the reason why the value of pollination services is increasing is due to the 300% increase in the production of pollination dependent agricultural crops in the last 50 years (Aizen & Harder 2009). However, honey bee colonies around the world have only increased by 45% and natural pollinators are declining (Aizen & Harder 2009).

Figure 2 Fallow fields in Chillan, Chile just west of the Andes Mountains. Chillan is a district of high agricultural production fit for agricultural crops similar to those used in the Willamette Valley of Oregon, USA. These crops include small fruits such as blueberries and raspberries, as well as apples, pears, and wine grapes. Many of these types of crops benefit from honey bee pollination

While honey bee colonies around the world may have been increasing, there have been significant regional losses throughout the last couple decades in the United States and Europe (UNEP 2010; Potts et al 2010). Considering the immense importance honey bees hold, these losses have raised concern about the sustainability of current beekeeping. There are a number of concerns in regards to sustainable health that include pests, pathogens, pesticides, poor nutrition, Colony Collapse Disorder, and climate change (UNEP 2010). Many studies are underway in order to understand the impact of these threats.

A particularly controversial research area has been the non-target effects of a class of insecticide known as neonicotinoids on pollinators (Yang et al. 2008; Ramirez-Romero et al. 2012; Decourtye et al. 2004). Neonicotinoids were first registered in the United States and the United Kingdom in the early 1990's and have since then been highly valued for their insect specificity and effectiveness (Sheets 2001). This class of insecticide is insect specific as it targets acetylcholine, or nicotinic, receptors (Jeschke & Nauen 2008). The binding of this chemical to these receptors results in spontaneous nerve firing which then leads to neuron signaling failure. Binding is permanent in insects and eventually leads to death (Buckingham et al. 1997; Cassida

& Quistad 2004; Jeschke & Nauen 2008). While mammals also have a type of neotinoic receptors, the insecticide cannot permanently bind to them, nor are these receptors as critical in mammal neurology as they are in insects (Sheets 2001; Cassida & Quistad 2004). Neonicotinoids are also systemic which means the chemical will spread throughout all plants parts including pollen and nectar.

Figure 3 A couple of hobbyist honey bee colonies within miles of the mass bumble bee die off that occurred in Wilsonville, Oregon over the summer 2013. Backyard beekeeping has been gaining in popularity in the United States in the recent years.

Enough concern surrounding the safety of these insecticides for honey bees has resulted in restriction of their use in France, the UK, and for application on *Tilia* species in Oregon (USDA ARS 2012). The motivation for banning cosmetic applications on *Tilia* species of this insecticide in Oregon was due to the 2013 mass bumblebee die off in the city of Wilsonville from the use of neonicotinoids to control aphids on linden trees (Black & Vaughan 2013). This was the largest bumblebee death ever documented and spurred intense public concern over the use of neonicotinoids. However, honey bee deaths were much smaller than bumblebee deaths and it seems clear that the insecticide impacts these two insects differently (Figure 3). It is

hypothesized that excessive die off was due to the synergistic effects of neonicotinoids and the linden tree nectar of which bumblebees consumed.

Laboratory studies have shown the negative impact of neonicotinoids on honey bees, but a number of these studies are criticized for using higher concentrations of neonicotinoids than concentrations predicted in real world settings (Decourtye et al. 2003; Decourtye et al. 2004; Yang et al. 2008). Another controversial aspect is that countries that have banned neonicotinoids have not observed a significant rebound in honey bee populations (USDA 2012). Similarly, there are countries that currently use neonicotinoids and have not had any significant colony loss (UNEP 2010). The countries used as case studies in defense of neonicotinoids include Canada and Australia as they currently use neonicotinoids without honey bee colony loss. However, the differences between average beekeeping styles amongst different countries, makes it extremely complex to draw conclusions from correlations such as these. For example, a major crop for pollination in Canada is canola oil seed. According to Canola Market Access Plan, canola contributes $19.3 billion per year to the Canadian economy and has been deemed "Canada's most valuable crop" by the Canola Council of Canada. In comparison to almonds, the major crop for pollination in the United States, canola plants have very nutritious pollen and nectar (Somerville 2001; Stace 1996). Almonds, on the other hand, provide a lower protein source for honey bees. The situation in Australia differs from the United States in that the major pest of United States' colonies, the varroa mite, *Varroa destructor*, has yet to arrive on Australian shores. The varroa mite arrived in the United States in 1987 bringing devastating impacts to United States' honey bee colonies (Wenner & Bushing 1996). Thus, comparisons of honey bee health between different countries are difficult.

An illustration of the dramatic differences in beekeeping practices occurs in the comparison between the United States and Thailand. These countries differ culturally, geological, agriculturally, and economically so it may be no surprise their relationship with honey bees differs as well. To begin, it is important to note how different the ecologies of these two countries are. The Unites States contains the biomes of temperate grassland, temperate shrub land, temperate deciduous forest, and a bit of temperate evergreen forest (Cain et al. 2014). In comparison, Thailand is situated much closer to the equator and is mostly tropical rainforest (Cain et al. 2014).

Figure 4 This beginning permaculture garden in Chiang Mai, Thailand, will be dependent on the numerous natural pollinators in the surrounding jungles. Development and increased use of pesticides may change the health of the jungles and pollinators in coming years.

In terms of biodiversity, tropical rainforests contain 50% of all species on earth despite covering only 11% of the land on the planet (Dirzo & Raven 2003). This biodiversity is reflected in Thailand's entomological diversity as well. Therefore, the amount of wild pollinators endemic to Thailand is quite large. As a result of this, the agricultural demand for pollination services is already filled by a saturation of natural pollinators (Figure 4). In the United States however, the demand for paid pollination services practically funds the beekeeping industry in the country.

Bee keepers in Thailand make their wages by selling honey for general food consumption or selling other honey bee products, mostly royal jelly, to traditional Chinese medicine markets (Burgett, personal interview). Despite the lack of scientific evidence of the medicinal value of royal jelly, the demand is extensive enough to fetch comfortable living conditions for beekeepers selling this product.

Figure 5 & 6 Farmer's markets in Chiang Mai, Thailand offer an assortment of wild collected plants, herbs, and insects. Market's such as these are often a venue for the sale of hunted honey.

In fact, there are a number of honey bee species native to Thailand including the giant honey bee, *Apis dorsata* and the Asian honey bee, *Apis ceranae* (Caron 1998). There are no honey bees native to the United States and only one species, *Apis mellifera*, or the western honey bee, has been introduced. Traditional beekeeping practices also exist in Thailand though not in the United States. These traditional Thai beekeeping practices make use of the Asian honey bee, *Apis ceranae*. Operations are typically small scale and utilize a fixed frame approach (Burgett, personal interview). In this style, the beekeeper usually ends up terminating the colony during

honey harvesting. Another way honey is collected in Thailand is through honey hunting. In this case, the honey hunters search for the single comb of the giant honey bee in forested areas to harvest honey from. This approach can also sometimes terminate the colony. There is more reliance on naturally harvested goods in Thailand than in the United States (Figures 5 & 6).

In the Unites States, beekeeping is much larger in scale and income is predominately driven by pollination rentals. Beekeepers truck honey bees across the country to bring their colonies to the crops that need them throughout the crop blooming season. This migratory beekeeping practice is fairly unique to United States because of the large-scale monoculture structure of agriculture established in the country.

By considering the difference in beekeeping styles across different countries, it is possible to better understand patterns of decline and health of honey bees globally. In the United States, it seems evident that the lack of genetic diversity, high demand for pollination in monoculture crops, migratory beekeeping practices, high pesticide exposure, and a number of fairly new pests and pathogens, may all be contributing to colony declines. When investigating the potential causes of colony decline in this country and around the world, it is important to consider the multifaceted nature of the problem. With global perspectives, it is possible to notice this multidisciplinary nature of honey bee health.

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Chapter 2

Fungicide sensitivity of honey bee associated fungi

Fungicide sensitivity of honey bee associated fungi

Abstract - Honey bees are exposed to a wide range of pesticides while pollinating agricultural crops. These pesticides residues are found in beeswax, nectar and pollen in the hive. Some of the pesticides found are fungicides which are not generally considered harmful to insects. However, these fungicides may negatively impact mutualistic fungi associated with honey bees causing indirect harm to colony health. Fungi are implicated for helping break down complex protein molecules and therefore allowing the honey bees to attain more nutrients from the food source. The objective of this experiment was to identify if honey bee associated fungi were affected by fungicides that honey bees are exposed to. In order to achieve this objective, a number of fungi were isolated from bee bread and the honey bee digestive tract. Five of these isolates were genetically identified by sequencing of the internal transcriber spacer regions 1 and 4 and selected for fungicide sensitivity testing. Each fungal isolate was subject to five treatments with replications. With the exception of *Mucor hiemalis,* all fungal species growth was significantly reduced ($p \leq 0.05$) by the fungicides chlorothalonil, iprodione, and boscalid at day 2 of exposure. This research suggests that while fungicides may not directly affect the honey, they may disrupt the normal balance of fungal associates in the hive. This imbalance may result in decreased nutritional attainment from bee bread thus indirectly affecting the health or behavior of the honey bee. More research is needed to identify the functional roles of fungi in honey bees.

Honey bees / fungicides / bee bread / microbiome / fungi

INTRODUCTION

Increased attention has been on honey bee health in the United States in the light of Colony Collapse Disorder and unsustainable overwintering loses in the last decade (Pettis & Delaplane, 2010; Ellis, J., Evans, J.D., Pettis, J.S. 2009; Aizen M. A. 2009). Recent research reveals that the causes for these colony losses is a complex problem and multifactorial although strong focus has been placed on the role pesticides in honey bee health (UNEP, 2010). Insecticides, namely neonicotinoids, have received much of the attention for honey bee decline yet studies have been conflicting and laboratory results have been difficult to apply to a field setting. This class of insecticide has been shown to have a damaging correlation with pollinators and has recently been shown to be addictive to bumblebees suggesting some pollinators actually seek out this apparently harmful chemical (Rundlof et al., 2015). However, France banned neonicotinoids in 1999 and still experiences Colony Collapse Disorder (USDA, 2012). In 2012, the European Commission also banned neonicotinoids and will review the impacts later this year (EPA, 2013).

Neonicotinoids are not the only pesticides honey bees are exposed to while pollinating conventional crops. Other pesticides such as fungicides, were generally considered safe for insects until recently due to a new understanding of the insect microbiome. There is a new appreciation for the symbiotic relationships between insects and microbes. Ants, close relatives of honey bees, use antibiotic producing fungi to help protect their food sources from decay bacteria (Chapela, S. Rehner, T. Schultz, U. Mueller, 1994). It is now hypothesized that fungi associated with honey bees fulfill a similar role (DeGrandi-Hoffman et al. 2012). Fermented pollen known as bee bread, provides honey bees with a number of nutritional needs such as protein and vitamins. A vast array of fungi and bacteria are associated with bee bread representing an important microbial signature. Thus far, these bee bread associated microbes

have been implicated for the pre-digestion of pollen allowing honey bees to more effectively acquire nutrients from the food source (DeGrandi-Hoffman et al. 2012). As with fungi related to ants, microbes in the bee bread have also been hypothesized to protect pollen stores from undesirable decomposition microbes.

About 70 million pounds of fungicides are applied in the United States annually according to the Environmental Protection Agency of the United States. These chemicals are important for the control of a number of fungal plant pathogens and can significantly increase crop yield. In the California almond crop, fungicides are used to control Alternaria Leaf Spot, Anthracnose, Brown Rot, Green Fruit Rot, Leaf Blight, Rust, and Scab according to CropLife Foundation. According to the same source, 82% of almonds acres in California are treated with fungicides equating to 571,000 acres receiving 1.8 million pounds of fungicide.

The California almond bloom is the largest pollination event in the United States requiring over 1.5 million honey bee colonies. These pollination efforts help Californian almond growers in producing 80% of the world's almond supply according to the Almond Board of California. It is estimated that a majority of honey bee colonies in the United States are brought to California for this pollination event exposing them to a number of similar chemicals. It is evident that honey bees are exposed to fungicides and other pesticides and inadvertently bring them back to the colony. These chemicals can be found contaminating wax, pollen, and honey. According to a recent study, there are over 150 different pesticides can be found in honey bee hives (Mullin et al., 2010).

Ironically, the highest residual fungicide concentrations are found in pollen where the role of fungi is perhaps the most important compared to any other honey bee product (Johnson et al. 2012). For this research project, we wanted to investigate how fungi isolated from bee bread

and honey bees react to some of the fungicides found in the honey bee hive. We focused on the fungicides boscalid, chlorothalonil, and iprodione. Boscalid is a generation II succinate dehydrogenase inhibitor that has a broad spectrum range against fungal diseases (McKay et al. 2011; Avenot & Michailides 2010). By inhibiting the succinate dehydrogenase enzyme, the fungus is unable to carry certain biochemical pathways in the citric acid cycle. This chemical commonly sold in a pre-mix with the fungicide pyraclostrobin and thus rarely applied to crops alone (Avenot & Michailides 2010). Iprodione works by inhibiting the germination of spores through the prevention of DNA and RNA synthesis. Chlorothalonil inhibits multiple metabolic enzymes, prevents spore germination, and disrupts cell membranes of fungi.

Of the numerous fungi isolated, we selected the species that we genetically identified and are known for producing chemically active secondary metabolites that could perhaps play a role in the bee bread fermentation or protection from decay bacteria. These species from the geneses of *Penicillium, Fusarium* and *Trichoderma*. Also selected for testing was *Mucor hiemalis*, a fungus in the zygomycota phyla. This fungal taxa is known for containing insect pathogens.

METHODS

Isolations of Asymptomatic Honey Bee Microorganisms

For the isolation of fungal strains from the honey bee digestive system, individual bees were rendered unconscious through momentary freezing environmental conditions and then dissected. The digestive system was then surface sterilized and separated by sterile scalpel into honey crop, midgut, and rectum. Each section was then cut open under aseptic conditions and the exposed material was swiped and transferred to a plate of Potato Dextrose Agar (PDA). Plates were monitored for growth and within 72 hours, new fungal growth was transferred and isolated to new plates of PDA. For isolation of fungi from bee bread, pellets were collected fresh from an incubated frame and transferred immediately to sterile petri dish for transportation to laminar flow hood. Once under aseptic conditions, bee bread samples were then further dissected and sterile needle point forceps were used to transfer small bits of fermented pollen to the center of a fresh petri plate filled with PDA. Between 48 and 72 hours of incubation, individual fungi were transferred to new plates until pure.

Molecular Identification of Fungi

Isolated fungi were grown out on Potato Dextrose Broth (PDB). Mycelium bodies were then separated from media broth by vacuum filtration. The mycelium bodies were then each freeze-dried with liquid nitrogen and crushed to a fine powder with a mortar and pestle. From these samples. DNA was extracted using DNAesay Plant. The DNA was then amplified through polymerase chain reaction (PCR) using primers ITS1 and ITS4 (Internal Transcribed Spacer) and products were visualized on an agarose gel. PCR products were cleaned using ExoSapIT and sequenced at the Oregon State University Center for Gene Research and Biotechnology. Sequence data was aligned using the BLAST tool on the NCBI website for identification of fungal isolates.

Fungicide Sensitivity Test

Fungicides were obtained in pure form at the following purity levels, Boscalid 99.9%, Iprodione 97%, Chlorothalonil 99.3%. Concentration of fungicide exposure to the fungal isolates was based on literature values of the concentration of these fungicides found in beeswax (Table I). Mass of the pure fungicide was measured on analytic balance and then dissolved in 3 ml of acetone. The fungicide acetone mixture and then added to autoclaved liquid Potato Dextrose Agar media at 50˚C in flow hood and agitated by swirling for two minutes to evaporate acetone and thoroughly suspend fungicide in media. Amended media was then poured into 15 x 100 mm petri plates at 20 ml of media per plate. Mycelium plugs 0.4cm^3 of 72 hour old fungal isolates were placed in the center of each plate. Each isolate to treatment plate was replicated four times and five fungal isolates were tested.

Data Collection and Analysis

The radial growth of each isolate was measured after 48 hours and also 6 days after inoculation. There were 3 measurements of radial growth taken per isolate for a more accurate understanding of growth vigor. Per each fungal isolate, data were compared between the radial growths of the treated fungi verse the radial growths of the control fungi. These statistical comparisons were made using a 2-Sample t-Test.

Table I. Concentrations of fungicides used in experiment.

Fungicide	Mass used in amended media (mg/L)	ppb (adjusted to purity)	Detection levels of fungicide in bee pollen (ppb)*	Solubility (water)
Chlorothalonil (99.3%)	0.7 mg	695	98,900	0.81 mg/L
Iprodione (97%)	5.8 mg	5,626	5,511	13.9 mg/L
Iprodione (97%)	13.9 mg	13,483	5,511	13.9 mg/L
Boscalid (99.9%)	4.6 mg	4,595	962	4.6 mg/L

*According to Johnson et al. 2012

RESULTS

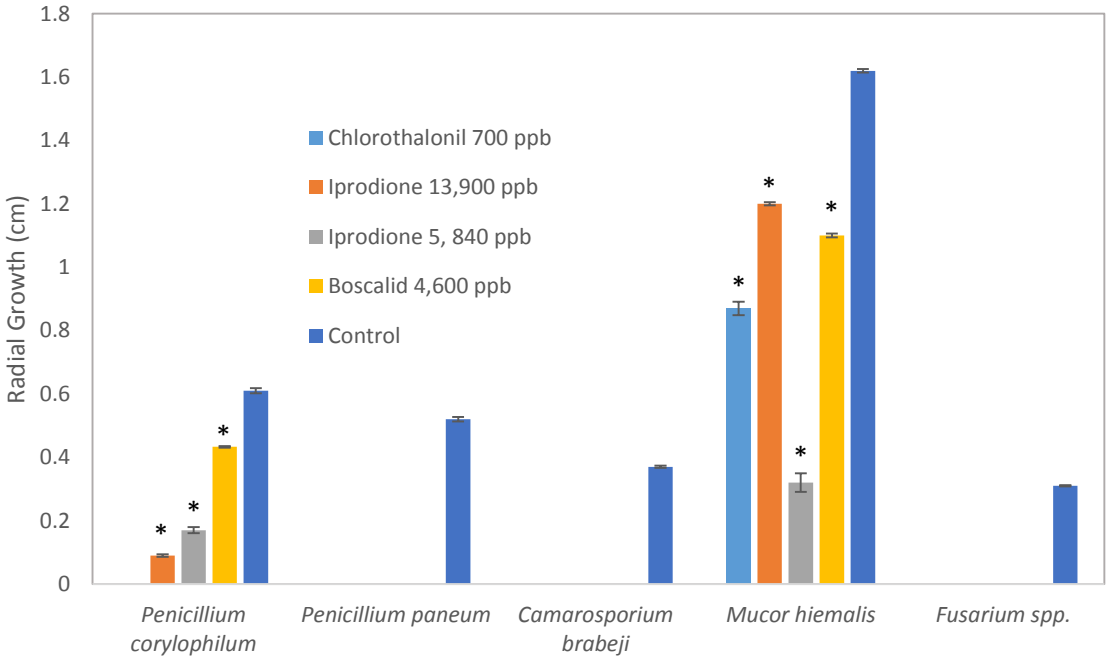

Figure 1 Difference in radial growth of fungal isolate between fungicidal treatments at 24 hours after mycelial plug inoculation on amended media plate. *Statistically significant ($p<0.05$)

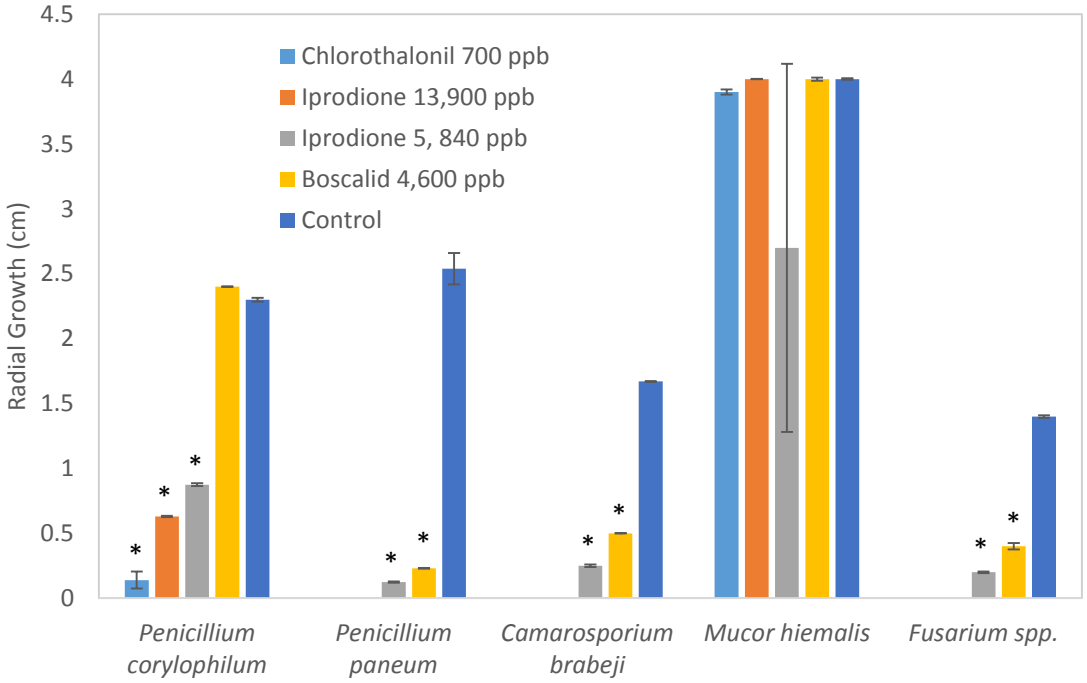

Figure 2 Difference in radial growth of fungal isolate between fungicidal treatments at 6 days after mycelial plug inoculation on amended media plate. *Statistically significant ($p<0.05$)

DISCUSSION

Based on the significant reduction of radial growth in fungal isolates when exposed to boscalid, chlorothalonil, and iprodione, it appears that fungicide exposure to honey bees could reduce the germination and growth of fungi in the honey bee colony. By day two, every fungicide level completely inhibited the growth of each *Fusarium*, *Penicillium paneum*, and *Camarosporium brabeji* (Figure 1). By day 6, radial growth inhibition was statistically significant with chlorothalonil 700 ppb and iprodione at 13,900 ppb for all of the isolates except for *M. hiemalis* (Figure 2). Boscalid at 4,600 ppb was the least inhibitory to all fungal isolates.

Considering *Mucor hiemlis* was the most resistant to the fungicides and the only one that is considered a potentially pathogenic fungus out of the either strains, there is concern that fungicide exposure may cause an unbalance of microbial communities and disrupt regular beebread fermentation processes. However, the role of symbiotic fungi in the honey bee hive is currently controversial and new evidence suggests that beebread does not use microbes for fermentative, nutrient conversion purposes (Anderson et al. 2014). Therefore, it may be difficult to fully understand the effects of fungal growth inhibition due to fungicides until their functional role is elucidated. This research supports the need to further explore the functional role in fungi.

There is still concern regarding synergistic effects between insecticides and fungicides on honey bee biology (Hooven 2013). Most of the concern is targeted at sub-lethal effects that may accumulate over time making honey bees more susceptible to infection or collapse (Pettis et al. 2012). Thus, the effects of honey bee exposure to fungicides is a legitimate concern that should be investigated in future work. The results of this work will be very helpful for growers and beekeepers to better understand the impact of chemical application on pollination dependent

crops. Perhaps, the spraying of crops in full bloom should be avoided to mitigate potential exposure to honey bees and other pollinators. Pollination dependent cropping practices developed with a better understanding of the effects of agricultural chemicals on honey bee fungal associates may help prevent potentially damaging pesticide effects in managed honey bee colonies. Further work on the microbiome of beebread and the honey bee digestive system will also be invaluable for understanding how pesticides might indirectly impact honey bee health.

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Chapter 3

Tylosin & Fumagillin - Impacts on honey bee midgut bacterial symbionts

Tylosin & Fumagillin - Impacts on honey bee midgut bacterial symbionts

Abstract- Concern for the managed honey bee population has been developing after drastic losses in the past decade. Suspected causes include new pests, pathogens, pesticides, migratory beekeeping, and nutritional deficiencies from a monoculture diet. To avoid the increasing threat of microbial pathogens, some beekeepers treat hives annually with tylosin and fumagillin, antibiotic compounds. Emerging research has identified the honey bee microbiome as an important aspect of colony health. It is unclear whether annual prophylactic treatments of tylosin and fumagillin applications deplete symbiotic microorganisms in the honey bee digestive tract. The purpose of this research was to gain an initial understanding if tylosin and fumagillin treatments significantly alter composition of *Lactobacillus* species in the honey bee midgut microbiome. Using a laboratory based, *in vitro* well-diffusion test and a caged *in vivo* experiment, four treatment groups consisting of 1) tylosin application, 2) fumagillin application, 3) tylosin with fumagillin application and 4) no chemical application as the control, were each replicated four times. Honey bees were sampled before treatment application, 5 days after treatment and 12 days after treatment. Midgut contents were then homogenized and concentration of *Lactobacilli* spp. in each midgut was assessed with a plate dilation series on MRS selective media. Colony Formation Units (CFU) were counted after plate inoculation and 72 hours of incubation. Proportions of high, medium and low CFU data were compared between treatment groups. Results suggest a need to further investigate the impact of annual antibiotic treatments and for a better understanding of the honey bee microbiome.

Apis mellifera* / fumagillin / tylosin / microbiome / *Lactobacillus

INTRODUCTION

New research concerning the honey bee microbiomes have revealed significant impacts on colony health (DeGrandi-Hoffman et al. 2012). High genetic diversity in a colony is correlated with a more diverse microbiome and a healthier overall colony (Mattila et al. 2012; Tarpy 2002). Lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* are theorized to play important functional roles such as pathogen inhibition and nutrient conversion (Evans & Armstrong 2006; Audisio et al. 2010). Honey bees are found to harbor a robust and unique set of *Lactobacillus* species in their honey crop (Vásquez et al. 2012) suggesting mutualistic relationships between honey bees and these microbes.

The human microbiome has likewise gained mounting attention for its influence on immunity, metabolism, and gene regulation (Greer et al. 2013). Interestingly, certain bacterial taxa have been found to be heritable and are associated with a significantly lower Body Mass Index in humans (Goodrich et al. 2014). When fecal transplants containing *Christinallaceae* were given to obese mice, significant weight loss occurred in the mice the following weeks (Goodrich et al. 2014). This study highlights the significant impact of the bacterial microbiome as well as the novelty of microbiome studies. Disruptions in the human microbiome composition has medical implications as it is an important regulator of immunity, metabolism, and gene expression (Dethlefsen et al 2008; Dethlefsen & Relman 2011). These medically significant disruptions are often caused by antibiotic use (Dethlefsen et al 2008; Dethlefsen & Relman 2011).

Concern for the use of antibiotics in non-human species is emerging as well. The effects of antibiotic use on the composition of microbiota in dogs has also begun to be explored (Suchdolski et al. 2009). In dogs, the effects of the antibiotic veterinary drug tylosin were studied and found to cause complex reactions in the composition of a number of bacterial taxa. This antibiotic is a macrolide antibiotic although its specific mode of action is unclear (Suchdolski et al. 2009; Reybroeck et al 2012). While some of the changes in taxa composition returned to normal over time, some taxa never returned to the dog's microbiome.

Tylosin is also a drug used in honey bee management and is used for the treatment of the disease American Foulbrood (Reybroeck et al. 2012). It was approved in 2005 after the other commonly used antibiotics, tetracyclines, became ineffective against resistant strains of the causative agent *Paenibacillus larvae* (Reybroeck et al. 2012). Honey bees are also exposed to number of other different compounds used for the purpose of pathogen control and management. Fumagillin is another antibiotic frequently used in honey bee colonies. Fumagillin inhibits methionine aminopeptidase 2 (Zhang et al. 2006) and is used for the treatment of nosemosis (Reybroech et al. 2012). Nosemosis is caused by the microsporida *Nosema apis* and *Nosema ceranae*. Microsporidia are groups of fungi that live intracellularly and disrupt normal cell functions. *Nosema* infects the cells in the honey bee digestive tract causing energetic stress and decreased colony productivity (Higes et al. 2013).

In light of the beneficial role that *Lactobacillus* species play in the honey bee systems and the mounting evidence of the ill effects of antibiotic use in other species, this research was conducted to identify if the antibiotics tylosin and fumagillin change the proportion of *Lactobacillus* species in the honey bee midgut. To our knowledge, there is currently no documentation on how these compounds affect the honey microbiome. This information could

provide insight on improving beekeeper practices to minimize potential side effects from antibiotic treatment or if treatment with these chemicals does any harm to the normal microbial associates of honey bees. We tested each chemical alone as well as the two chemicals together at concentrations that honey bees would be exposed to during treatments.

METHODS

Well diffusion test for in vitro sensitivity to tylosin and fumagillin

Using a 6 mm diameter cork borer, a well was cut out of the center of 4 cm diameter petri plates filled with solidified MRS media. The wells were then filled with 50 µl of either the treatments of 1) water control, 2) tylosin, 3) fumagillin, or 4) tylosin and fumagillin combined. Concentrations used were calculated based on the concentrations recommended by the antibiotic manufacturer and assumed 30,000 bees in a colony. The concentrations were is 1.19 mg/mL for fumagillin and 0.14 mg/mL for tylosin. After 48 hours, the zone of inhibition was measured from the edge of the well to the bacterial field of growth.

Marking test honey bees and inoculating with hive microflora

Frames of developing bees were collected 24 hours prior to anticipated bee emergence and placed in a ventilated but closed nucleus hives. Frames were then incubated overnight in nucleus hives inside a laboratory growth chamber replicating hive conditions. The newly emerged bees were brushed off the comb into a painting chamber (Figure 1) where they were hand painted with a dot of paint on their thorax (Figure 2). Painted bees were then released into a parent hive to be inoculated with the colony microbiota prior to treatment.

Filling cages and chemical application

After 48 hours of hive microbiota exposure, painted bees were collected through gentle vacuuming (Figure 3) and brought to lab. The painted bees collected from the parent hive were then counted and separated out randomly into individual cages of approximately 120 bees per cage. The four treatments were 1) control, 2) tylosin, 3) fumagillin, and 4) tylosin and fumagillin combined. Each treatment was replicated four times with a separate cage per replicate. Cages were kept in a growth chamber with conditions representing hive conditions at 33°C and 55% relative humidity. Cages were fed sugar syrup at equal parts water and sugar. Each cage was also provided a water source through a vial attached on top of the cage.

Sampling honey bees and dilution plating

For each replicate and treatment group, 10 bees are removed from cage to be sampled for each sampling time point. Individual bees were rendered unconscious through momentary freezing environmental conditions and then dissected. The alimentary canal was then surface sterilized and separated by sterile scalpel into honey crop, midgut, and rectum. Individual midguts were placed in a 1.5 mL sterile centrifuge test and then homogenized in 1 mL sterile water via a fitted pestle. This represented the stock dilution which was then vortexed and diluted by 10^{-3} two times for a total of three dilution series. A total of 10 µL of each dilution was streaked onto MRS plates under aspectic conditions. Plates were then incubated in the dark at 33°C and 55% humidity to replicate hive conditions. After 72 hours, Colony Formation Units were counted per dilution per sample. There were 2 plate replicates per sample which were averaged before data analysis.

Analyzing Colony Formation Unit data

The CFU data was organized into groups: 1) low occurrence where CFU$\leq$99, 2) medium occurrence where CFU=100-199, and 3) high occurrence where CFU$\geq$200. The frequencies of each of these groups were compared between the time points and treatment groups using a Chi-squared (X^2) test.

Figure 1 Newly emerged bees from incubated frame are brushed into box to be painted.

Figure 2 After newly emerged painted bees spent 48 hours in a mother colony to be inoculated with normal hive microflora, they were removed from hive and brought back to lab. (Photo by Stephen Ward)

RESULTS

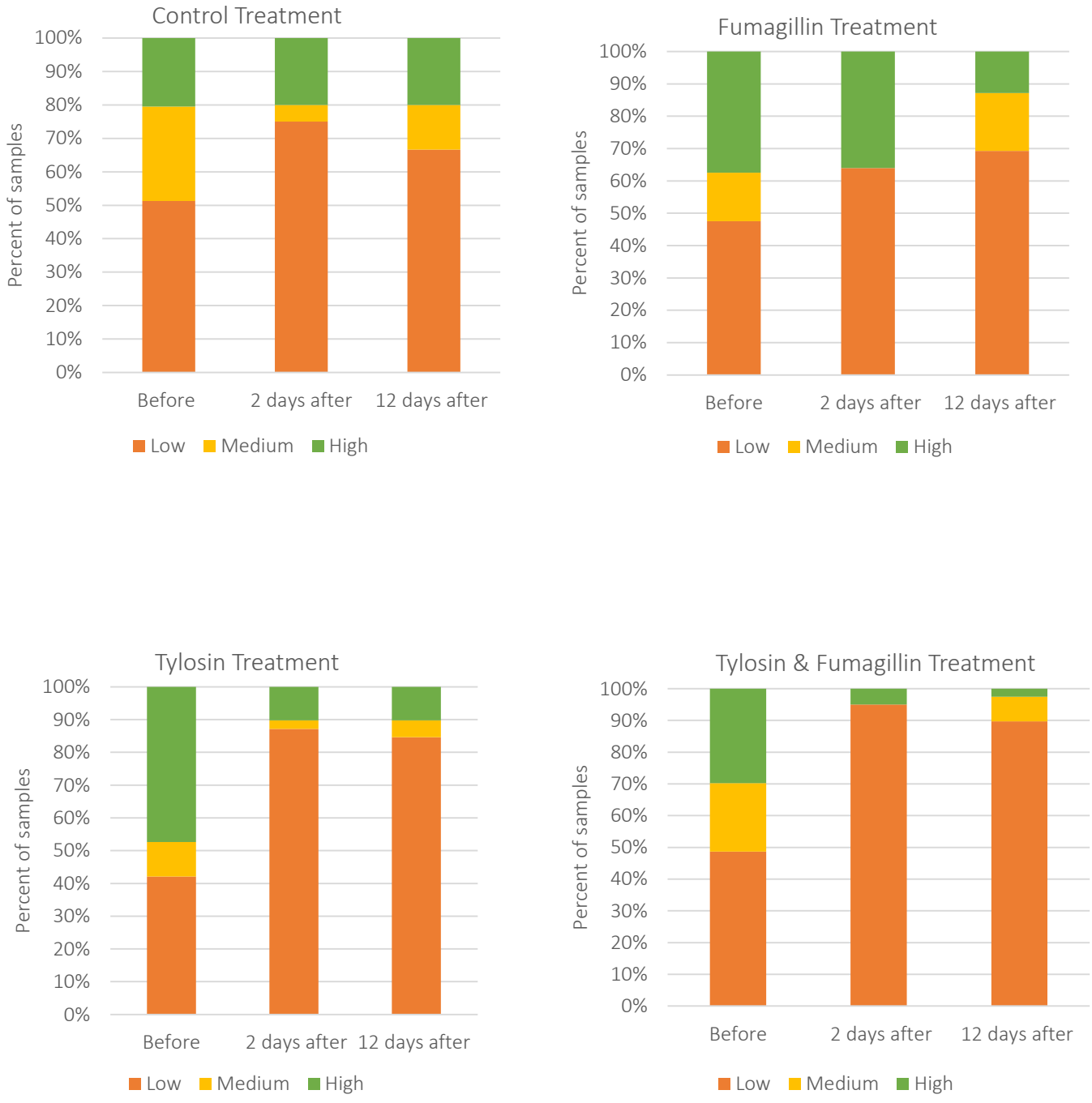

Figure 3 The proportion and change in category of Colony Formation Units (Low, Medium, or High) per treatment group over time points

Table I. Chi-Squared statistics per category of CFU frequency change between time points per treatment group. *Statistically significant at $p \leq 0.05$

Treatment	p value	X^2
Control	0.208	5.882
Fumagillin	0.026*	11.01
Tylosin	<0.001*	24.67
Fumagillin & Tylosin	<0.001*	23.44
Before proportions of all treatments	0.204	8.492

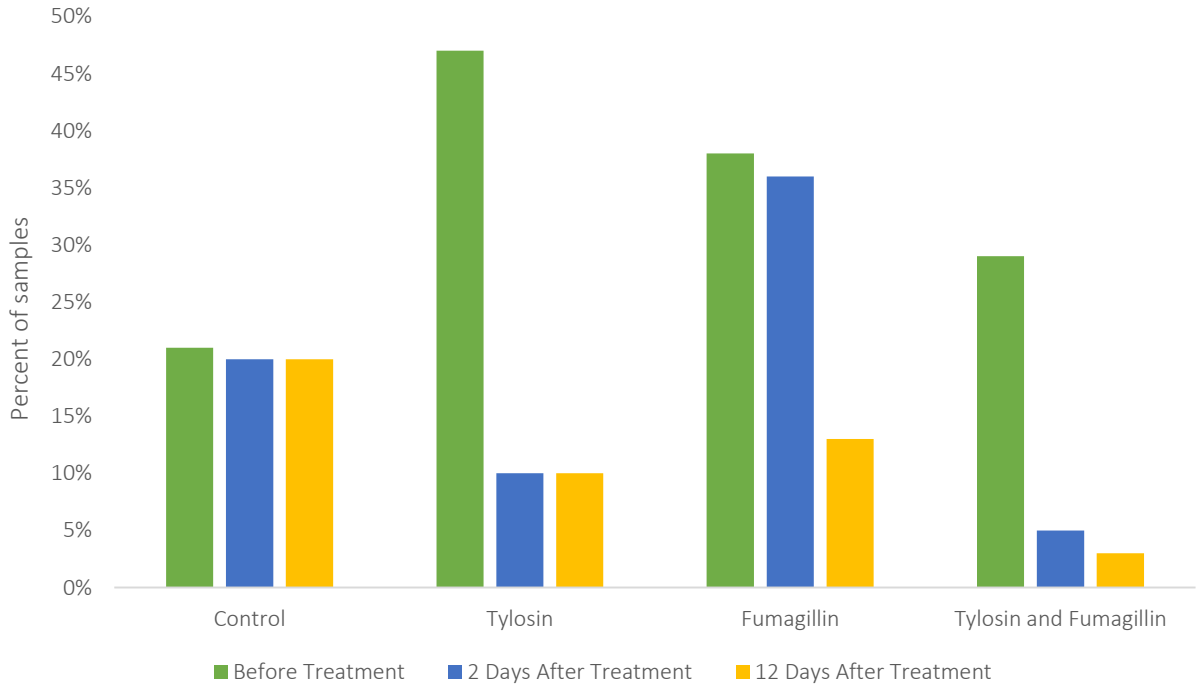

Figure 4 The change in the proportion of high CFU samples between treatment groups and time points of before chemical treatment, 2 days after treatment and 12 days after treatment.

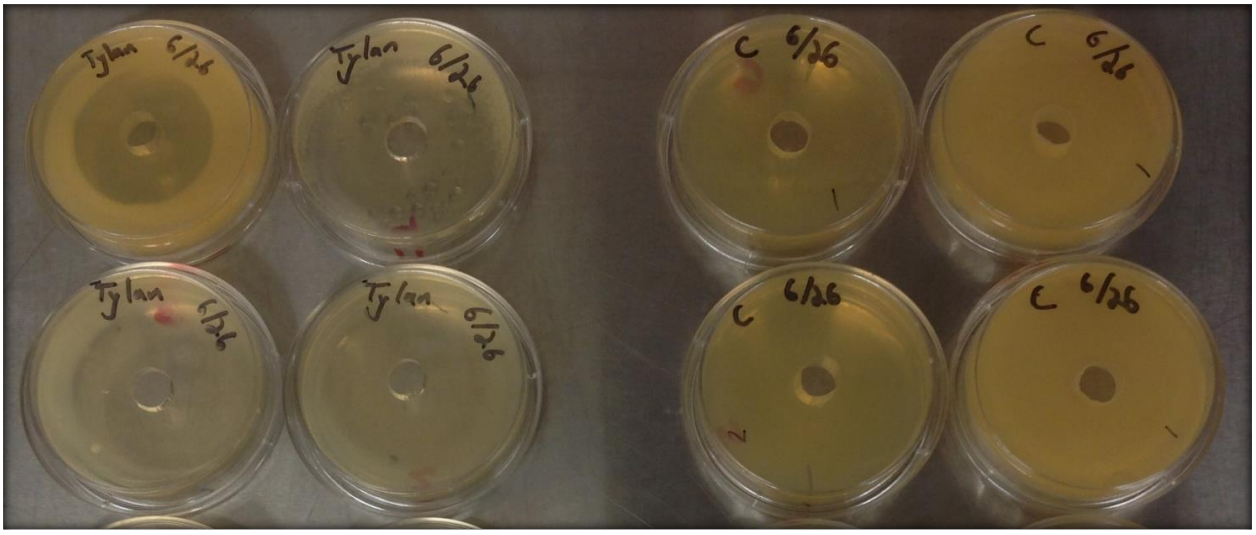

Figure 5 Preliminary *in vitro* results showed that tylosin was able to inhibit honey bee midget bacterial.

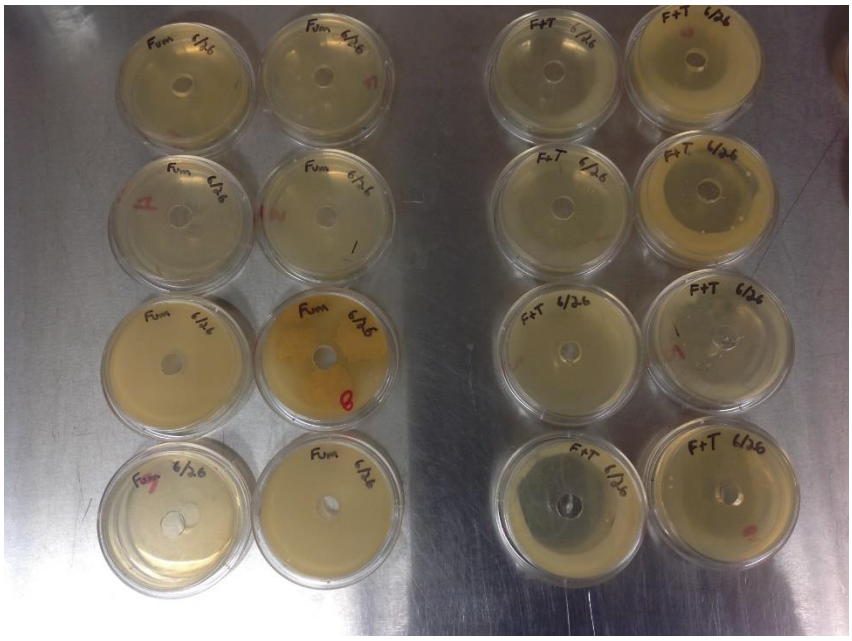

Figure 6 Preliminary *in vitro* results suggested there were no synergistic effects between tylosin and fumagillin.

DISCUSSION

According to *in vitro* well diffusion results, tylosin is capable of significant growth inhibition in *Lactobacillus* species (Figure 5 & 6). According to the *in vivo* results, tylosin also significantly alters in the proportion of *Lactobacillus* species in the honey bee midgut (Figure 3 & 4; Table I). This may suggest that tylosin decreases the concentration of *Lactobacillus* species in the honey bee midgut Whether or not this inhibition is detrimental to the honey bee health short or long term is still unclear. While fumagillin did not have any effect on *Lactobacillus* species *in vitro* (Figure 6), there appeared to be a synergistic effect with tylosin *in vivo* (Figure 4) suggesting the need to further understand if tylosin and fumagillin should or should not be used in treatment at the same time (Figure 3; Table I).

While these results provide some important initial insight into microbiome and chemical dynamics in the honey bee midgut, there is further research that needs to be conducted about this complex topic. It is still unknown how fumagillin and tylosin treatments affect the honey bee midgut mircobiome in field and real world environments. Also unknown is how exactly vary levels of *Lactobacillus* species in the midgut affect honey bee health in the long term. What exact functional roles these microbes fulfill in the midgut are also unknown.

Understanding the honey bee microbiome will be important for the advancement of honey bee health. Ideally, research on the honey bee microbiome can help develop effective pest and pathogen control protocols and ensure maximum honey bee metabolic abilities.

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