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Potential for probiotics to mitigate environmental stress in Western honey bees

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Abstract

The use of probiotics, particularly strains of lactobacilli, presents a promising strategy for addressing key threats to *Apis mellifera*, such as *Varroa* mites and *Paenibacillus larvae*. Lactobacilli are part of the honey bee hindgut microbiota, and some probiotic strains have been shown to enhance host immunity and buffer against pathogens. In addition, certain *Lactobacillus* strains can inhibit *P. larvae* growth, potentially preventing outbreaks. There is also some evidence that lactobacilli increase resistance against *Varroa* mites through immune system modulation or hive environment alteration. The objectives of this thesis were to: (1) review the complex interactions between *P. larvae* and other microorganisms within the bee gut microbiota; (2) perform an empirical study delivering probiotics to honey bee colonies across diverse landscapes. The field study revealed that oral administration of LX3 via protein patties significantly reduces mite infestations relative to a no-LX3 patty control across all environments, whereas topical spray applications do not yield the same success. This approach could serve as an environmentally friendly, sustainable method to improve bee health, crucial for maintaining ecological balance and supporting global agriculture.

Keywords

Apis mellifera, honey bee, lactobacilli, probiotics, *Varroa destructor*, mites, *Paenibacillus larvae*, American foulbrood, patty, microbiota.

Summary for Lay Audience

Honey bees, vital pollinators in the agricultural sector, are increasingly threatened by pests and pathogens, specifically *Varroa* mites, which are the number one cause of overwinter failure in Canadian beekeeping, and *Paenibacillus larvae*, the pathogenic bacterium that causes American foulbrood (AFB) disease. Beekeepers use increasingly sophisticated pest management strategies but with diminishing returns. There is therefore a sector-wide need for innovation in honey bee pest control and pathogen management. One approach, which I will refer to as microbial therapeutics, is to supply large numbers of bees within colonies a dose of living beneficial bacteria ('probiotics') that can potentially bolster the bee's immune and defense mechanisms. In this thesis I explore this idea in two ways. First, I present a review and deep synthesis of the literature to offer support and explore the limitations of using microbial therapeutics to control AFB. I conclude that there is a need to shift away from managing AFB strictly with antibiotics, and create a more sustainable approach, involving the integration of the entire microbial ecology associated with honey bees. Second, I conducted an empirical study that puts these ideas to the test. Specifically, I tested whether the supplementation of hives with lactobacilli, either orally through a patty or topically through a spray, in three different environments – agricultural, forage-rich and urban – could reduce their susceptibility to *Varroa* and *P. larvae* under the varied conditions that beekeepers might face. I found that the oral administration three-strain lactobacilli consortium (LX3) via the BioPatty helps to keep *Varroa* mite infestations low within treated colonies. It is noteworthy that the continuous use of a vehicle patty, which is common practice in commercial apiaries, increases *Varroa* mite infestations.

In total, my thesis highlights that carefully selected and thoroughly researched probiotic bacteria have the potential to mitigate honey bee pathogens. Their application can contribute to sustainable integrated pest management in the beekeeping industry. My research therefore contributes to pure and applied aspects of bee biology and suggests a new direction for managing bee health. Rather than targeting a single pathogen, we must address the entire microbial environment of bees, including all harmful microbes, to ensure a sustainable future for honey bees, the beekeeping industry, and crop pollination.

Co-Authorship Statement

The experiments and data analyses within this thesis were primarily conceived, performed, and analyzed by Andrew Pitek with supervision and guidance from Graham Thompson. The manuscripts presented were written by Andrew Pitek with input from the co-authors.

Chapter 1: General introduction.

Andrew Pitek and Graham Thompson conceived the concepts of the manuscript.

Chapter 2: 2 Disentangling the microbial ecological factors impacting honey bee susceptibility to *Paenibacillus larvae* infection

Andrew Pitek, Brendan Daisley, Elizabeth Mallory, Anna Chernyshova, Emma Allen-Vercoe, Gregor Reid and Graham Thompson contributed to the conceptualization of the ideas presented. Andrew Pitek and Brenda Daisley drafted the manuscript and generated the figures. All authors helped revise the manuscript.

Chapter 3: Probiotic effects on ectoparasitic mite infestations in honey bees (*Apis mellifera*) are modulated by environmental conditions and route of administration

Andrew Pitek and Graham Thompson conceived the experiments. Andrew Pitek performed experiments, collected data, and analyzed data for all experiments with minor exceptions. Gurpreet Dhami performed the real-time PCR analysis with DNA template that I supplied to her. John Chmiel and Anna Chernyshova assisted with the data collection. John Chmiel and Brendan Daisley provided scientific input and assisted with data analysis.

Chapter 4: General Conclusion.

Andrew Pitek prepared this section with input from Graham Thompson and others.

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Glossary of Terms

AFB (AMERICAN FOULBROOD)	A highly contagious bacterial disease affecting honey bee larvae, caused by Paenibacillus larvae.
ANTIBIOTICS	Chemical substances used to kill or inhibit the growth of bacteria.
ANTIMICROBIAL	An agent that kills or inhibits the growth of microorganisms, including bacteria, viruses, fungi, and parasites.
***APIS MELLIFERA* (WESTERN HONEY BEE)**	A species of honey bee known for its role in pollination and honey production.
BACTERIOPHAGES	Viruses that infect and replicate within bacteria, sometimes used as a treatment against bacterial diseases like AFB.
BIFIDOBACTERIUM	A genus of Gram-positive, non-motile, often branched anaerobic bacteria that are a common part of the gut flora in mammals.
BIOPATTY	A type of patty infused with a probiotic consortium used in beekeeping to support bee health.
BIOSPRAY	A spray form containing probiotics applied to beehives to deliver beneficial bacteria to the bees.
CFU (COLONY-FORMING UNITS)	A unit used to estimate the number of viable bacteria or fungal cells in a sample, where each unit represents a cell or group of cells capable of forming a colony.
COLONY COLLAPSE DISORDER (CCD)	A phenomenon involving the sudden loss of a honey bee colony's worker bee population, leading to colony failure.
DYSBIOSIS	An imbalance in the microbial community that can lead to health issues.
ECTOPARASITE	An organism that lives on the outside of the host, such as the Varroa mite on honey bees.
EFB (EUROPEAN FOULBROOD)	A bacterial disease affecting honey bees, not as lethal as AFB but still a concern for bee health.

ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS (ERIC) TYPES I-V	Genotypes of *Paenibacillus larvae* identified by repetitive element-PCR fingerprinting, each with different virulence genes.
FAO (FOOD AND AGRICULTURE ORGANIZATION)	A specialized agency of the United Nations that leads international efforts to defeat hunger and improve agriculture.
GILLIAMELLA	A genus of Gram-negative, often rod-shaped bacteria within the family Orbaceae, found in the digestive tracts of bees and involved in the digestion of their pollen-based diet.
GRAM-POSITIVE BACTERIA	Bacteria that give a positive result in the Gram stain test, which is traditionally used to quickly classify bacteria into two broad categories according to their type of cell wall.
HEMOCOEL	The primary body cavity of insects where blood circulates, and in the context of bees, a site of infection during the later stages of AFB.
HEMOLYMPH	The fluid equivalent to blood in most invertebrates, circulating within their open circulatory system and performing similar functions such as nutrient transport and immune defense.
LACTIC ACID BACTERIA (LAB)	A group of beneficial bacteria used in the production of fermented foods and potentially as probiotics for honey bees.
LACTOBACILLUS	A genus of Gram-positive, rod-shaped bacteria that are a major part of the lactic acid bacteria group, known for fermenting sugars into lactic acid and commonly used in the production of fermented dairy and vegetable products.
MICRO-THERAPEUTICS	Treatments that involve the use of microorganisms to confer health benefits, such as probiotics.
MICROBIOTA	The community of microorganisms (including bacteria, fungi, and viruses) living in a particular environment, such as the gut of honey bees.

OMAFRA (ONTARIO MINISTRY OF AGRICULTURE, FOOD & RURAL AFFAIRS)	The provincial ministry responsible for the agriculture sector in Ontario, Canada.
OXYTETRACYCLINE	An antibiotic commonly used in beekeeping to prevent infections like AFB, which acts by inhibiting protein synthesis in bacteria.
PAENIBACILLUS LARVAE	A Gram-positive, spore-forming bacterium that causes American Foulbrood disease in honey bee larvae.
PATTY	A food supplement for bees, often made from pollen substitute, which can be infused with probiotics.
PBS (PHOSPHATE BUFFER SALINE)	A buffer solution used in biological research, including the preparation of probiotics for bees.
PROBIOTICS	Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.
QPCR (QUANTITATIVE POLYMERASE CHAIN REACTION)	A laboratory technique used to amplify and simultaneously quantify a targeted DNA molecule.
SERRATIA MARCESCENS	An opportunistic bacterium that can infect bees and is associated with increased bee mortality.
SNODGRASSELLA	A genus of Gram-negative bacteria from the family Neisseriaceae, found in the gut of honey bees and playing a role in their nutrition and immunity.
TROPHALLAXIS	The process by which social insects, like bees, share food and nutrients within the colony, which can also spread pathogens.
TYLOSIN	An antibiotic commonly used in beekeeping to prevent infections like AFB, which acts by inhibiting protein synthesis in bacteria.
VAIRIMORPHA CERANAE (PREVIOUSLY NOSEMA CERANAE)	A fungal pathogen that mainly affects honey bee species.
***VARROA DESTRUCTOR* (VARROA MITE)**	A parasitic mite that attacks honey bees, leading to colony decline.

Chapter 1

1.1 Goals of the thesis

The use of probiotics, particularly specific strains of lactobacilli, as a treatment for the key threats to *Apis mellifera*, such as *Varroa* mites and *Paenibacillus larvae*, represents a promising avenue in apicultural management. Many strains of lactobacilli are part of the natural microbiota of the honey bee hind gut and have been identified for their role in enhancing host immunity and otherwise buffering bees from pathogenic invasion via the gut-hemolymph barrier (Evans & Armstrong, 2006). Fundamental to this discussion has been that specific strains of *Lactobacillus* can inhibit the growth of *P. larvae*, potentially preventing outbreaks or otherwise mitigating the worse symptoms of this destructive disease (Forsgren et al., 2010). Additionally, the application of lactobacilli has been linked to increased resistance against the parasitic *Varroa* mite, presumably through the modulation of the bee's immune system or alteration of the hive environment that affects mite reproduction (Vilarem et al., 2023). These findings, together with those presented in my own thesis chapters below, suggest that probiotics could serve as an environmentally friendly and sustainable method to improve bee health and longevity, addressing both bacterial and parasitic challenges without the use of antibiotics or chemical acaricides, which can accumulate and have deleterious effects on bee colonies (Johnson et al., 2013). The exploration of lactobacilli as a biological control agent thus offers a dual benefit of managing disease while promoting the overall health of bee populations, crucial for maintaining the ecological balance and supporting global agriculture.

The objectives of my thesis are to:

(i) Build upon previous studies that have shown three probiotic strains *(Lactiplantibacillus plantarum* Lp39*, Lacticaseibacillus rhamnosus* GR-1, and *Apilactobacillus kunkeei* BR-1 have the potential to mitigate environmental stress in honey bee (*A. mellifera*) colonies (Daisley et al., 2019) and be an alternative treatment option for honey pests and pathogens through different modes of delivery (Daisley et al., 2023). ;

(ii) Conduct an empirical study that assesses two distinct modes of delivering probiotic strains to honey bee colonies in three diverse landscapes to potentially mitigate environmental stressors.

1.1.1 Literature review

In Chapter Two, I aim to unravel the intricate microbial ecological dynamics that render honey bees susceptible to infection by *Paenibacillus larvae*, the causative agent of AFB, a lethal larval disease (Genersch, 2010). Despite the disease's extensive history and the multitude of research efforts directed at understanding its pathogenesis, the transition from dormant spore to active vegetative growth of *P. larvae*, which heralds the onset of AFB symptoms, largely remains an enigma. My research sheds light on this critical transition phase, emphasizing the often-overlooked roles of the honey bee microbiome. I explore the 'competitive' and 'collaborative' interactions between *P. larvae* and other microorganisms within the bee environment, providing fresh perspectives on AFB pathogenesis. The investigation also delves into the potential detrimental health impacts of chronic antibiotic treatment on honey bees (Daisley et al., 2020b) and proposes innovative strategies for sustainable disease management through probiotics and microbiota management, aiming for a more holistic approach to AFB control.

The current approaches to eliminating *P. larvae* include the use of antibiotics and alternative methods such as bioactive essential oils, antimicrobial plant extracts, and bacteriophage therapy. The prophylactic application of antibiotics, particularly oxytetracycline in the USA and Canada, serves as the primary preventative method against *P. larvae*. Oxytetracycline, a broad-spectrum bacteriostatic compound, functions by suppressing vegetative *P. larvae* through reversible binding to its 30S ribosomal subunit, thereby preventing aminoacyl tRNA from attaching to the ribosomal receptor site (Chukwudi, 2016). This action inhibits protein translation and cellular replication, forcing the pathogen back into a dormant spore state. While intermittent application of oxytetracycline effectively prevents AFB symptoms, its use has been linked to adverse health effects, including reduced capped brood counts, weakened immune gene expression, and induction of gut microbiota dysbiosis (Daisley et al., 2020b).

Furthermore, antibiotic exposure has been associated with increased susceptibility to opportunistic infections and potential toxic effects on honey bee physiology.

The review underscores the critical need for a paradigm shift in managing AFB, moving beyond traditional methods that solely focus on eradicating *P. larvae*. My findings suggest that the complex interplay between *P. larvae*, the honey bee microbiome, and environmental factors significantly influences disease dynamics. By highlighting the potential of probiotic applications to harness the natural defense mechanisms of honey bees (Chmiel et al., 2021; Daisley et al., 2019; Evans & Lopez, 2004), I propose a sustainable and less invasive strategy to combat AFB. This approach not only targets *P. larvae* directly but also aims to restore and maintain a healthy microbiome within bee colonies, thereby enhancing innate immunity and resilience against AFB. My research opens new avenues for future investigations into the role of microbiota in honey bee health and disease, emphasizing the importance of microbial ecology in developing effective and sustainable disease management strategies.

1.1.2 An empirical study and test of probiotics

I investigate the efficacy of using beneficial bacteria, specifically a three-strain lactobacilli consortium (LX3), to combat *Varroa destructor* mites and *Paenibacillus larvae* pathogens in honey bees. The strains (*Lactobacillus rhamnosus* GR-1, were selected for their ability to interfere with pathogens, modulate the bee's immune system and restore the host's microbiome. Conducted across diverse environmental settings—urban, agricultural, and forage-rich habitats—my research aims were to discern the influence of these factors and the method of probiotic delivery, via edible patty and aerosol spray, on treatment outcomes.

The study revealed that oral administration of LX3 via protein patties significantly reduces mite infestations across all environments, relative to untreated patty controls, whereas topical spray applications do not yield the same success. Intriguingly, colonies that receive a protein patty devoid of LX3 exhibit the highest mite proliferation, particularly in the forage-rich setting, suggesting that supplemental feeding without probiotic intervention can inadvertently exacerbate mite issues. However, the LX3

treatment has no discernible impact on *P. larvae* loads, likely due to the low baseline levels of this pathogen in the asymptomatic colonies studied.

My findings highlight the nuanced relationship between environmental conditions, supplemental feeding practices, and the effectiveness of probiotic treatments in beekeeping. The marked reduction in *Varroa* mite levels through oral LX3 application underscores the potential of probiotics as a natural and effective means to bolster honey bee health, offering a viable alternative to traditional chemical treatments. Furthermore, the unintended facilitation of mite growth by protein patties alone calls for a re-evaluation of current supplemental feeding strategies, emphasizing the need for incorporating probiotic elements to mitigate potential adverse effects. As the beekeeping industry grapples with the dual challenges of environmental stressors and pathogen pressures, the insights gained from this study could inform more holistic and sustainable approaches to honey bee management and conservation. Moving forward, I aim to explore the broader applicability of probiotic treatments in addressing other pressing bee health concerns, ultimately contributing to the resilience and vitality of honey bee populations critical to agriculture and natural ecosystems alike.

1.1.3 General conclusions

In my fourth chapter I summarize my research and provide suggestions for future studies. One such suggestion is that future studies should consider using established colonies to enhance the accuracy and relevance of data, particularly concerning the presence and impact of *P. larvae*. Older colonies tend to have more stabilized microbiota and immune responses, potentially providing clearer insights into the interactions between bee health and bacterial pathogens (Johnson et al., 2013). Furthermore, considering the promising role of lactobacilli in managing bee health, I propose that future studies should explore the delivery of lactobacilli strains through a novel spray method. This approach could allow for a more direct and uniform application, potentially increasing the effectiveness of probiotics against threats like the *Varroa* mite (Daisley et al., 2023). Observing direct mite mortality following such treatments could offer valuable data on the efficacy of probiotics delivered in this manner, contributing to more sustainable beekeeping practices and improved colony health (Forsgren et al., 2010).

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Chapter 2

2 Disentangling the microbial ecological factors impacting honey bee susceptibility to *Paenibacillus larvae* infection

The material in this chapter has been partially adapted from a review article published in the international, peer-reviewed journal *Trends in Microbiology*. On that article I am joint-first author. The content license is reprinted in Appendix A.

*Pitek, A. P., *Daisley, B. A., Mallory, E., Chernyshova, A. M., Allen-Vercoe, E., Reid, G., & Thompson, G. J (2023) Disentangling the microbial ecological factors impacting honey bee susceptibility to *Paenibacillus larvae* infection. *Trends in Microbiology, 31*(5), 521-534

* Denotes equal contribution.

2.1 Abstract

Paenibacillus larvae is a spore-forming bacterial entomopathogen and causal agent of the important honey bee larval disease, American foulbrood (AFB). Active infections by vegetative *P. larvae* are often deadly, highly transmissible, and incurable for colonies but, when dormant, the spore form of this pathogen can persist asymptomatically for years. Despite intensive investigation over the past century, this process has remained enigmatic. Here, we provide an up-to-date synthesis on the often-overlooked microbiota factors involved in the spore-to-vegetative growth transition (corresponding with onset of AFB disease symptoms) and offer a novel outlook on AFB pathogenesis by focusing on the 'collaborative' and 'competitive' interactions between *P. larvae* and other honey bee-adapted microorganisms. Furthermore, we discuss the health trade-offs associated with chronic antibiotic exposure and propose new avenues for the sustainable control of AFB via probiotic and microbiota management strategies.

2.2 Overview of AFB pathogenesis in honey bees

Crop pollination is a key pillar to agricultural success and sustainability of international food supplies. However, honey bees (*Apis mellifera*) and other critical pollinators are rapidly declining due to a combination of factors including the continuous spread of infectious diseases within agroecosystems (Daisley et al., 2022). One important enzootic pathogen is *Paenibacillus larvae*, which is a spore-forming Gram-positive bacterium and the causal agent of American foulbrood (AFB) – a highly contagious intestinal infection that is lethal to honey bee larvae and contributes substantially to colony loss in at least 200 countries (Boncristiani et al., 2021).

Notably, the pathogenesis of AFB has been intensively studied over the past century, yet despite marked advancement in our understanding of *P. larvae* molecular mechanisms, it remains unclear what triggers asymptomatic-to-symptomatic disease transition under natural conditions and there has been meager improvement in terms of developing effective control measures. These shortcomings may be explained by the fact that current reductionist treatment approaches focus solely on eliminating *P. larvae* without adequate consideration of the greater microbial communities found in association with honey bees.

Accordingly, this chapter will focus on establishing a systems-level understanding of the complex interplay between the honey bee microbiome, *P. larvae* prevalence, AFB disease occurrence, and the microbial ecological impacts of standard disease management strategies.

2.3 A primer on the infectious cycle of *P. larvae*, and its control via antibiotics

2.3.1 Initial stages of infection

The overall biology of *P. larvae* and its virulence mechanisms have been reviewed in-depth by others (Poppinga & Genersch, 2015; Müller et al., 2015; Ebeling et al., 2016) and thus only the most relevant concepts will be highlighted here. Briefly, the infection cycle begins when honey bee larvae (usually first or second instars exhibiting a weak immune response) ingest *P. larvae* spores from contaminated food sources (Figure 2-1A).

Once in the larval midgut, the spores (inactive) undergo germination to vegetative cells (active) in response to environmental stimuli (e.g. L-tyrosine and uric acid (Alvarado et al., 2013)) causing rapid proliferation of *P. larvae*. At this stage, a chitin-degrading enzyme (PlCBP49, key virulence factor) is secreted to digest the protective peritrophic matrix and thereby gain access to the intestinal epithelium. Additionally, an S-layer protein (SplA) (Poppinga et al., 2012), several ADP-ribosylating AB-toxins (Plx1, Plx2 and C3larvin) (Fünfhaus et al., 2013; Krska et al., 2015), and a wide range of other predicted virulence factors (Erban et al., 2019) also play important roles depending on the genotype of *P. larvae*. Five of these genotypes have been identified (ERIC types I-V) based on repetitive element-PCR fingerprinting (Genersch et al., 2006; Beims et al., 2020), each possessing a slightly different set of virulence genes with a similar purpose of breaching the midgut epithelial barrier. Following subsequent invasion of the larval hemocoel, infection proceeds via the extensive secretion of metalloproteases that digest internal tissue and enable continued growth (Antúnez et al., 2011). Notably, a recent proteomics study revealed that a neutral metalloproteinase (UniProt: V9WB82) was the most important virulence factor associated with larval decay (Erban et al., 2022).

2.3.2 AFB disease symptoms

It is during the active vegetative growth period of the *P. larvae* infection cycle that AFB disease symptoms arise. These include scattered irregular brood capping and conspicuous dark, sunken and commonly punctured caps that emit a ‘foul’ odor (de Graaf et al., 2006). When the decaying larvae are pulled out from their brood comb, they form a characteristic ‘ropey’ thread that is easily identifiable during field inspections (de Graaf et al., 2006). In the final stage of infection after complete digestion, *P. larvae* transitions back into its spore state and the dead larval mass ultimately dries out to form what are known as comb ‘scales’. These scales contain billions of spores that can lay dormant (for decades in some cases (Haseman, 1961)) or be a direct source for new infections. Exacerbating the spread of disease, nurse bees – adult caste members that are behaviorally specialized for rearing larvae in the brood chamber (Amdam & Omholt, 2003) – unintentionally act as vectors of *P. larvae* dispersal through trophallaxis and physical contact associated with feeding activities. An initial infection can thus quickly

manifest into a catastrophic situation that overwhelms the colony's hygienic and immune defenses. Moreover, AFB outbreaks tend to spread quickly to neighboring colonies through robbing, colony drift, shared floral sources, and the use of contaminated hive equipment (Figure 2-1B) (Lindström et al., 2008). Such events typically lead to the simultaneous collapse of many colonies in an apiary, and as a result of the risk to those in close proximity, AFB is considered a notifiable disease in most countries.

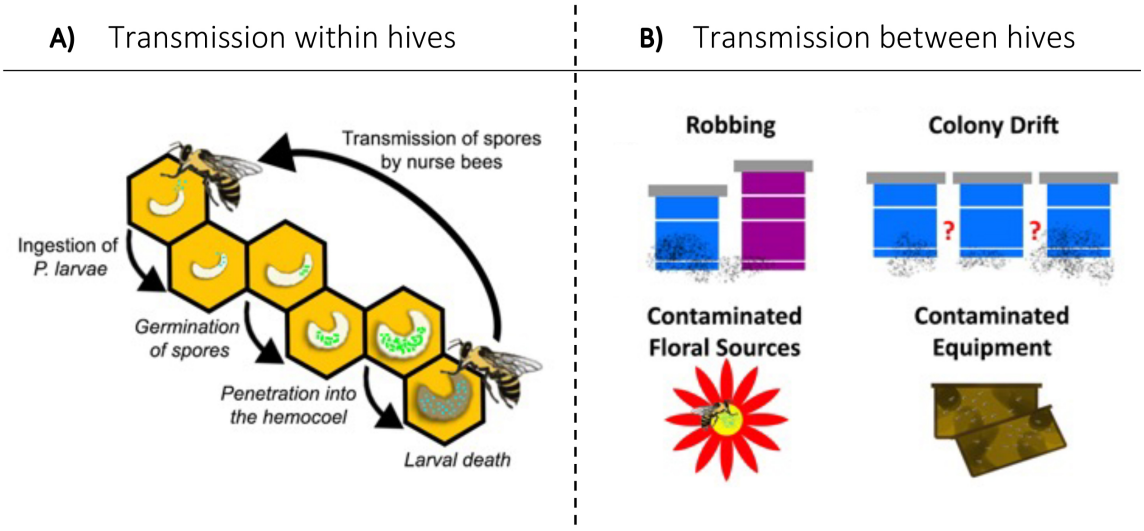

Figure 2-1. Pathogen transmission dynamics of *Paenibacillus larvae* in honey bees.

A The spread of *P. larvae* within hives can be exacerbated by nurse bees transferring spores from dead larvae (blue dots) to fresh young larvae. These spores are ingested by the larvae via trophallaxis and then germinate in the midgut lumen (green dots), eventually penetrating and invading the hemocoel. This results in larval death, after which a nurse bee will clean the cell and the cycle completes when spores are once again transferred to a fresh larva. **B** The spread of *P. larvae* between hives can occur in many ways, including robbing of other infected colonies, bees drifting from one colony to another, contact with a contaminated floral source, and contact with contaminated beekeeping equipment.

2.3.3 AFB prevention methods

Since AFB is considered to have no cure once clinical symptoms appear, the main priority is to prevent disease outbreaks from occurring in the first place. Prophylactic application of antibiotics is the primary preventative method used in most areas of the world, except for certain European countries where apicultural antibiotic use is banned (Sneeringer et al., 2019). In the US and Canada, the most common antibiotic is oxytetracycline – a broad-spectrum bacteriostatic compound that suppresses vegetative *P. larvae* via reversible binding to its 30S ribosomal subunit and subsequent prevention of aminoacyl tRNA attachment to the ribosomal receptor site (Chukwudi, 2016). Ultimately, this prevents protein translation and cellular replication, thereby forcing the pathogen back into a dormant spore state (Roberts, 1996). Antibiotic use may accordingly mask symptoms of AFB but does not eliminate *P. larvae* from an infected colony. Although disease symptoms can return once treatment stops, evidence supports that intermittent application of oxytetracycline is effective for preventing symptoms of AFB (Alippi et al., 1999) and conveniently, other bacterial diseases such as European foulbrood (EFB) as well (Budge et al., 2010).

Often overlooked are the adverse health effects associated with chronic antibiotic use in apiculture. For example, prophylactic administration of oxytetracycline (routine 14-day treatment) in asymptomatic hives is associated with reduced capped brood counts, weakened immune gene expression, and induction of gut microbiota dysbiosis (Daisley et al., 2020a). Moreover, Raymann et al. (2017) showed that tetracycline-based antibiotics can contribute to bee mortality by indirectly increasing susceptibility to other diseases, such as opportunistic infection by *Serratia marcescens*. Powell et al. (2021) also observed near identical findings for tylosin (another AFB control antibiotic) in terms of increasing susceptibility to opportunistic *S. marcescens* infection. In a similar manner, exposure to penicillin and streptomycin can increase susceptibility to the fungal pathogen *Vairimorpha* (previously *Nosema*) *ceranae* (Li et al., 2017), presumably via off-target inhibitory effects of the antibiotic on pathogen-excluding gut symbionts that otherwise protect against infection. It is noteworthy to mention that at high concentrations, some antibiotics can exert toxic effects on honey bee physiology (Pettis et al., 2004). Although,

a more pertinent concern in most cases is the off-target effects on microbial symbionts and the subsequent impacts this can have on bee metabolism and immunity (Daisley et al., 2020b). Given the well-known digestive roles of the gut microbiota (Bonilla-Rosso & Engel, 2018), it is conceivable that antibiotic exposure could also interfere with honey bee nutritional status.

In response to these potential dangers (as well as the issues of environmental contamination and spread of antibiotic resistance elements) the Food and Agriculture Organization (FAO) of the United Nations recently implemented more stringent regulatory policies, which has led to a worldwide reduction in antibiotic use by beekeepers since 2018 (FAO, 2021). However, over the same period (2018 to 2022), trends from the US and Canada show that annual colony loss rates increased from ~41% to 50% (USDA, 2022) and ~25% to 45% (CAPA, 2022), respectively. Available data from Canada further confirms that oxytetracycline usage declined from ~60% to 30% on average over the four years (CAPA, 2022). While it is difficult to ascertain the exact involvement of AFB, these data suggest that antibiotic withdrawal after decades of use could have negative consequences on colony health outcomes. Altered transgenerational immune priming could be one factor involved in the case of AFB (Dickel et al., 2022; Hernández López et al., 2014), although findings have been inconsistent or context-dependent for other infectious bacterial diseases (Ory et al., 2022) as well as viral diseases (Lang et al., 2022). Determining how repeat exposure to antibiotics (as well as pesticides with antimicrobial properties (Daisley et al., 2022)) affects immune function and long-term health trajectory of honey bees under controlled conditions is a topic worthy of future investigation.

A broad range of alternative methods have been tested for AFB control (e.g. bioactive essential oils, antimicrobial plant extracts, bacteriophage therapy, inactivated *P. larvae* bacterins, hygienic breeding, and beneficial bacteria), which are outside of the scope of this article but have been thoroughly discussed in a review (Alonson-Salces et al., 2017). An exception is that of lactic acid bacteria (LAB) supplementation explored in the following sections. Notably, while some alternative approaches hold great promise from a theoretical standpoint, large-scale field trial data is required to validate their efficacy

and at current antibiotics remain as the only FDA-approved treatment against AFB (Richards et al., 2021). Given the declining trends in antibiotic usage, efficacious alternatives as well as novel strategies for improved management of AFB are needed now more than ever.

2.4 Microbiota dysbiosis and AFB disease – an overlooked connection?

Despite the extreme contagiousness and lethality of active AFB outbreaks, quantitative PCR-based detection methods have shown that low levels of *P. larvae* are frequently present in hives lacking any visual signs of the disease (D'Alvise et al., 2019). That is, asymptomatic infections are likely far more widespread than currently recognized. The fact that spores can lay dormant for such long periods – 35 years or longer in some cases (Haseman, 1961) – without causing any signs of active AFB suggests that the process of germination is either being actively inhibited or that the molecular signals initiating germination (e.g. L-tyrosine and uric acid (Alvarado et al., 2013)) are absent in the larval gut during these asymptomatic periods. One potential scenario is that *P. larvae* germination is influenced by either collaborative or competitive interactions with other microbes found in the honey bee gut or hive environment. Supporting this notion, recent evidence suggests that the bacterial causal agent of EFB, *Melissococcus plutonius*, produces disease in a microbiota-dependent manner (Floyd et al., 2020). It is thus conceivable that the underlying microbial ecology of the larval gut, adult gut, or whole hive may similarly dictate whether other diseases, such as AFB, can or will occur. By focusing only on host physiology, rather than the superorganism including all of its co-adapted symbionts as proposed by the holobiont theory (Guerrero et al., 2013), a large component of the underlying etiology of AFB may be overlooked.

As shown in Figure 2, the larval intestinal tract (during a healthy state) is colonized by a protective layer of symbionts that acts as a physical barrier to symptomatic infection via blocking *P. larvae* access to the host epithelium. Conversely, there is an incremental depletion in symbiont abundance (and likely diversity) in early- and late-stage AFB disease during which *P. larvae* transitions from its spore state to vegetative growth and rapidly proliferates. It remains unclear, however, whether these associations are cause or

effect related. One explanatory scenario could be that bacteriocins produced by *P. larvae* (e.g., paenilamicin/paenilarvin/W2EAN2) act as drivers of symbiont depletion via their antimicrobial effects (Erban et al., 2022; Hertlein et al., 2016; Müller et al., 2014). This is unlikely the case, since bacteriocins are not produced by dormant spore-state *P. larvae* (i.e., during asymptomatic infection) and only appear after-the-fact once vegetative growth has ensued (i.e., during symptomatic infection). This suggests that multi-site microbiota dysbiosis in the hive may represent a predisposing factor to AFB, though the situation is complicated by other compounding factors (including immune suppression and co-infecting pathogens) that can simultaneously regulate disease dynamics (Figure 2-2). We explore these interactions in the following sections as they specifically relate to *P. larvae* spore-to-vegetative state disease transition.

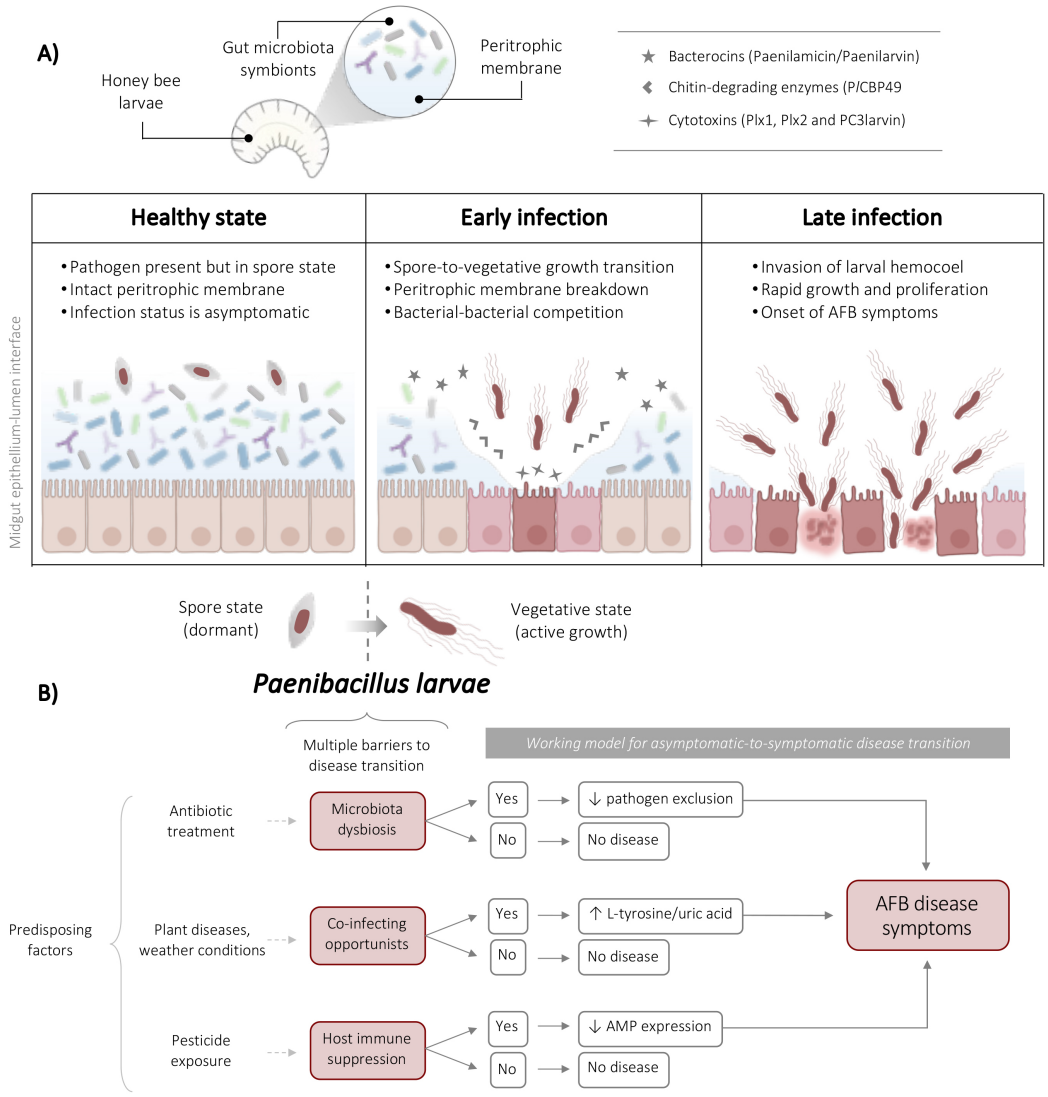

Figure 2-2. Impacts of the honey bee microbiota, coinfections, and immunity on AFB disease transition.

A The molecular mediators that govern *P. larvae* infection status in honey bee larvae are visually summarized based on a recent in-depth review (Ebeling et al. 2016). **B** Proposed barriers needing to be overcome for *P. larvae* to transition from asymptomatic-to-symptomatic infection status. Microbiota dysbiosis or depletion of symbionts in the larval gut (e.g., antibiotic exposure), as well as immune suppression (e.g., pesticide exposure) may increase susceptibility to *P. larvae*. However, for AFB to fully develop, it is likely that co-infecting opportunists capable of producing germinants (e.g., L-tyrosine, uric acid) essential to *P. larvae* growth is necessary. Abbreviations: AFB, American foulbrood; AMP, antimicrobial peptide.

2.4.1 Competitive interactions between *P. larvae* and bacterial symbionts

Riessberger-Galle et al. (2001) showed that heat-inactivated midgut extracts from adult nurse bees could, in a dose-dependent manner, inhibit the growth of vegetative *P. larvae* and suppress germination of its spores *in vitro*. These experimental findings support that concept that competitive interactions in the honey bee microbiota play a role in resisting AFB disease. They also suggest that cellular components of the microbiota contain molecules antagonistic to the pathogen. Furthermore, the total concentration of bacterial symbionts seems to be an important factor affecting *P. larvae* growth dynamics. Thus, in addition to the higher immune gene expression (inhibitory to *P. larvae*) in adults relative to larvae (Chan et al., 2009), higher loads of bacterial symbionts may also contribute (via pathogen exclusion properties) to disease resistance.

In terms of pure isolates, bacterial symbionts from both adults and larvae (including *Apilactobacillus kunkeei*) have demonstrated inhibitory effects on *P. larvae in vitro* (Evans & Armstrong, 2005; Forsgren et al., 2010), indicating that the larval microbiota also has potential to protect against infection. It is possible then, that the depletion or delayed acquisition of these bacteria – for example, due to antibiotic exposure, pesticides, or environmental factors (Daisley et al., 2020b) – may allow for *P. larvae* germination and subsequent AFB disease transition. During a natural AFB outbreak in Ontario, Daisley et al. (2019) found that third-to-fifth instar larvae from colonies presenting clinical signs and symptoms possessed almost undetectable levels of *A. kunkeei* (~10^2 or fewer target gene copies based on qPCR quantification with species-specific primers). In a later study from the same region, however, Daisley et al. (2020a) discovered that third-to-fifth instar larvae from healthy hives (i.e., asymptomatic for AFB symptoms but still colonized by *P. larvae*) had over 100-fold higher levels of *A. kunkeei* (~5x10^4 target gene copies equating to ~10,000 cells of *P. larvae* per individual). While it is tempting to speculate that depletion of *A. kunkeei* (and likely other symbionts) could be responsible for stimulating AFB disease outbreaks, these association-based findings do not allow us to decipher between cause and effect (e.g., AFB disease could be driving the decreased colonization of *A. kunkeei* rather than the other way around).

Recent evidence from experimental infection models, however, suggests that *P. larvae* inoculation does not induce any obvious change in the larval gut microbiota during the initial stages of infection, beyond altering structural compositionality due to the increase in its own relative abundance (Panjad et al., 2021). Altogether, these observations suggest that the total abundance (rather than necessarily the presence or absence) of bacterial symbionts in the larval gut may influence the permissibility of AFB disease transition. How the relative abundance of gut microbes influences health and disease in bees is an understudied topic.

2.4.2 Collaborative interaction between *P. larvae* and environmental bacteria

Even if competitive interactions were reduced or eliminated by a lack of bacterial symbionts, *P. larvae* disease transition is still dependent on the presence of certain stimuli for germination to occur (e.g. L-tyrosine and uric acid (Alvarado et al., 2013)). One possibility is that *P. larvae* relies on other bacteria for production of these germinants. *Escherichia coli* is an environmental opportunistic pathogen with many strains showing the capacity to synthesize high levels of L-tyrosine as well as secrete xanthine dehydrogenase (Crane et al., 2013) – a protein-catabolizing enzyme that can facilitate oxidative metabolism of purines to uric acid. Consistently, Daisley et al. (2019) observed that *E. coli* loads increased by over 10-fold in adult nurse bee guts during asymptomatic to symptomatic AFB disease transition under natural conditions – a phenomenon that co-occurred alongside a 10- to 100-fold decrease in Alpha, Beta, and Gamma phylotype microbiota members.

Consumption of *E. coli* contaminated pollen is a conceivable route through which adult bees could have been exposed to high levels of *E. coli* and this may have induced food-borne illness symptoms, similar to the gastrointestinal distress and dysbiosis events seen in humans (Ferens & Hovde, 2011). It is feasible that the horizontal transmission of host-adapted bacterial symbionts from adults to larvae decreased while the transmission of *E. coli* and other opportunistic species increased. Ultimately, such events could create the perfect conditions for *P. larvae* to germinate, rapidly proliferate in its vegetative growth phase, and cause AFB signs and symptoms to arise. Other opportunistic environmental

bacteria, such as *S. marcescens*, have also been isolated from diseased larvae in the past (El Sanousi et al., 1987), whereas oral supplementation with *Proteus, Enterobacter*, and *Morganella spp*. under laboratory conditions have each been shown to independently induce more than 80% mortality in honey bee larvae (Al-Ghamdi et al., 2018). Notably, *S. marcescens* is also known to be enriched in the adult gut microbiota during times of dysbiosis (e.g. induced by antibiotic exposure) and can directly elevate host mortality rates by ~10-15% after invading the hemolymph (Raymann et al., 2017).

2.4.3 Immune-microbiota interactions that regulate disease outcomes

Honey bees are not defenseless to hive invasion by environmental bacteria. Immune responses, including the up-regulation of antimicrobial peptide (AMP) gene expression (Evans, 2004), can selectively kill opportunistic pathogens while posing minimal harm to symbionts. For example, the honey bee AMP apidaecin exerts a minimum inhibitory concentration (MIC) of 1.56 µg ml1 on *E. coli*, whereas the MIC against 'core' bacterial symbionts (such as *Bifidobacterium, Lactobacillus, Snodgrassella*, and certain *Gilliamella spp.* found in social bees (Kwong & Moran, 2016; Daisley & Reid, 2021) exceeds >50 µg ml1 in most cases (Kwong et al., 2017). This preferential immune response is thought to play a key role in helping to shape their gut microbiota (Horak et al., 2020), which in effect provides an added layer of protection against infectious disease. Such synergy is especially crucial in cases where pathogens can evade one but not both systems.

Highlighting the extremes, certain *S. marcescens* strains can evade the honey bee immune system but be repelled by microbiota-derived metabolites (Raymann et al., 2018), whereas certain *E. coli* strains appear to be less affected by the pathogen excluding properties of the microbiota yet they are highly susceptible to host immune effectors such as AMPs (Daisley et al., 2019; Kwong et al., 2017). Interestingly, despite being highly host-adapted, *P. larvae* is susceptible to both honey bee AMPs (e.g., Defensin-1) and metabolites from bacterial symbionts (Cornman et al., 2013), perhaps explaining why it has evolved to specifically attack first-to-second instar larvae that have underdeveloped immune systems and inconsistent microbiota profiles (Ebeling et al., 2016). It is

noteworthy to mention that bacterial symbionts (especially *Lactobacillus* and *Snodgrassella spp.*) play a crucial role in honey bee immune regulation, and thus maintenance of healthy immune signalling and microbiota functioning is in reality a bidirectional process biologically favouring healthy homeostasis and disease resistance (Daisley et al., 2020b). Cumulatively, this indicates that an optimal way to improve resistance to AFB (and potentially other bacterial diseases) is to support the natural immune and microbiota defense systems of honey bees.

2.5 Probiotics as a sustainable solution for AFB prevention

It is clear that consideration of the honey bee microbiota must be integrated into disease management strategies. As noted above, antibiotics are the most widely used control strategy for AFB, although there is substantial evidence to suggest this can have serious long-term consequences on host health via off-target deleterious effects on their gut microbiota (Daisley et al., 2020b). This presents a difficult situation for the beekeeping industry since real-world statistics (based on available data from Canada) suggest that antibiotics do help prevent annual colony loss (CAPA, 2022). There could be many factors affecting these trends, but assuming the association is valid, these observations could indicate that commercial bee stocks (chronically exposed to antibiotics in the past) have evolved a dependency on antibiotics. Operations deciding to forgo any antibiotic treatment may thus render their colonies vulnerable to disease risk, as predicted under a Darwinian model (Neumann & Blacquière, 2017). Some naturalist theories suggest that antimicrobial plant-derived extracts and essential oils could be adequate replacements for pharmaceutical-grade antibiotics (Flesar et al., 2010; González & Marioli, 2010). But these strategies are also problematic since they too are single-purposed – to kill a pathogen of interest (e.g., *P. larvae*) – with their non-selective impacts on honey bee symbionts largely ignored. A more sensible approach may be the application of honey bee-tailored probiotics.

2.5.1 Probiotics as a potential solution for sustainable control of AFB

Probiotics are considered "live microorganisms that when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). While guidelines surrounding probiotics have faced intense scrutiny in their development and regulation, those intended for honey bees have not. So far, the goal of candidate probiotics in apiculture research has mainly focused on improving colony performance and combatting widespread pathogens that affect both larvae and adult honey bees (Alberoni et al., 2016). In relation to AFB, at least 250 strains of mostly LAB derived from bees, plants, or fermented foods have been screened for probiotic potential *in vitro* based on their *P. larvae*-inhibiting properties (Evans & Armstrong, 2005; Babrud et al., 2019; Iorizzo et al., 2020; Kačániová et al., 2018; Ramos et al., 2019; Yoshiyama et al., 2013). Despite many strains with capacity to inhibit spore germination or vegetative growth of *P. larvae*, their mechanisms of action to confer health benefits remain poorly understood. The intrinsic ability of LAB strains to produce lactic acid (which can lower pH and thereby exclude the growth of many pathogens) has been proposed as a prerequisite for *P. larvae* inhibition (Mudroňová et al., 2011). However, the differential inhibition properties between closely related LAB suggest that other strain-specific factors may also be involved (Audisio et al., 2011). Of note, the potential host-mediated effects of probiotics (e.g. effects on immune system, microbiome dysbiosis, and nutrition) would not be observed during *in vitro*-based inhibition assays; this area of research would benefit from further study.

2.5.2 Inconsistencies between laboratory and field data

An interesting set of findings from Forsgren et al. (2010) showed that 12 LAB strains derived from the honey bee crop [*Lactobacillus* (=*Apilactobacillus*) *kunkeei*, *L.* (=*Bombilactobacillus*) *mellis*, *L. kimbladii*, *L. kullabergensis*, *L. helsingborgensis*, *L. melliventris*, *L. apis*, *L.* (=*Bombilactobacillus*) *mellifer*, *Bifidobacterium asteroides* and *Bifidobacterium coryneforme = indicum*] possessed negligible inhibition properties when tested individually, but when combined, the strains completely inhibited all tested ERIC types (I, II, III and IV) of *P. larvae*. This suggests that synergetic interactions are at play,

which is intriguing given that all strains were isolated from the same niche habitat (the adult honey bee intestinal tract). In the same study, supplementation of the 12 LAB strains in larval food during a *P. larvae* infection assay (at 48 hours post-infection) rescued survival deficits by ~30% (Forsgren et al., 2010). In a follow-up study on the 12 LAB strains (plus one additional strain – *A. apinorum* Fhon13N), agar diffusion assays demonstrated that most, if not all, of the inhibitory effect was associated with the extracellular fraction (i.e., secretome) of the 13 combined LAB strains when mixed together in a metabolically active state (Lamei et al., 2019). The presence of a putative bacteriocin (Helveticin J produced by *L. helsingborgensis* Bma5N) as well as the secretion of other *P. larvae*-inhibiting small molecules was proposed but not verified. Nonetheless, the findings do not explain why such compounds required all strains to be incubated together for the inhibitory effect to be conveyed.

Next, in a four-month field trial, hive supplementation with the 13 LAB strains showed no effect on the incidence of AFB symptoms, which led the authors to conclude (via Bayesian mathematical modelling) that their discrepant findings must simply be due to the complex interactive nature of the hive (Stephan et al., 2019). A closer look at the methodology used, however, indicates that most or all the ~109 total LAB cells supplemented to the hive were likely dead since they were suspended in a standard sucrose syrup mixture and incubated overnight at 40°C prior to use. Sucrose syrup is an osmotic stressor that induces cell lysis in bacteria, and past studies show >90% reduction in LAB cell viability by four days even at the conservative temperature of 30°C (Ptaszyńska et al., 2016). A follow-up study (six-month field trial) with these 13 LAB strains and same delivery method (effectively supplementing dead cell-fraction postbiotics) again reported no effect on *P. larvae* loads in the treated hives (Lamei et al., 2020).

It is perhaps not surprising that supplementation of the cell fractions failed to elicit a beneficial effect in either of the field trials when in fact the desired inhibitory effect on *P. larvae* was originally characterized to be secreted by metabolically active cells (Lamei et al., 2019). Most studies aiming to test probiotics for other purposes in honey bees have also used the sucrose-syrup delivery method, and thus the validity of the findings should

be considered carefully, and indeed strains that do not confer a health benefit do not meet the criteria for a probiotic and products should not use that term.

Notably, bacteria need not always be alive to produce a beneficial effect on the host – for example, live and heat-killed *S. alvi* can improve survival after *S. marcescens* infection, albeit to different degrees (Horak et al., 2020). The preparation of such inanimate microorganisms and/or their components that provides a health benefit to the host is known as a postbiotic (Salminen et al., 2021). An additional advantage of administering inanimate bacteria is the reduced risk of potential adverse effects when screening for probiotic candidates. nonetheless, to re-emphasize, to be labelled a 'probiotic', microorganisms must be living and provide a clear benefit to host health (Hill et al., 2014).

2.5.3 Importance of probiotic delivery method

Ensuring supplementation of viable probiotics to the hive requires careful consideration of the delivery method. One way to offer added nutritional benefits to a beehive is through delivery in a pollen patty. Already widely used in industry, pollen patties (i.e. convenient for beekeepers) facilitate adequate probiotic distribution in the hive as a result of nutrient flow between nurse bees (which consume the supplemented product) and larvae (which consume nurse bee-secreted brood food) in the hive (Corby-Harris et al., 2014). Emphasizing the crucial importance of delivery method, Daisley et al. (2019; 2020b) showed that the direct inhibitory effects of *Lactiplantibacillus plantarum* Lp39*, Lacticaseibacillus rhamnosus* GR-1, and *Apilactobacillus kunkeei* BR-1 (LX3) on *P. larvae*. These effects were similarly mediated by extracellular metabolite production and were consistently observed under laboratory conditions as well as during multiple field trials in which viable bacterial cells (~10^9 CFU per strain) were supplemented to the hive using a pollen patty-based delivery system. Furthermore, it was confirmed that the LX3 strains could reach the intestinal tracts of intended adult and larval targets within the hive, as detected via qPCR using species-specific primers. Validation experiments are not yet common in honey bee studies (Yoha et al., 2021) but are useful for determining probiotic distribution in the hive (or lack thereof) as well as the effectiveness of different delivery methods.

2.5.4 Effects of probiotics in hives symptomatic for AFB

The Ontario field trial of Daisley et al. (2019) describes how hives supplemented with the LX3 candidate probiotic prior to a natural AFB outbreak exhibited a ~100-fold reduction in *P. larvae* loads (~5x10^4 CFU/g infected larvae) relative to the control group (~5x10^6 CFU/g infected larvae) that received only a vehicle pollen patty. Notably, infected larvae from the control group were near devoid of *A. kunkeei* and LX3 supplementation (containing *A. kunkeei* BR-1) could effectively increase colonization levels by 1000-fold or more. Beyond direct cell-to-cell inhibition of *P. larvae*, the LX3 strains also upregulated larval gene expression of *Def-1* and *Pcbd*, encoding an AMP with strong activity against *P. larvae* (Khilnani & Wing, 2015) and a peritrophin/chitin-binding protein involved with structural maintenance of peritrophic matrix (Comman et al., 2013), respectively. These immunomodulatory effects were likely derived from either *L. plantarum* Lp39 or *L. rhamnosus* GR-1, or both, based on past modelling in *Drosophila melanogaster* (Chmiel et al., 2019; Daisley et al., 2017). Thus, the findings together support the notion that certain probiotics, such as the LX3 strains, can offer multifactorial protection against AFB via direct (e.g. microbial competitive interactions) and indirect (e.g. modulation of host gene expression) mechanisms, as depicted in Figure 3.

Furthermore, infection assays under controlled conditions verified that *P. larvae* BMR43-81 (field isolate from diseased hives in the same study) caused ~30% mortality and that LX3 supplementation in larval food (prophylactically) could almost completely rescue survival deficits (Daisley et al., 2019). It is therefore tempting to speculate from this data that the LX3 supplemented hives might have been able to recover from AFB. Although this would be a highly desirable effect, no long-term monitoring was possible to confirm this due to local laws mandating apiary shutdown following inspection by authorities (Daisley et al., 2019).

2.5.5 Effects of probiotics in hives asymptomatic for AFB

In a second Ontario field trial by Daisley et al. (2020a), the same LX3 strains were tested as an adjunctive therapy (four weeks) following standard oxytetracycline treatment (two weeks) for purposes of lowering *P. larvae* levels in asymptomatic hives and preventing

AFB. The findings showed that LX3 supplementation could suppress *P. larvae* loads significantly below that of the antibiotic alone, and to almost undetectable levels by the final timepoint ($<10^2$ copies of *P. larvae* 16S rRNA gene detected in larval samples). In addition, LX3 supplementation showed a time-dependent reduction of *P. larvae* loads in adult nurse bees – an effect that did not occur in response to antibiotic treatment alone (Daisley et al., 2020a). This together suggests a unique benefit of LX3 since adult bees are considered passive carriers of infection (Erban et al., 2017) with their *P. larvae* loads showing a direct relationship with appearance of clinical AFB symptoms in larvae (Kačániová et al., 2018).

Notably, LX3 supplementation also helped to ameliorate some of the well-known side effects of oxytetracycline including reductions to queen egg laying, hemolymph killing capacity, and microbiota dysbiosis in adult workers (Daisley et al., 2020a). The latter effect was characterized by a significant enrichment of all core microbiota members (e.g., *Gilliamella, Snodgrassella, Bifidobacterium,* and *Lactobacillus* Firm-4/Firm-5 spp.) during the antibiotic recovery period, alongside a depletion in opportunistic environmental bacteria – which may help reduce AFB risk through suppressing collaborative interactions (Figure 2-3). Cumulatively, these findings support that the timely use of probiotics can aid in re-establishing a healthy microbiota following antibiotic exposure. The evidence also concurs with the proposition that immunomodulatory LAB probiotics can re-set dysbiotic microbiota phenotypes in bees (Daisley et al., 2020b).

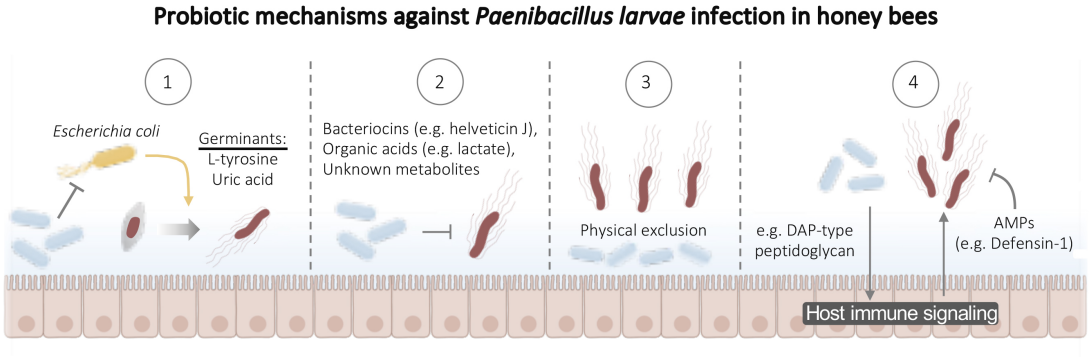

Figure 2-3. Probiotic mechanisms supporting American foulbrood (AFB) disease prevention.

Four scenarios by which probiotics have the potential to prevent symptomatic infection by *Paenibacillus larvae*. **(1)‘**Collaborative’ interactions with environmental opportunist pathogens may play a role in the spore-to-vegetative growth transition of *P. larvae* (e.g., *E. coli* can produce essential germinants needed for *P. larvae* growth). Inhibition of these coinfecting opportunists by probiotics may help to resist AFB disease by reducing the bioavailability of germinants found in the honey bee midgut. **(2)** Probiotics have the potential to directly kill or inhibit *P. larvae* during vegetative growth via bacterocins, organic acids, and other unknown anti-*P. larvae* metabolites. **(3)** In the absence of healthy microbiota colonization by honey bee symbionts, probiotics may help to physically exclude *P. larvae* from the midgut epithelium and thus from entering the hemocoel and causing AFB disease symptoms to arise. **(4)** Immunomodulatory cell wall components found in some probiotics [e.g., diaminopimelic (DAP)-type peptidoglycan] have the potential to upregulate honey bee antimicrobial peptides (AMPs) including *Defensin-1*, which has selective activity against *P. larvae* and other pathogens, without harming honey bee symbionts.

2.5.6 Considerations of supplementing live bacteria to honey bees

While ample studies have been performed *in vitro*, relatively few investigations at the field level have tested probiotics as a preventative measure against AFB. The two field trials from Ontario described above (Daisley et al., 2019; Daisley et al., 2020b) suggest great potential (at least for the LX3 strains tested), although follow-up studies in different geographic regions and with larger sample sizes will help to substantiate reproducibility of the findings prior to commercial usage.

As with any application of live bacteria, there are potential dangers to the host that must be considered. For example, in one study, supplementation of *S. alvi* wkB2 (a honey bee symbiont) to protect against protozoal infection by *Lotmaria passim* unexpectedly showed a worsening of infection, increased protozoal loads, elevated stress markers, as well as decreased expression of key detoxification genes (Schwarz et al., 2016). The underlying mechanism is thought to be related to complex biofilm interactions, although this is based on limited knowledge. Whether *in vitro* studies could have been predictive of this outcome is also not known. In this case, the symbiont was not probiotic.

Another counterintuitive finding related to protozoal infections comes from a microbiota transplantation study in bumble bees (*Bombus terrestris*) (Mockler et al., 2018). Specifically, the results show that wild bee microbiota, in some cases, is less effective than commercial bee microbiota (exposed to chronic antibiotics) in providing resistance to *Crithidia bombi* infection when transferred to a naïve host (Mockler et al., 2018). These results highlight that while the complete transfer of microbial communities from a healthy hive to a diseased hive indeed might hold promise for re-establishing lost symbionts (Daisley et al., 2020b), we should not assume that the absence of antibiotic exposure (or other anthropogenic stressors) guarantees a healthy microbiota phenotype. Likewise, it cannot be assumed that microbiota transfer (from wild or conventional bees) will be effective for an intended purpose. Future studies should explore this expanding area of research with careful attention, in order to identify potential risks as well as variability of success when transplanting donor microbiota.

Most LAB are renowned for their safety profiles in animals and as such are widely used as probiotics in humans (Hill et al., 2014). Nonetheless, the use of LAB in honey bees has been reported to be deleterious in rare cases. For example, Ptaszyńska et al. (2016) reported that co-administration of *Lactobacillus acidophilus, Lactobacillus delbrueckii*, and *Bifidobacterium bifidum* could increase pathogenic *Nosema (=Vairimorpha) ceranae* loads during experimental infection. While these findings do warrant concern, it is difficult to ascertain their biological relevance since survival was not measured and because emerging evidence now suggests that *V. ceranae* may promote longevity in honey bees – at least in the absence of nutritional deficits or any other health concerns (Zhang et al., 2021). To note, some study details were also not included (e.g. strain type of the bacteria used and their anti-pathogen properties). Future studies should seek to provide this information, and likewise the strain of pathogens used for infection assays since there are often differences in virulence factors, as is the case for *P. larvae* genotypes (Genersch et al., 2006).

Another criticism of using probiotics is their potential to permanently replace the host's indigenous organisms and thereby change the natural ecosystem. No evidence of this has been found and as probiotic strains do not colonize the host, they must be re-applied to convey their desired effects.

2.6 Concluding remarks

American foulbrood disease has long hampered the apicultural industry and, despite well-known protocols for its detection (government or designated inspectors), prevention (oxytetracycline and other antibiotics) and containment (burning of hives), it remains a perennial concern to beekeeping operations. Current management strategies in the fight against AFB remain stagnant; beekeepers lack a choice in treatment options and research innovations on this front have been slow to develop. Seizing upon a broader realization within the biological sciences that the health and well-being of living organisms critically depends upon their symbiotic relationships with microbes, some pioneering studies have begun to describe and test how gut microbe diversity changes with the age, caste and geographic region. The prospect of supplementing managed colonies with beneficial bacteria purposefully selected for their strain-level abilities to complement core

microbiota functional capacities or to stimulate bee metabolic performance is limited to a few key studies so far. Altogether, the reviewed material cumulatively suggests that AFB disease management strategies need to move beyond targeting a single organism (e.g., *P. larvae*) and should instead expand their focus to include the entire ecology of honey bee-associated microbial communities to ensure a sustainable future for the beekeeping industry.

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Chapter 3

3 Probiotic effects on ectoparasitic mite infestations in honey bees (*Apis mellifera*) are modulated by environmental conditions and route of administration

The material in this chapter has been partially adapted from an article that is currently under peer-review to assess its suitability for publication in the journal *Applied and Environmental Microbiology*. On that article I am first author.

Pitek AP, Daisley BA, Chmiel JA, Chernyshova AM, Dhami G, Reid G, Thompson GJ. (Submitted ms: AEM00834-24) Probiotic effects on ectoparasitic mite infestations in honey bees (*Apis mellifera*) are modulated by environmental conditions and route of administration. Applied and Environmental Microbiology.

3.1 Abstract

Mounting evidence suggests that beneficial bacteria can improve the health of managed honey bees (*Apis mellifera*) via immune system support as well as direct inhibition of pathogens. However, our understanding is limited about how environmental factors and different delivery methods impact treatment outcomes. Here, we evaluated how supplementation of a three-strain lactobacilli consortium (LX3) to honey bee hives in three apiaries (15 colonies per apiary; three colonies x five treatment groups), in either edible (protein patty) or topical (spray) forms, affects common ectoparasitic mite (*Varroa destructor*) and bacterial pathogen (*Paenibacillus larvae*) disease burdens under real-world conditions across three distinct habitats (forage-rich, agricultural, and urban). Results demonstrate a reduction in mite infestation levels following oral LX3 administration via patty delivery, whereas spray methods were ineffective. Notably, a control group that received only an uninoculated patty (*i.e.*, no LX3) carried more mites than any other group, suggesting excess protein within hives is a catalyst for mite proliferation. This effect, whereby the excess-protein group had the highest parasite load, was pronounced in the most natural (forage-rich) environment location, indicating a

significant habitat-by-treatment interaction. No influence of LX3 on *P. larvae* loads was observed in the asymptomatic colonies studied, which is likely attributable to the already low levels of pathogenic spores present and challenges associated with detection limits. In summary, this multi-habitat field study suggests that a protein patty is an effective vehicle for delivering probiotic bacteria to commercial honey bee colonies and controlling *Varroa destructor* infestation levels.

3.2 Introduction

The Western honey bee (*Apis mellifera*) is an economically valuable insect whose natural ability to forage for pollen and nectar has rendered it an efficient pollinator of flowering crops (Hung et al., 2018). The strategic use of honey bees in the pollination services sector is thought to contribute to the production of as much as 35% of the world's produce (Klein et al., 2007). *Apis mellifera* has, however, become increasingly difficult to manage in areas where agriculture is intense and the bee's natural resilience to environmental stress is pushed to a limit (Goulson et al., 2015). Regulators have long warned of a "pollination crisis" that warrants a coordinated response above the level of individual beekeepers (Aizen & Harder, 2009). In colder climes, including regions of North America, the rate of overwinter colony loss is high enough to routinely trigger public concern in news media (e.g., Hoye & Pauls, 2022) and this rate of loss has renewed interest in monitoring and best practice within the beekeeping community.

The widespread plight of honey bee populations throughout North America likely stems from a mixture of stressors, such as parasitic mites (Spivak et al., 2011), an array of bacterial pathogens (Fünfhaus et al., 2018), pesticides and other environmental toxins (Daisley et al., 2022; Traynor et al., 2021) as well as habitat loss (Goulson et al., 2015; Youngsteadt et al., 2015). The beekeeping industry is replete with guidelines on how best to avoid or mitigate some of these stressors. Common among the prescribed remedies is the application of pharmaceutical treatments (e.g., oxytetracycline, tylosin, etc.) against specific pests and pathogens (Raymann, 2021). The use of regulated medications can however be expensive, impractical, perceived as 'unnatural' and even ineffective if their long-term use inadvertently selects for resistance (Obshta et al., 2023; Piva et al., 2020). Antibiotics may also cause dysbiosis and, paradoxically, render colonies more susceptible to secondary infection (Daisley et al., 2020b; Deng et al., 2022; Raymann et al., 2017). Consequently, the industry seeks new strategies to complement or even replace current practice (Croppi et al., 2021; Steinhauer et al., 2021). Ideally, such alternatives will be effective, affordable and without side effects.

One approach that warrants more testing is the relatively new field of microbial therapeutics – that is, the deliberate formulation of microbial supplements that support or enrich the bee's own native microbiome to beneficial effect (Abdi et al., 2023; Alberoni et al., 2016; Alonso-Salces et al., 2017; Motta et al., 2022). Unlike antibiotics or other medicated treatments that attack pathogens directly, 'probiotics' (Hill et al., 2014), as applied to beekeeping, complement the host's health and immune system to help guard against disease and potentially buffer individuals against this and other forms of environmental stress (Chmiel et al., 2020). Despite much promise, the effectiveness of probiotics within a commercial beekeeping environment has not been widely tested (Chmiel et al., 2021; Damico et al., 2023). Previous studies have, however, shown that strains of lactobacilli, among other possibilities, can modulate the immune response, help to assimilate nutrients and prevent intestinal disorders (Pachla et al., 2018; Rana et al., 2024). This lactic acid-producing group of bacteria is therefore a potential source of strains suited for bee-friendly microtherapeutic treatments (Rodríguez et al., 2023).

A study by our research group tested a combination of three strains – namely, *Lactiplantibacillus plantarum* Lp39, *Apilactobacillus kunkeei* BR-1 and *Lacticaseibacillus rhamnosus* GR-1, which are collectively referred to as 'LX3' (Daisley et al., 2019) – for their ability to inhibit growth of *Paenibacillus larvae*, the bacteria that cause American foulbrood disease (Genersch, 2010). The study showed that supplementation of colonies with LX3 lowered pathogen load in the guts of worker bees, and conferred other benefits, including stimulated worker immunity and queen egg laying (Daisley et al., 2020). These findings were, however, limited to a single apiary. Further studies have since tested the effectiveness of this (Daisley et al., 2023b) and other probiotic formulations (Truong et al., 2023) at a larger scale. However, because the application of putatively beneficial bacteria to bee colonies is so early in its development, the most effective method of delivery has not been determined. Intuitively this would be in edible form, to target the gut directly, but spray-based delivery to large numbers of workers on hive brood frames might also be practical and potentially better suited to ectoparasites like *Varroa* mites that are clearly not situated in the gut. Importantly, using a spray method could deliver living bacterial cells since some strains remain viable for

months in phosphate-buffered saline (PBS) (Liao & Shollenberger, 2003). Spray delivery therefore offers an alternative way to efficiently distribute living bacteria to a large number of workers within hives (Daisley et al., 2023).

In this study, the objective was to test how delivery of LX3 in oral and spray forms affects pathogen (*P. larvae*) and, for the first time, parasite (*Varroa destructor*) load, and do so across different habitats in the Southwestern Ontario (Canada) region.

3.3 Methods

3.3.1 Culturing

Three strains of lactobacilli were cultured: *Lactoplantibacillus plantarum* Lp39 (American Type Culture Collection [ATCC] 14917), *Lactocaseibacillus rhamnosus* GR-1 (ATCC 55826), and *Apilactobacillus kunkeei* BR-1. Briefly, each strain was grown under microaerophilic conditions at 37 °C using de Man, Rogosa, and Sharpe (catalog number: 288130, BD Difco) broth or agar supplemented with 10 g/L D-fructose (catalog number: F-3510, Sigma-Aldrich; MRS-F). For both BioPatty- and spray-based LX3 treatments, bacterial cells were collected in a similar manner; following overnight incubation on fresh streak plates, a single colony of each strain was used to inoculate multiple broth cultures, using a separate colony for each culture. The cultures were then uniformly incubated at 37 °C for 24 hours using sterile 50 mL polypropylene conical tubes (catalog number: 339652, Thermo Scientific; MRS-F filled to 50 mL, lids tightly closed). Cells were collected by centrifugation at 5,000 g for 10 minutes (at 4 °C), washed with 0.01 M PBS, and centrifuged again at 5,000 g for 10 minutes (at 4 °C). Finally, each strain containing 5×10^{10} colony-forming units (CFU) was combined at equal cell densities into a final concentrated volume of 4 mL 0.01 M PBS.

3.3.2 Patty and spray treatment recipes

Pollen patties are used in commercial beekeeping to promote colony growth and stimulate population expansion by providing a readily accessible source of protein and nutrients. These supplements, often formulated with a mixture of soy flour, sucrose, and brewer's yeast, are designed to mimic the natural pollen that bees collect, thus supporting

the nutritional needs of the hive (DeGrandi-Hoffman et al., 2020). The administration of pollen patties can significantly boost brood rearing and overall colony strength, particularly during periods when natural pollen sources are scarce (Daisley et al., 2019). However, this practice is not without risks, as it may inadvertently increase *Varroa* mite or other parasite infestations, which exploit the patty itself or the brood that results from patty-stimulated laying (Traynor et al., 2020). It appears therefore that there may be a trade-off between helping the bees and inadvertently helping the parasites. Consequently, integrating probiotics into pollen patties has been suggested to mitigate these risks by enhancing the bees' natural defenses against parasites and pathogens, thereby promoting a healthier and more resilient colony (Vilarem et al., 2023).

A 250 g patty was made using a standard pollen substitute recipe consisting of 28.5 g soy flour, 74.1 g granulated sucrose, 15.4 g debittered brewer's yeast, and 132.1 g of a simple sucrose-based syrup solution (2:1[w/v]). For the LX3-infused BioPatty (previously described in Daisley et al., 2019), a concentrated suspension of LX3 strains was added to 0.01 M PBS and mixed until the infusion was visibly homogenous, resulting in a final concentration of 2 x 10^8 CFU/g for each strain. For vehicle patties, an equivalent volume of sterile 0.01 M PBS was added that did not contain any live bacterial suspension. Each patty was positioned between two sheets of wax paper (30 cm x 45 cm) and, and within 24-hrs of production, each patty was placed on top of frames in the brood chamber of Langstroth bee hives. For the LX3-infused BioSpray (previously described in Daisley et al., 2023), the concentrated LX3 suspension was added to 28 mL of 0.01 M PBS in a sterile spray bottle to obtain a diluted concentration of 1.6 × 10^9 CFU/mL per strain. The nozzle of the bottle discharges 2 mL per spray, so 32 mL of the LX3-containing suspension was administered into the hive via 16 standardized spray actions (2 mL front and 2 ml back of each brood frame, for 8 brood frames per hive). The same spray sequence was used for the vehicle spray, but with 32 mL of sterile 0.01 M PBS added to a sterile spray bottle instead.

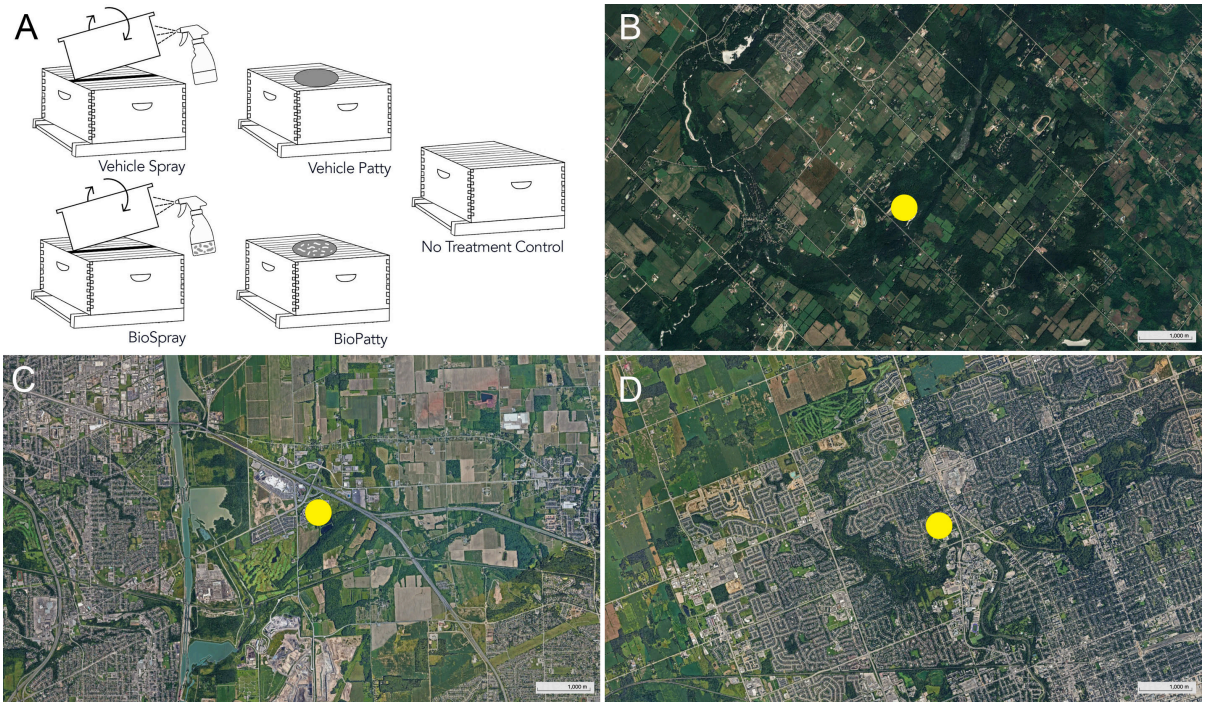
3.3.3 Treatment groups, apiary setup, and sampling procedure

The field trials consisted of two treatments (patty and spray) and their respective treatment-specific controls, as well as a full control with no association to treatment or its delivery

vehicle (Figure 3-1A). This design was replicated across three habitats, capturing the response in a naturally forage-rich area (near Milton, ON), a predominantly agricultural habitat (Niagara-on-the-Lake, ON), and a distinctly urban setting in the City of London, ON (Figure 3-1B-D). These land use designations roughly correspond to the 'natural land', 'secondary agricultural forage' and 'developed land' of Sobkowich et al.'s (2022) Ontario Varroa survey.

Within each of the three apiaries, 15 colonies (three colonies × five treatment groups) were set up with new, standard wooden 10-frame Langstroth hive boxes placed on a stand, approximately 12 inches off the ground. Each colony was provided with a naturally mated queen of mixed Italian background (A. mellifera ligustica), two frames of capped brood, one frame of honey, one frame of empty wax comb, six empty frames of foundation (for a total of ten frames per box). In addition, the equivalent of four full frames worth of bees were supplied. All colonies were treated with oxytetracycline 28 days before the start of the experiment. Treatments were administered once every two weeks for eight weeks (July-August 2020). Each colony was equipped with a queen excluder and a medium box with 10 frames above the excluder to allow for additional honey stores as needed. Treatments were assigned to colonies at random as: i) BioPatty group (n=3 hives), ii) vehicle patty control group (n=3 hives), iii) BioSpray group (n=3 hives), iv) vehicle spray control group (n=3 hives), and v) no treatment control (NTC) group (n=3 hives). A total of 45 colonies were used for the experiment (15 colonies × three locations).

Figure 3-1. Field study Design. A There are a total of five treatment groups in this study: BioSpray, vehicle spray, BioPatty, vehicle patty and no-treatment control. Each group was assigned three hives, for a total of n=15 hives for each of three apiaries (N=45 colonies in total). The apiaries capture three nominal habitat types in southwestern Ontario: **B** forage-rich areas with a high diversity of floral sources, including those in forest patches and feral fields, **C** agricultural areas dominated by monoculture crops such as corn and soybean, and **D** urban areas near a city with industrial areas and limited forage availability.



3.3.4 Estimating *Varroa* load

Varroa mite infestation levels were estimated from each colony at weeks 1, 4 and 8 using an alcohol wash technique (Dietemann et al., 2013). Briefly, approximately 300 bees (1/2 cup) were collected from brood frames into a container filled with 70% alcohol. To ensure consistency of this approach, we validated this measure by manually counting the bees for a subsample. This method is inexact but consistent and allows us (and others) to sample rapidly with minimal disturbance to the hive. The container was shaken for two minutes to dislodge the mites from the bodies of the worker bees. The mites were then isolated from the wash by filtering through a 1/8-inch mesh wire screen, allowing the mite to flow with the alcohol into a separate container. From mite counts, the average level of infestation by habitat and treatment was estimated.

3.3.5 Estimating *Paenibacillus larvae* load from quantitative PCR of pathogen DNA

To determine the level of *P. larvae* infection in colonies, a total of n = 30 adult nurse-age bees (less than 15 days old) were collected from each colony bi-weekly. The bacterial pathogen does not infect the worker caste (it infects larvae; Genersch, 2010) but workers are nonetheless vectors for the bacterial spores and inadvertently infect larvae when tending to them (Powell et al., 2014). Collected workers were placed into a 50 mL falcon tube and placed on dry ice immediately in the field before transferring to a -80 ^{o}C freezer to preserve their tissue for molecular analysis. The samples were thawed on ice for 5 minutes then, using forceps, the entire digestive tract was removed by gently pulling on the rectum just above the stinger at the end of the abdomen. Samples that gave the appearance of a pollen-based diet of nurse bees (digestive tracts that were yellow-orange in colour) were used for the analysis, while those reflecting a nectar-based diet of forager bees (with an uncoloured semi-transparent appearance) were discarded.

DNA was extracted from the samples using a CTAB method as described previously (Powell et al., 2014). A spectrophotometer was used to estimate the concentration of extracted DNA based on 260/280 and 260/230 ratio of absorbance values. Finally, qPCR was used to quantify pathogen load from bacterial DNA. First, extracted DNA was

diluted 10-fold to use as a starting template. Next, a SYBR Green-based qPCR kit (Applied Biosystems) was used with one primer specific to a region of the *16S rRNA* gene of *P. larvae* and another for universal bacterial (341F to 805R) quantification (Klindworth et al., 2013; Martínez et al., 2010), as well as primers specific to the *ribosomal protein S5* gene (Evans, 2006) and the *ß-actin* gene (Zhang et al., 2014) as endogenous controls (Table 3-1). All qPCR reactions were performed in DNase- and RNase-free 384-well plates using a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Amplification data were analyzed using QuantStudio Design and Analysis software and used the $2^{-\Delta\Delta}$ Ct method to estimate fold-change relative to an endogenous control gene (*ß-actin*).

Table 3-1. List of oligonucleotide primers and their target loci. *Beta actin* (host endogenous control gene*), Ribosomal protein S5* (host endogenous control gene), universal bacteria (targeting the conserved region 341-805bp of the *16S rRNA* gene of any bacteria), and *Paenibacillus larvae 16S* (targeting positions 30-407bp of the *16S rRNA* gene that is specific to *P. larvae*).

Primer target	**Reference**	**Sequence (5'-3')**
Beta actin	Zhang et al., 2014	F: ATGCCAACACTGTCCTTTCTGG R: GACCCACCAATCCATACGGA
Ribosomal protein S5	Evans, 2006	F: AATTATTTGGTCGCTGGAATTG R: TAACGTCCAGCAGAATGTGGTA
Universal bacteria 16S	Klindworth et al., 2013	F: CCTACGGGNGGCWGCAG R: GACTACHVGGGTATCTAATCC
Paenibacillus larvae 16S	Martínez et al., 2010	F: CGGGAGACGCCAGGTTAG R: TTCTTCCTTGGCAACAGAGC

3.3.6 Statistical analysis

Statistical analysis of the data was performed using R (version 4.3.1) and GraphPad (version 10.2.0). For all comparisons, data were analyzed using mixed-effects modelling, whereby treatment (categorical), time (continuous) and their interaction were predictors with location (categorical) as a random variable. Analysis of Variance (ANOVA) was used to assess global comparisons and emtrends (from the R package EMMEANS version 1.10.0). This was used to estimate and compare the marginal means of the linear trends. For all comparisons in GRAPHPAD, data was separated by site and analyzed using mixed-effects modelling and Tukey Post-Hoc test.

3.4 Results

Baseline levels of parasite and pathogen load were determined for all colonies at each site at the outset of the experiment. Mite populations were estimated at below 1% (0 – 3 mites per 300 worker bees) on Day Zero for all colonies in the experiment. A slight increase in mite levels in the NTC group occurred throughout the timeframe of the experiment. Specifically, at Day Zero, the NTC value was between 0 – 0.33% mite infestation, rising to between 0 – 1.33% after eight weeks (Figure 3-2A). Time had a significant effect on mite levels ($F_{1, 123} = 45.88$, $P < 0.001$), with nearly all colonies showing an increase at the urban, forage-rich and agricultural settings (Figure 3-2B-D).

When looking at the treatment effect, and considering the variation associated with site, time ($F_{1, 123} = 45.88$, $P < 0.0001$) and the interaction between time and treatment ($F_{4, 123} = 3.01$, $P = 0.021$) were significantly different by ANOVA (Table S1). This suggests that the effect of treatment on mite infestation changes over time. Further, slopes of the interaction between time and treatment were compared, there was a significant difference between the BioPatty and vehicle patty groups (T-ratio = -3.25; $P = 0.013$; Table S5), meaning that of all possible pairwise comparisons these two are the most different.

The forage-rich location was strongly affected by treatment ($F_{4,10} = 7.859$, $P = 0.004$), time ($F_{2,20} = 17.24$, $P < 0.0001$) and their interaction ($F_{8,20} = 3.856$, $P = 0.007$). Here, the

vehicle patty had elevated mite loads relative to all other treatments (Figure 3-2C), including at eight weeks the NTC ($P < 0.0001$), vehicle spray ($P < 0.0001$), BioSpray ($P = 0.0001$) and BioPatty ($P < 0.0001$; Table S3). The overall mite count was also highest at the Agricultural site (Figure 3-2D), where, again, the vehicle patty had the highest mite loads relative to several other treatments (Table S4). By contrast, the BioPatty treatment resulted in the lowest final mite counts in all three settings (Figure 3-2B-D). Indeed, the single greatest difference in mite count was BioPatty vs vehicle patty at the forage-rich habitat (the highest mite infestation by Week 8 being 0.33% for the BioPatty group and 2.33% for vehicle patty (Figure 3-2C). The BioSpray treatment increased the mite count in the urban hives (Figure 3-2B).

For pathogen load, the normalized relative expression of the 16S gene transcript was used to estimate the total bacterial load and specifically the *P. larvae* load. The total bacterial load on a worker bee was consistently ~7 $\log_{10}$ gene copies (range 2.0 - 8.2 $\log_{10}$) throughout all the hives at each setting (n=45) across all timepoints (n = 3), while the *P. larvae* load per worker bee was ~2 $\log_{10}$ gene copies (range 2.0 - 4.5). These molecular estimates of bacterial numbers were low and suggest that the asymptomatic colonies in our apiaries had negligible levels of foulbrood infection and the loads for total bacteria were quite stable over time for all three locations (Figure 3-3; Supplementary Tables S6-S8), with the Forage-rich habitat being the most variable. Across all treatment groups and habitats, *P. larvae* was low or not detectable, with some variability in the Forage-rich habitat (Figure 3-4). The American foulbrood-causing bacterium showed no significant difference over time (Tables S9-S11) or as a function of treatment.

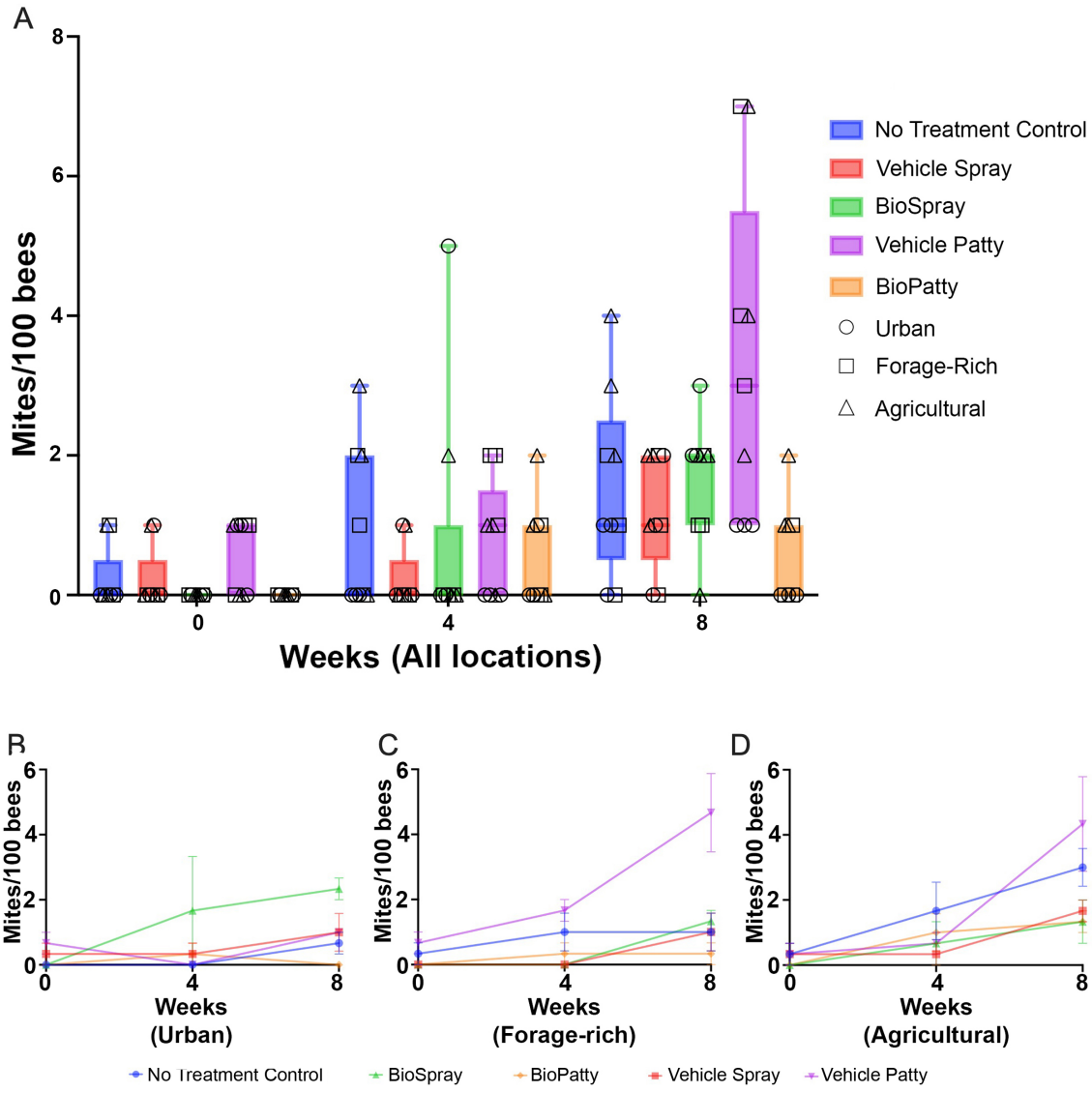


Figure 3-2. Varroa mite count per 300 bees at each timepoint (weeks 0, 4 and 8) for each treatment group. A Average mite count per treatment group (n=5 groups) for all three landscapes (**B** urban, **C** forage-rich, **D** agricultural) and timepoints (weeks 0, 4 and 8). Data represents the median (line in box), inter-quartile range (box), and minimum/maximum (whiskers) of mite count.

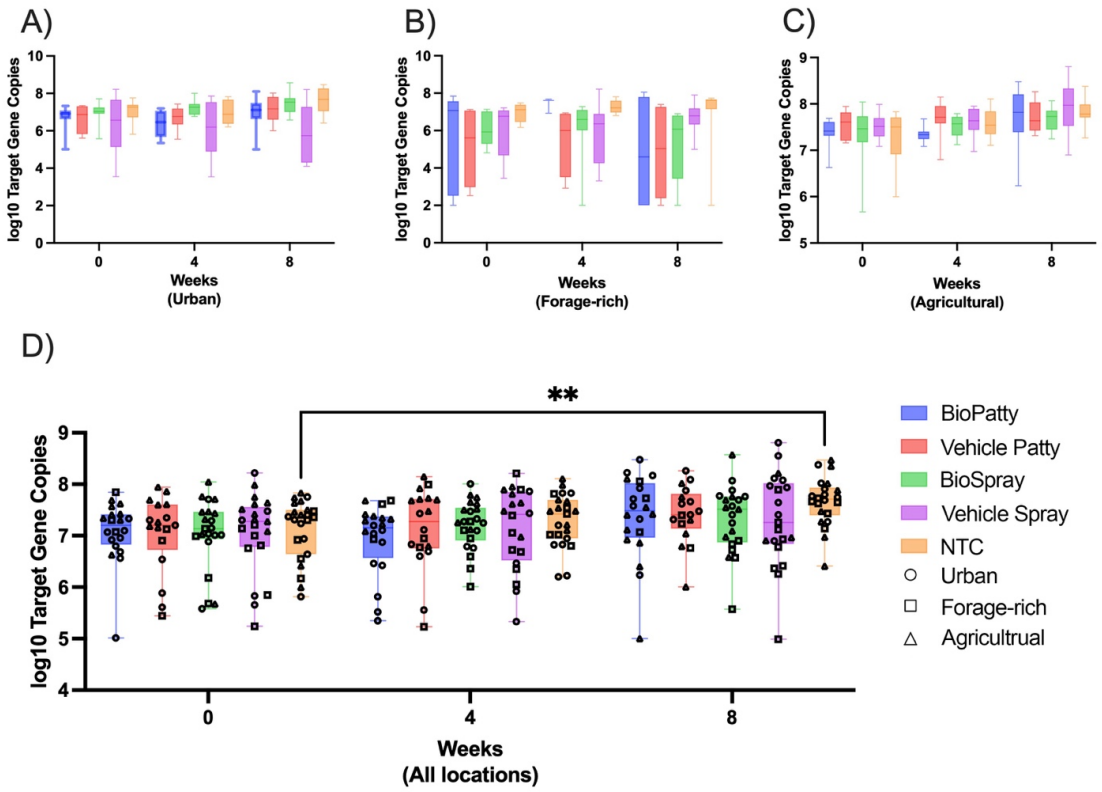


Figure 3-3. Total bacterial loads over a span of eight weeks. Total bacterial loads estimated for the **A** urban (London), **B** forage-rich (Milton) and **C** agricultural (Niagara) habitats across three timepoints (weeks 0, 4 and 8). **D** Combined total bacterial loads for all three habitats over time. A mixed-effects model and Tukey Post-Hoc test indicated a significant increase in bacterial load in the no-treatment control group ($P < 0.005$; Table S1 and S5). Data represent the median (line in box), inter-quartile range (box), and minimum/maximum (whiskers) of total bacterial load.

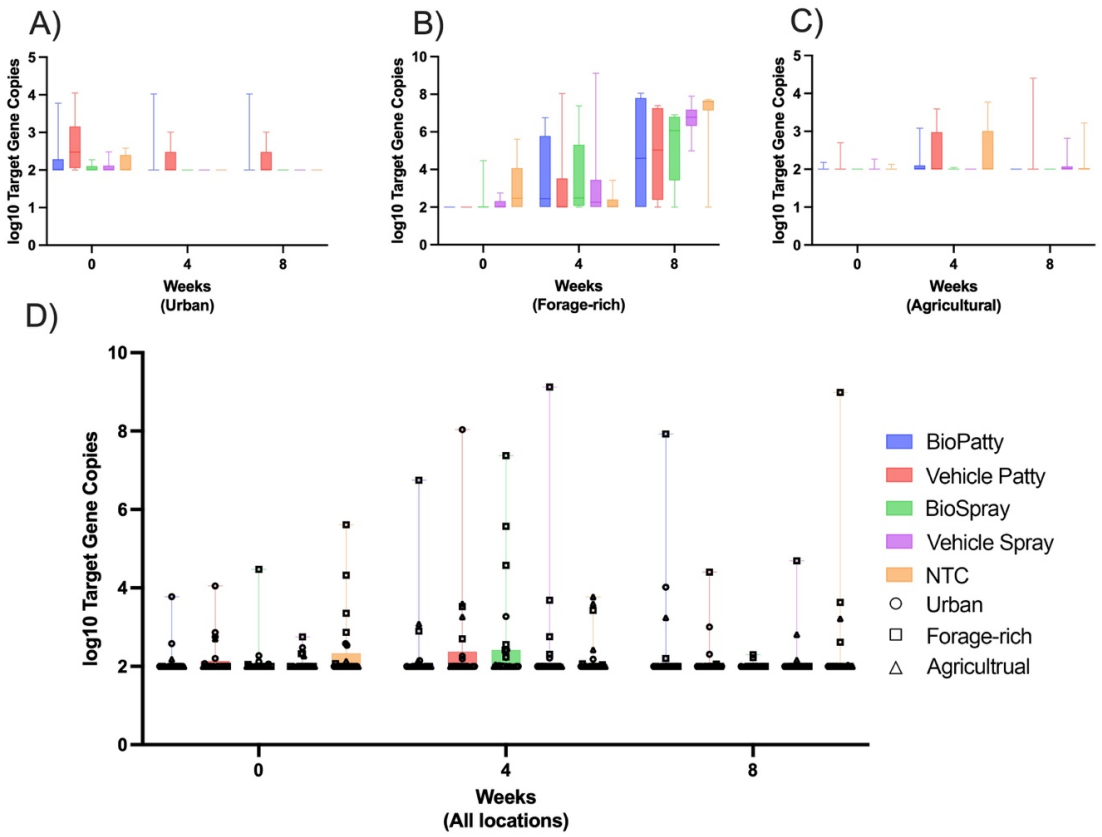


Figure 3-4. *Paenibacillus larvae* loads over a span of eight weeks. *P. larvae* loads estimated for the **A** urban (London), **B** forage-rich (Milton) and **C** agricultural (Niagara) habitats across three timepoints (weeks 0, 4 and 8). **D** Combined *P. larvae* loads for all three habitats over time. Data represents the median (line in box), inter-quartile range (box), and minimum/maximum (whiskers) of *P. larvae* loads.

3.5 Discussion

This study used a multi-habitat field trial to test how oral vs spray-based delivery of a three-strain consortium of immunostimulatory lactobacilli could influence honey bee resistance to environmental stress, as measured through susceptibility to a specific parasite and pathogen known to afflict managed honey bees in the study region. The study showed that oral delivery of the LX3 formulation developed by Daisley et al. (2019) in the form of a BioPatty was effective at keeping *Varroa* mite loads low, despite a tendency for *Varroa* to increase in untreated control colonies over the course of the experiment. This suppressive effect was observed across three separate sites that reflected local versions of urban, agricultural and relatively undisturbed naturally forage-rich areas, with the effect being particularly strong at the latter site, near Milton Ontario. *Varroa* is an ectoparasite that is increasingly common in these regions (Sobkowich et al., 2022) but our spray-based application of LX3 appeared ineffective in comparison to the patty, which underscores the importance of delivery when applying microbial therapeutics. No influence of LX3 on *P. larvae* loads was observed in the asymptomatic colonies studied, which is likely attributable to the already low levels of pathogenic spores present and challenges associated with detection limits. These results therefore show the potential for widespread utility of this particular lactobacilli infused BioPatty across a range of beekeeping landscapes.

The level of *Varroa* infestation in the study populations was stable and for most colonies was just below a critical threshold (three or more mites per 100 bees) that usually prompts miticide treatment. The presence of mites in a majority of colonies indicates that there remains a persistent region-wide threat from these devastating, virus-carrying mites in the study region, as first reviewed by Guzmán-Novoa et al. (2010). *Varroa* mites are naturally adapted to the Eastern honey bee *A. cerana* and cause considerably more harm to its newfound host, *A. meliffera* (Morfin et al., 2023; Traynor et al., 2020). In the present study, mite loads did vary as a function of time and treatment, with the maximum recorded loads nearly quadrupling in no-treatment control colonies (from 1 to 4 mites per 300 sampled bees) over a span of eight weeks, and increasing roughly seven-fold in the vehicle patty group (from 1 to 7 mites per 300 samples bees). The common use of protein

patties as a feed supplement to support colony growth may inadvertently increase mite loads, as reported for at least one other study (DeGrandi-Hoffman et al. 2020).

The undesirable effect of the patty on mite load may be a by-product of increased opportunities for mites to reproduce and develop if, for example, feeding patties to hives results in more drone cells, which are favoured by the mites (Güneşdoğdu et al., 2021; Traynor et al., 2020). Regardless, this apparent trade-off between colony growth and disease may alert the beekeeping community to limit supplemental feeding with patties in areas where mites are common or, alternatively, formulate patties with the addition of probiotic strains that support the natural resiliency of honey bees against this parasite and other forms of environmental stress. The elevated mite load at the forage-rich site may be related to local variation in bee nutrition, population density or spatial structuring of the mite, as had been discussed for Ontario (Sobkowich et al., 2022) and for other *Varroa* surveys (Dolezal et al., 2016; Stevenson et al., 2005). The oral administration of LX3 via the BioPatty was helpful against ectoparasites, possibly because of delivery to larvae by worker bees, rendering larvae more resistant, or lactic acid itself may inhibit the mite's ability to attach or move within the colony growth (Vilarem et al., 2023). The BioPatty can potentially be a viable mid-season treatment option against *Varroa* mites, especially since the only other available treatment in Ontario is formic acid (46.7% concentration) (OMAFRA, 2012). Finally, both the BioSpray and vehicle spray sometimes reduced mite levels (in two of three habitats), possibly as a by-product of grooming behaviors induced from spraying.

The current test of the BioPatty against the bacterial pathogen causing American foulbrood was not strong, simply because the presence of *P. larvae* was too light in the colonies to further test this effect. The low baseline level of *P. larvae* is probably due to the condition of the colonies, which began as nucleus colonies in entirely new equipment. Previous studies have, however, shown BioPatty efficacy in controlling *P. larvae* (Daisley et al., 2019) and helping to restore the gut microbiome following antibiotic perturbation (Daisley et al., 2020b). BioSpray, by contrast, may nonetheless be effective at controlling microbial brood pathogens like *Melissococcus plutonius* and *Ascosphaera*

apis (Daisley et al., 2023). This leaves beekeepers with a dilemma since the LX3 formulation is not yet commercially available in patty or spray form and alternative commercial ‘probiotic’ products are not typically tested to prove their effectiveness (Chmiel et al., 2021; Damico et al., 2023; Motta et al., 2022). Nonetheless, microbial therapeutics can leverage the bee's natural abilities to stave off some stress via the gut-microbe barrier. The application of probiotics as a strategic treatment to colonies in stress has the potential to become an integral component of a comprehensive disease management strategy. Based on current research, we advocate for both patty and spray-based delivery mechanisms that involve LX3 or other therapies shown to be effective (e.g., Powell et al., 2021). In each case, however, clear protocols need to be established that link to a beekeeper's specific needs so that potentially useful therapies are not administered incorrectly or in vain. The implications of microbial therapeutic research include the possibility of enhancing the resilience of managed honey bee colonies against diseases and thus helping to futureproof apiculture and pollination services.

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3.7 Supplementary

Table 3-2. Two-way analysis of variance for mite infestation for all locations (n=3 sites), weeks (n=3 timepoints), and treatments (n=5 groups).

Source of Variation	**SS**	**DF**	**MS**	**F $_{(DFn, DFd)}$**	***P* value**
Treatment	0.382	4	0.960	$F_{4, 123} = 0.906$	$P = 0.985$
Time (Weeks)	48.400	1	48.400	$F_{1, 123} = 45.88$	$P < 0.0001$
Interaction	12.711	4	3.178	$F_{4, 123} = 3.013$	$P = 0.0201$

Table 3-3. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of *Varroa destructor* mite infestation levels for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the urban location. Tukey HSD test results are bolded for any significant difference between treatment groups within a single timepoint.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	No Treatment Control vs. Vehicle Spray	-0.333	-2.392 to 1.725	0.990
	No Treatment Control vs. BioSpray	0.000	-2.059 to 2.059	0.999
	No Treatment Control vs. Vehicle Patty	-0.667	-2.725 to 1.392	0.879
	No Treatment Control vs. BioPatty	0.000	-2.059 to 2.059	0.999
	Vehicle Spray vs. BioSpray	0.333	-1.725 to 2.392	0.990
	Vehicle Spray vs. Vehicle Patty	-0.333	-2.392 to 1.725	0.990
	Vehicle Spray vs. BioPatty	0.333	-1.725 to 2.392	0.990
	BioSpray vs. Vehicle Patty	-0.667	-2.725 to 1.392	0.879
	BioSpray vs. BioPatty	0.000	-2.059 to 2.059	0.999
	Vehicle Patty vs. BioPatty	0.667	-1.392 to 2.725	0.879
Week 4	No Treatment Control vs. Vehicle Spray	-0.3333	-2.392 to 1.725	0.990
	No Treatment Control vs. BioSpray	-1.667	-3.725 to 0.3920	0.158
	No Treatment Control vs. Vehicle Patty	0.000	-2.059 to 2.059	0.999
	No Treatment Control vs. BioPatty	-0.3333	-2.392 to 1.725	0.990
	Vehicle Spray vs. BioSpray	-1.333	-3.392 to 0.7253	0.350
	Vehicle Spray vs. Vehicle Patty	0.3333	-1.725 to 2.392	0.990
	Vehicle Spray vs. BioPatty	3.331e-016	-2.059 to 2.059	0.999
	BioSpray vs. Vehicle Patty	1.667	-0.3920 to 3.725	0.158
	BioSpray vs. BioPatty	1.333	-0.7253 to 3.392	0.350
	Vehicle Patty vs. BioPatty	-0.3333	-2.392 to 1.725	0.990
Week 8	No Treatment Control vs. Vehicle Spray	-0.3333	-2.392 to 1.725	0.990
	No Treatment Control vs. BioSpray	-1.667	-3.725 to 0.3920	0.159
	No Treatment Control vs. Vehicle Patty	-0.3333	-2.392 to 1.725	0.990
	No Treatment Control vs. BioPatty	0.6667	-1.392 to 2.725	0.880
	Vehicle Spray vs. BioSpray	-1.333	-3.392 to 0.7253	0.350
	Vehicle Spray vs. Vehicle Patty	0.000	-2.059 to 2.059	0.999
	Vehicle Spray vs. BioPatty	1.000	-1.059 to 3.059	0.627
	BioSpray vs. Vehicle Patty	1.333	-0.7253 to 3.392	0.350
	BioSpray vs. BioPatty	2.333	0.2747 to 4.392	**0.020**
	Vehicle Patty vs. BioPatty	1.000	-1.059 to 3.059	0.627

Table 3-4. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of *Varroa destructor* mite infestation levels for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the forage-rich location. Tukey HSD test results are bolded for any significant difference between treatment groups within a single timepoint.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	No Treatment Control vs. Vehicle Spray	0.333	-1.535 to 2.202	0.985
	No Treatment Control vs. BioSpray	0.333	-1.535 to 2.202	0.985
	No Treatment Control vs. Vehicle Patty	-0.333	-2.202 to 1.535	0.985
	No Treatment Control vs. BioPatty	0.333	-1.535 to 2.202	0.985
	Vehicle Spray vs. BioSpray	0.000	-1.868 to 1.868	0.999
	Vehicle Spray vs. Vehicle Patty	-0.667	-2.535 to 1.202	0.837
	Vehicle Spray vs. BioPatty	0.000	-1.868 to 1.868	0.999
	BioSpray vs. Vehicle Patty	-0.667	-2.535 to 1.202	0.837
	BioSpray vs. BioPatty	0.000	-1.868 to 1.868	0.999
	Vehicle Patty vs. BioPatty	0.667	-1.202 to 2.535	0.837
Week 4	No Treatment Control vs. Vehicle Spray	1.000	-0.8682 to 2.868	0.538
	No Treatment Control vs. BioSpray	1.000	-0.8682 to 2.868	0.538
	No Treatment Control vs. Vehicle Patty	-0.667	-2.535 to 1.202	0.837
	No Treatment Control vs. BioPatty	0.667	-1.202 to 2.535	0.837
	Vehicle Spray vs. BioSpray	0.000	-1.868 to 1.868	0.999
	Vehicle Spray vs. Vehicle Patty	-1.667	-3.535 to 0.2015	0.098
	Vehicle Spray vs. BioPatty	-0.333	-2.202 to 1.535	0.985
	BioSpray vs. Vehicle Patty	-1.667	-3.535 to 0.2015	0.098
	BioSpray vs. BioPatty	-0.333	-2.202 to 1.535	0.985
	Vehicle Patty vs. BioPatty	1.333	-0.5348 to 3.202	0.259
Week 8	No Treatment Control vs. Vehicle Spray	0.000	-1.868 to 1.868	0.999
	No Treatment Control vs. BioSpray	-0.333	-2.202 to 1.535	0.985
	No Treatment Control vs. Vehicle Patty	-3.667	-5.535 to -1.798	**<0.0001**
	No Treatment Control vs. BioPatty	0.667	-1.202 to 2.535	0.837
	Vehicle Spray vs. BioSpray	-0.333	-2.202 to 1.535	0.985
	Vehicle Spray vs. Vehicle Patty	-3.667	-5.535 to -1.798	**<0.0001**
	Vehicle Spray vs. BioPatty	0.667	-1.202 to 2.535	0.837
	BioSpray vs. Vehicle Patty	-3.333	-5.202 to -1.465	**0.0001**
	BioSpray vs. BioPatty	1.000	-0.8682 to 2.868	0.538
	Vehicle Patty vs. BioPatty	4.333	2.465 to 6.202	**<0.0001**

Table 3-5. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of *Varroa destructor* mite infestation levels for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the agricultural location. Tukey HSD test results are bolded for any significant difference between treatment groups within a single timepoint.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	No Treatment Control vs. Vehicle Spray	-2.220e-016	-2.420 to 2.420	0.999
	No Treatment Control vs. BioSpray	0.333	-2.087 to 2.754	0.994
	No Treatment Control vs. Vehicle Patty	-2.220e-016	-2.420 to 2.420	0.999
	No Treatment Control vs. BioPatty	0.333	-2.087 to 2.754	0.994
	Vehicle Spray vs. BioSpray	0.333	-2.087 to 2.754	0.994
	Vehicle Spray vs. Vehicle Patty	0.000	-2.420 to 2.420	0.999
	Vehicle Spray vs. BioPatty	0.333	-2.087 to 2.754	0.994
	BioSpray vs. Vehicle Patty	-0.333	-2.754 to 2.087	0.994
	BioSpray vs. BioPatty	0.000	-2.420 to 2.420	0.999
	Vehicle Patty vs. BioPatty	0.333	-2.087 to 2.754	0.994
Week 4	No Treatment Control vs. Vehicle Spray	1.333	-1.087 to 3.754	0.510
	No Treatment Control vs. BioSpray	1.000	-1.420 to 3.420	0.752
	No Treatment Control vs. Vehicle Patty	1.000	-1.420 to 3.420	0.752
	No Treatment Control vs. BioPatty	0.667	-1.754 to 3.087	0.929
	Vehicle Spray vs. BioSpray	-0.333	-2.754 to 2.087	0.994
	Vehicle Spray vs. Vehicle Patty	-0.333	-2.754 to 2.087	0.994
	Vehicle Spray vs. BioPatty	-0.667	-3.087 to 1.754	0.929
	BioSpray vs. Vehicle Patty	0.000	-2.420 to 2.420	0.999
	BioSpray vs. BioPatty	-0.333	-2.754 to 2.087	0.994
	Vehicle Patty vs. BioPatty	-0.333	-2.754 to 2.087	0.994
Week 8	No Treatment Control vs. Vehicle Spray	1.333	-1.087 to 3.754	0.510
	No Treatment Control vs. BioSpray	1.667	-0.7537 to 4.087	0.292
	No Treatment Control vs. Vehicle Patty	-1.333	-3.754 to 1.087	0.510
	No Treatment Control vs. BioPatty	1.667	-0.7537 to 4.087	0.292
	Vehicle Spray vs. BioSpray	0.333	-2.087 to 2.754	0.994
	Vehicle Spray vs. Vehicle Patty	-2.667	-5.087 to -0.2463	**0.025**
	Vehicle Spray vs. BioPatty	0.333	-2.087 to 2.754	0.994
	BioSpray vs. Vehicle Patty	-3.000	-5.420 to -0.5796	**0.009**
	BioSpray vs. BioPatty	0.000	-2.420 to 2.420	0.999
	Vehicle Patty vs. BioPatty	3.000	0.5796 to 5.420	**0.009**

Table 3-6. Pairwise comparisons of estimated marginal mean of the slope of treatment groups for *Varroa destructor* mite infestation levels. Confidence level used: 0.95; *P*-value adjustment: Tukey method for comparing a family of five estimates.

Comparison	Estimate	SE	df	T-ratio	Adjusted *P* value
NTC vs. BioPatty	0.097	0.086	123	1.136	0.787
NTC vs. BioSpray	-0.042	0.086	123	-0.487	0.988
NTC vs. Vehicle Patty	-0.181	0.086	123	-2.11	0.223
NTC vs. Vehicle Spray	0.042	0.086	123	0.487	0.988
BioPatty vs. BioSpray	-0.139	0.086	123	-1.623	0.486
BioPatty vs. Vehicle Patty	-0.278	0.086	123	-3.246	**0.013**
BioPatty vs. Vehicle Spray	-0.056	0.086	123	-0.649	0.967
BioSpray vs. Vehicle Patty	-0.139	0.086	123	-1.623	0.486
BioSpray vs. Vehicle Spray	0.083	0.086	123	0.974	0.867
Vehicle Patty vs. Vehicle Spray	0.016	0.086	123	2.597	0.077

Table 3-7. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of total bacterial loads for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the urban location. Tukey HSD test results are bolded for any significant difference between treatment groups within a single timepoint.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	BioPatty vs. Vehicle Patty	0.067	-1.252 to 1.386	0.999
	BioPatty vs. BioSpray	-0.244	-1.460 to 0.9723	0.981
	BioPatty vs. Vehicle Spray	0.377	-0.9424 to 1.696	0.932
	BioPatty vs. NTC	-0.343	-1.559 to 0.8730	0.935
	Vehicle Patty vs. BioSpray	-0.310	-1.662 to 1.041	0.968
	Vehicle Patty vs. Vehicle Spray	0.310	-1.135 to 1.755	0.975
	Vehicle Patty vs. NTC	-0.410	-1.761 to 0.9420	0.917
	BioSpray vs. Vehicle Spray	0.620	-0.7313 to 1.972	0.707
	BioSpray vs. NTC	-0.099	-1.351 to 1.152	1.000
	Vehicle Spray vs. NTC	-0.720	-2.071 to 0.6320	0.578
Week 4	BioPatty vs. Vehicle Patty	-0.285	-1.604 to 1.034	0.975
	BioPatty vs. BioSpray	-0.827	-2.043 to 0.3894	0.330
	BioPatty vs. Vehicle Spray	0.328	-0.9912 to 1.647	0.958
	BioPatty vs. NTC	-0.565	-1.781 to 0.6513	0.697
	Vehicle Patty vs. BioSpray	-0.541	-1.893 to 0.8103	0.799
	Vehicle Patty vs. Vehicle Spray	0.613	-0.8317 to 2.058	0.763
	Vehicle Patty vs. NTC	-0.279	-1.631 to 1.072	0.979
	BioSpray vs. Vehicle Spray	1.154	-0.1972 to 2.506	0.131
	BioSpray vs. NTC	0.262	-0.9894 to 1.513	0.977
	Vehicle Spray vs. NTC	-0.892	-2.244 to 0.4591	0.359
Week 8	BioPatty vs. Vehicle Patty	-0.177	-1.496 to 1.142	0.996
	BioPatty vs. BioSpray	-0.492	-1.708 to 0.7237	0.793
	BioPatty vs. Vehicle Spray	1.122	-0.1972 to 2.441	0.134
	BioPatty vs. NTC	-0.630	-1.846 to 0.5860	0.603
	Vehicle Patty vs. BioSpray	-0.315	-1.666 to 1.037	0.967
	Vehicle Patty vs. Vehicle Spray	1.299	-0.1458 to 2.744	0.099
	Vehicle Patty vs. NTC	-0.453	-1.804 to 0.8989	0.884
	BioSpray vs. Vehicle Spray	1.614	0.2625 to 2.966	**0.011**
	BioSpray vs. NTC	-0.138	-1.389 to 1.114	0.998
	Vehicle Spray vs. NTC	-1.752	-3.103 to -0.4002	**0.005**

Table 3-8. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of total bacterial loads for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the forage-rich location.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	BioPatty vs. Vehicle Patty	0.230	-5.646 to 6.105	0.999
	BioPatty vs. BioSpray	-0.594	-5.808 to 4.620	0.987
	BioPatty vs. Vehicle Spray	-0.500	-5.641 to 4.641	0.994
	BioPatty vs. NTC	-1.515	-6.844 to 3.813	0.741
	Vehicle Patty vs. BioSpray	-0.823	-6.226 to 4.579	0.943
	Vehicle Patty vs. Vehicle Spray	-0.730	-5.966 to 4.506	0.966
	Vehicle Patty vs. NTC	-1.745	-7.411 to 3.921	0.595
	BioSpray vs. Vehicle Spray	0.094	-1.786 to 1.974	1.000
	BioSpray vs. NTC	-0.922	-2.396 to 0.5532	0.283
	Vehicle Spray vs. NTC	-1.015	-2.681 to 0.6503	0.338
Week 4	BioPatty vs. Vehicle Patty	1.940	-2.763 to 6.643	0.411
	BioPatty vs. BioSpray	1.366	-1.240 to 3.972	0.412
	BioPatty vs. Vehicle Spray	1.535	-0.4191 to 3.489	0.147
	BioPatty vs. NTC	0.140	-1.173 to 1.453	0.984
	Vehicle Patty vs. BioSpray	-0.574	-4.867 to 3.720	0.985
	Vehicle Patty vs. Vehicle Spray	-0.405	-4.678 to 3.868	0.994
	Vehicle Patty vs. NTC	-1.800	-6.623 to 3.023	0.456
	BioSpray vs. Vehicle Spray	0.169	-2.632 to 2.970	1.000
	BioSpray vs. NTC	-1.226	-3.815 to 1.363	0.481
	Vehicle Spray vs. NTC	-1.395	-3.279 to 0.4894	0.178
Week 8	BioPatty vs. Vehicle Patty	-0.040	-5.418 to 5.337	0.999
	BioPatty vs. BioSpray	-0.408	-5.350 to 4.534	0.998
	BioPatty vs. Vehicle Spray	-1.839	-6.809 to 3.130	0.634
	BioPatty vs. NTC	-1.920	-6.954 to 3.114	0.702
	Vehicle Patty vs. BioSpray	-0.368	-4.501 to 3.765	0.998
	Vehicle Patty vs. Vehicle Spray	-1.799	-5.860 to 2.263	0.504
	Vehicle Patty vs. NTC	-1.880	-6.174 to 2.414	0.616
	BioSpray vs. Vehicle Spray	-1.431	-3.858 to 0.9966	0.348
	BioSpray vs. NTC	-1.512	-4.821 to 1.797	0.609
	Vehicle Spray vs. NTC	-0.081	-3.042 to 2.879	0.999

Table 3-9. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of total bacterial loads for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the agricultural location.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	BioPatty vs. Vehicle Patty	-0.167	-0.662 to 0.328	0.827
	BioPatty vs. BioSpray	0.052	-0.765 to 0.868	1.000
	BioPatty vs. Vehicle Spray	-0.138	-0.601 to 0.325	0.879
	BioPatty vs. NTC	0.078	-0.697 to 0.852	0.997
	Vehicle Patty vs. BioSpray	0.219	-0.591 to 1.028	0.902
	Vehicle Patty vs. Vehicle Spray	0.029	-0.408 to 0.466	0.999
	Vehicle Patty vs. NTC	0.245	-0.521 to 1.011	0.840
	BioSpray vs. Vehicle Spray	-0.190	-0.988 to 0.609	0.932
	BioSpray vs. NTC	0.026	-0.931 to 0.983	0.999
	Vehicle Spray vs. NTC	0.216	-0.538 to 0.970	0.880
Week 4	BioPatty vs. Vehicle Patty	-0.341	-0.800 to 0.117	0.185
	BioPatty vs. BioSpray	-0.183	-0.5054to 0.140	0.419
	BioPatty vs. Vehicle Spray	-0.285	-0.6660to 0.095	0.186
	BioPatty vs. NTC	-0.243	-0.635 to 0.150	0.337
	Vehicle Patty vs. BioSpray	0.158	-0.322 to 0.638	0.836
	Vehicle Patty vs. Vehicle Spray	0.056	-0.455 to 0.566	0.997
	Vehicle Patty vs. NTC	0.098	-0.419 to 0.616	0.975
	BioSpray vs. Vehicle Spray	-0.102	-0.514 to 0.309	0.935
	BioSpray vs. NTC	-0.060	-0.482 to 0.362	0.991
	Vehicle Spray vs. NTC	0.043	-0.417 to 0.502	0.998
Week 8	BioPatty vs. Vehicle Patty	-0.007	-0.912 to 0.897	0.999
	BioPatty vs. BioSpray	0.030	-0.862 to 0.922	0.999
	BioPatty vs. Vehicle Spray	-0.228	-1.208 to 0.753	0.947
	BioPatty vs. NTC	-0.116	-1.016 to 0.784	0.992
	Vehicle Patty vs. BioSpray	0.037	-0.436 to 0.510	0.999
	Vehicle Patty vs. Vehicle Spray	-0.220	-0.928 to 0.488	0.860
	Vehicle Patty vs. NTC	-0.109	-0.615 to 0.398	0.960
	BioSpray vs. Vehicle Spray	-0.257	-0.940 to 0.425	0.746
	BioSpray vs. NTC	-0.146	-0.598 to 0.306	0.847
	Vehicle Spray vs. NTC	0.112	-0.587 to 0.811	0.985

Table 3-10. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of *P. larvae* loads for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the urban location.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	BioPatty vs. Vehicle Patty	-0.394	-0.982 to 0.195	0.346
	BioPatty vs. BioSpray	0.205	-0.337 to 0.748	0.830
	BioPatty vs. Vehicle Spray	0.182	-0.407 to 0.771	0.911
	BioPatty vs. NTC	0.123	-0.420 to 0.665	0.970
	Vehicle Patty vs. BioSpray	0.599	-0.004 to 1.203	0.052
	Vehicle Patty vs. Vehicle Spray	0.576	-0.069 to 1.221	0.103
	Vehicle Patty vs. NTC	0.517	-0.087 to 1.120	0.130
	BioSpray vs. Vehicle Spray	-0.023	-0.626 to 0.580	0.999
	BioSpray vs. NTC	-0.083	-0.641 to 0.476	0.994
	Vehicle Spray vs. NTC	-0.060	-0.663 to 0.544	0.999
Week 4	BioPatty vs. Vehicle Patty	0.006	-0.583 to 0.594	0.999
	BioPatty vs. BioSpray	0.225	-0.318 to 0.767	0.779
	BioPatty vs. Vehicle Spray	0.225	-0.364 to 0.813	0.826
	BioPatty vs. NTC	0.225	-0.318 to 0.767	0.779
	Vehicle Patty vs. BioSpray	0.219	-0.384 to 0.822	0.851
	Vehicle Patty vs. Vehicle Spray	0.219	-0.426 to 0.864	0.879
	Vehicle Patty vs. NTC	0.219	-0.384 to 0.822	0.851
	BioSpray vs. Vehicle Spray	0.000	-0.603 to 0.603	0.999
	BioSpray vs. NTC	0.000	-0.559 to 0.559	0.999
	Vehicle Spray vs. NTC	0.000	-0.603 to 0.603	0.999
Week 8	BioPatty vs. Vehicle Patty	0.006	-0.583 to 0.594	0.999
	BioPatty vs. BioSpray	0.225	-0.318 to 0.767	0.779
	BioPatty vs. Vehicle Spray	0.225	-0.364 to 0.813	0.826
	BioPatty vs. NTC	0.225	-0.318 to 0.767	0.779
	Vehicle Patty vs. BioSpray	0.219	-0.384 to 0.822	0.851
	Vehicle Patty vs. Vehicle Spray	0.219	-0.423 to 0.864	0.879
	Vehicle Patty vs. NTC	0.219	-0.384 to 0.822	0.851
	BioSpray vs. Vehicle Spray	0.000	-0.603 to 0.603	0.999
	BioSpray vs. NTC	0.000	-0.559 to 0.559	0.999
	Vehicle Spray vs. NTC	0.000	-0.603 to 0.603	0.999

Table 3-11. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of *P. larvae* loads for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the forage-rich location.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	BioPatty vs. Vehicle Patty	0.000		
	BioPatty vs. BioSpray	-0.316	-1.420 to 0.788	0.838
	BioPatty vs. Vehicle Spray	-0.156	-0.460 to 0.149	0.451
	BioPatty vs. NTC	-1.027	-2.722 to 0.667	0.291
	Vehicle Patty vs. BioSpray	-0.316	-1.420 to 0.788	0.838
	Vehicle Patty vs. Vehicle Spray	-0.156	-0.460 to 0.149	0.451
	Vehicle Patty vs. NTC	-1.027	-2.722 to 0.667	0.291
	BioSpray vs. Vehicle Spray	0.160	-0.944 to 1.264	0.985
	BioSpray vs. NTC	-0.712	-2.512 to 1.089	0.719
	Vehicle Spray vs. NTC	-0.872	-2.564 to 0.821	0.433
Week 4	BioPatty vs. Vehicle Patty	0.331	-4.893 to 5.556	0.999
	BioPatty vs. BioSpray	-0.180	-5.363 to 5.003	0.999
	BioPatty vs. Vehicle Spray	0.151	-5.045 to 5.347	0.999
	BioPatty vs. NTC	1.156	-4.640 to 6.952	0.844
	Vehicle Patty vs. BioSpray	-0.511	-4.049 to 3.027	0.990
	Vehicle Patty vs. Vehicle Spray	-0.180	-4.002 to 3.642	1.000
	Vehicle Patty vs. NTC	0.825	-2.356 to 4.006	0.876
	BioSpray vs. Vehicle Spray	0.331	-3.173 to 3.835	0.998
	BioSpray vs. NTC	1.336	-1.227 to 3.900	0.443
	Vehicle Spray vs. NTC	1.005	-2.080 to 4.090	0.790
Week 8	BioPatty vs. Vehicle Patty	-0.040	-5.418 to 5.337	0.999
	BioPatty vs. BioSpray	-0.408	-5.350 to 4.534	0.998
	BioPatty vs. Vehicle Spray	-1.839	-6.809 to 3.130	0.634
	BioPatty vs. NTC	-1.920	-6.954 to 3.114	0.702
	Vehicle Patty vs. BioSpray	-0.368	-4.501 to 3.765	0.998
	Vehicle Patty vs. Vehicle Spray	-1.799	-5.860 to 2.263	0.504
	Vehicle Patty vs. NTC	-1.880	-6.174 to 2.414	0.616
	BioSpray vs. Vehicle Spray	-1.431	-3.858 to 0.9966	0.348
	BioSpray vs. NTC	-1.512	-4.821 to 1.797	0.609
	Vehicle Spray vs. NTC	-0.081	-3.042 to 2.879	0.999

Table 3-12. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test of *P. larvae* loads for all five treatment groups (n=3 hives per group) at each week (n=3 timepoints) for the agricultural location.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted *P* value
Week 0	BioPatty vs. Vehicle Patty	-0.065	-0.378 to 0.248	0.946
	BioPatty vs. BioSpray	0.022	-0.059 to 0.103	0.853
	BioPatty vs. Vehicle Spray	-0.007	-0.123 to 0.109	0.999
	BioPatty vs. NTC	0.008	-0.077 to 0.093	0.998
	Vehicle Patty vs. BioSpray	0.087	-0.226 to 0.400	0.849
	Vehicle Patty vs. Vehicle Spray	0.058	-0.256 to 0.372	0.967
	Vehicle Patty vs. NTC	0.073	-0.239 to 0.386	0.914
	BioSpray vs. Vehicle Spray	-0.029	-0.132 to 0.073	0.853
	BioSpray vs. NTC	-0.014	-0.063 to 0.035	0.857
	Vehicle Spray vs. NTC	0.016	-0.090 to 0.121	0.989
Week 4	BioPatty vs. Vehicle Patty	-0.244	-1.030 to 0.542	0.862
	BioPatty vs. BioSpray	0.146	-0.332 to 0.625	0.805
	BioPatty vs. Vehicle Spray	0.152	-0.326 to 0.630	0.785
	BioPatty vs. NTC	-0.269	-1.152 to 0.613	0.864
	Vehicle Patty vs. BioSpray	0.390	-0.341 to 1.121	0.414
	Vehicle Patty vs. Vehicle Spray	0.396	-0.336 to 1.127	0.402
	Vehicle Patty vs. NTC	-0.026	-1.016 to 0.965	0.999
	BioSpray vs. Vehicle Spray	0.005	-0.014 to 0.025	0.848
	BioSpray vs. NTC	-0.416	-1.257 to 0.426	0.480
	Vehicle Spray vs. NTC	-0.421	-1.263 to 0.420	0.469
Week 8	BioPatty vs. Vehicle Patty	-0.300	-1.375 to 0.774	0.848
	BioPatty vs. BioSpray	0.000		
	BioPatty vs. Vehicle Spray	-0.108	-0.419 to 0.203	0.752
	BioPatty vs. NTC	-0.157	-0.700 to 0.385	0.831
	Vehicle Patty vs. BioSpray	0.300	-0.774 to 1.375	0.848
	Vehicle Patty vs. Vehicle Spray	0.192	-0.883 to 1.267	0.969
	Vehicle Patty vs. NTC	0.143	-0.9567to 1.243	0.992
	BioSpray vs. Vehicle Spray	-0.108	-0.419 to 0.203	0.752
	BioSpray vs. NTC	-0.157	-0.700 to 0.385	0.831
	Vehicle Spray vs. NTC	-0.049	-0.615 to 0.516	0.999

Table 3-13. Tukey's multiple comparisons test of bacterial (BAC) and *Paenibacillus larvae* loads at week 0 for all locations (agricultural, forage-rich, and urban).

Week 0	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjust *P* Value
BAC	BioPatty vs. Vehicle Patty	0.007	-0.759 to 0.773	ns	0.999
	BioPatty vs. BioSpray	-0.260	-0.997 to 0.478	ns	0.869
	BioPatty vs. Vehicle Spray	0.035	-0.688 to 0.757	ns	0.999
	BioPatty vs. NTC	-0.460	-1.190 to 0.270	ns	0.415
	Vehicle Patty vs. BioSpray	-0.266	-1.033 to 0.500	ns	0.874
	Vehicle Patty vs. Vehicle Spray	0.028	-0.724 to 0.779	ns	0.999
	Vehicle Patty vs. NTC	-0.467	-1.225 to 0.292	ns	0.441
	BioSpray vs. Vehicle Spray	0.294	-0.428 to 1.016	ns	0.796
	BioSpray vs. NTC	-0.200	-0.930 to 0.529	ns	0.943
	Vehicle Spray vs. NTC	-0.494	-1.208 to 0.219	ns	0.318
P. larvae	BioPatty vs. Vehicle Patty	-0.129	-0.895 to 0.638	ns	0.991
	BioPatty vs. BioSpray	-0.020	-0.758 to 0.718	ns	0.999
	BioPatty vs. Vehicle Spray	0.026	-0.696 to 0.748	ns	0.999
	BioPatty vs. NTC	-0.136	-0.866 to 0.593	ns	0.986
	Vehicle Patty vs. BioSpray	0.109	-0.658 to 0.875	ns	0.995
	Vehicle Patty vs. Vehicle Spray	0.155	-0.597 to 0.906	ns	0.980
	Vehicle Patty vs. NTC	-0.008	-0.766 to 0.751	ns	0.999
	BioSpray vs. Vehicle Spray	0.046	-0.676 to 0.768	ns	1.000
	BioSpray vs. NTC	-0.116	-0.846 to 0.614	ns	0.992
	Vehicle Spray vs. NTC	-0.162	-0.876 to 0.552	ns	0.971

Table 3-14. Tukey's Multiple Comparisons test of bacterial (BAC) and *P. larvae* loads at week 4 for all locations (agricultural, forage-rich, and urban).

Week 4	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjust *P* Value
BAC	BioPatty vs. Vehicle Patty	0.029	-0.987 to 1.044	ns	0.999
	BioPatty vs. BioSpray	-0.040	-1.009 to 0.929	ns	0.999
	BioPatty vs. Vehicle Spray	0.351	-0.609 to 1.311	ns	0.852
	BioPatty vs. NTC	-0.356	-1.326 to 0.613	ns	0.850
	Vehicle Patty vs. BioSpray	-0.069	-1.051 to 0.914	ns	1.000
	Vehicle Patty vs. Vehicle Spray	0.322	-0.651 to 1.296	ns	0.892
	Vehicle Patty vs. NTC	-0.385	-1.368 to 0.597	ns	0.817
	BioSpray vs. Vehicle Spray	0.391	-0.534 to 1.316	ns	0.772
	BioSpray vs. NTC	-0.317	-1.251 to 0.618	ns	0.884
	Vehicle Spray vs. NTC	-0.708	-1.633 to 0.217	ns	0.222
P. larvae	BioPatty vs. Vehicle Patty	-0.259	-1.274 to 0.757	ns	0.956
	BioPatty vs. BioSpray	-0.178	-1.147 to 0.792	ns	0.987
	BioPatty vs. Vehicle Spray	-0.070	-1.030 to 0.890	ns	1.000
	BioPatty vs. NTC	0.111	-0.858 to 1.080	ns	0.998
	Vehicle Patty vs. BioSpray	0.081	-0.901 to 1.064	ns	0.999
	Vehicle Patty vs. Vehicle Spray	0.189	-0.784 to 1.162	ns	0.984
	Vehicle Patty vs. NTC	0.370	-0.613 to 1.353	ns	0.839
	BioSpray vs. Vehicle Spray	0.108	-0.817 to 1.033	ns	0.998
	BioSpray vs. NTC	0.289	-0.646 to 1.223	ns	0.915
	Vehicle Spray vs. NTC	0.181	-0.744 to 1.106	ns	0.983

Table 3-15. Tukey's Multiple Comparisons test of bacterial (BAC) and *P. larvae* loads at week 8 for all locations (agricultural, forage-rich, and urban).

Week 8	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjust *P* Value
BAC	BioPatty vs. Vehicle Patty	-0.022	-1.065 to 1.020	ns	0.999
	BioPatty vs. BioSpray	-0.124	-1.119 to 0.871	ns	0.997
	BioPatty vs. Vehicle Spray	-0.269	-1.264 to 0.726	ns	0.946
	BioPatty vs. NTC	-0.751	-1.756 to 0.255	ns	0.244
	Vehicle Patty vs. BioSpray	-0.102	-1.134 to 0.931	ns	0.999
	Vehicle Patty vs. Vehicle Spray	-0.247	-1.279 to 0.785	ns	0.965
	Vehicle Patty vs. NTC	-0.728	-1.771 to 0.314	ns	0.309
	BioSpray vs. Vehicle Spray	-0.145	-1.130 to 0.839	ns	0.994
	BioSpray vs. NTC	-0.627	-1.621 to 0.368	ns	0.416
	Vehicle Spray vs. NTC	-0.481	-1.476 to 0.514	ns	0.672
P. larvae	BioPatty vs. Vehicle Patty	0.339	-0.718 to 1.396	ns	0.903
	BioPatty vs. BioSpray	0.387	-0.608 to 1.382	ns	0.822
	BioPatty vsVehicle Spray	0.256	-0.739 to 1.250	ns	0.955
	BioPatty vs. NTC	0.256	-0.750 to 1.261	ns	0.956
	Vehicle Patty vs. BioSpray	0.048	-0.999 to 1.095	ns	0.999
	Vehicle Patty vs. Vehicle Spray	-0.084	-1.131 to 0.963	ns	1.000
	Vehicle Patty vs. NTC	-0.083	-1.140 to 0.974	ns	1.000
	BioSpray vs. Vehicle Spray	-0.131	-1.115 to 0.853	ns	0.996
	BioSpray vs. NTC	-0.131	-1.126 to 0.864	ns	0.996
	Vehicle Spray vs. NTC	0.000	-0.995 to 0.995	ns	0.999

Table 3-16. Mixed-Effects Analysis of bacterial (BAC) loads for all locations and treatment groups.

Fixed effects (type III)	*P* value	*P* value summary	Statistically significant (P < 0.05)?	$F_{(DFn, DFd)}$	Geisser-Greenhouse's epsilon
Week	0.0007	***	Yes	$F_{(1.601, 140.1)} = 8.901$	0.8005
Treatment	0.4686	ns	No	$F_{(4, 116)} = 0.8964$	
Week x Treatment	0.8895	ns	No	$F_{(8, 175)} = 0.4497$	

Table 3-17. Tukey's Multiple Comparisons test of bacterial (BAC) loads for all locations (n=3 sites) and treatment groups (n=5 groups).

	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Adjusted *P* Value
BioPatty	Week 0 vs. Week 4	0.144	-0.116 to 0.405	0.358
	Week 0 vs. Week 8	-0.282	-0.982 to 0.418	0.567
	Week 4 vs. Week 8	-0.426	-1.161 to 0.309	0.322
Vehicle Patty	Week 0 vs. Week 4	-0.089	-0.591 to 0.412	0.358
	Week 0 vs. Week 8	-0.372	-0.962 to 0.219	0.567
	Week 4 vs. Week 8	-0.282	-0.953 to 0.389	0.322
BioSpray	Week 0 vs. Week 4	-0.177	-0.461 to 0.106	0.274
	Week 0 vs. Week 8	-0.296	-0.718 to 0.127	0.202
	Week 4 vs. Week 8	-0.118	-0.448 to 0.212	0.639
Vehicle Spray	Week 0 vs. Week 4	-0.085	-0.475 to 0.304	0.840
	Week 0 vs. Week 8	-0.257	-0.877 to 0.363	0.539
	Week 4 vs. Week 8	-0.172	-0.945 to 0.602	0.835
NTC	Week 0 vs. Week 4	-0.168	-0.522 to 0.186	0.468
	Week 0 vs. Week 8	-0.540	-0.923 to -0.157	**0.005**
	Week 4 vs. Week 8	-0.372	-0.803 to 0.059	0.098

Table 3-18. Mixed-Effects Analysis of *P. larvae* loads for all locations (n=3 sites) and treatment groups (n=5 groups).

Fixed effects (type III)	*P* value	$F_{(DFn, DFd)}$	Geisser-Greenhouse's epsilon
Week	0.0925	$F_{(1.730, 302.8)} = 2.491$	0.8651
Treatment	0.9466	$F_{(4, 350)} = 0.1841$	
Week x Treatment	0.6035	$F_{(8, 350)} = 0.7993$	

Table 3-19. Tukey's multiple comparisons test of *Paenibacillus larvae* loads for all locations (n=3 sites) and treatment groups (n=5 groups).

	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Adjusted *P* Value
BioPatty	Week 0 vs. Week 4	-0.218	-0.854 to 0.418	0.666
	Week 0 vs. Week 8	-0.232	-0.959 to 0.495	0.704
	Week 4 vs. Week 8	-0.014	-0.941 to 0.913	0.999
Vehicle Patty	Week 0 vs. Week 4	-0.305	-0.775 to 0.164	0.249
	Week 0 vs. Week 8	0.081	-0.310 to 0.472	0.860
	Week 4 vs. Week 8	0.386	-0.317 to 1.090	0.367
BioSpray	Week 0 vs. Week 4	-0.489	-1.236 to 0.259	0.250
	Week 0 vs. Week 8	0.105	-0.147 to 0.357	0.558
	Week 4 vs. Week 8	0.594	-0.067 to 1.255	0.084
Vehicle Spray	Week 0 vs. Week 4	-0.331	-1.165 to 0.504	0.584
	Week 0 vs. Week 8	-0.046	-0.316 to 0.223	0.903
	Week 4 vs. Week 8	0.284	-0.522 to 1.09	0.656
NTC	Week 0 vs. Week 4	0.139	-0.204 to 0.482	0.571
	Week 0 vs. Week 8	-0.010	-0.854 to 0.834	1.000
	Week 4 vs. Week 8	-0.149	-0.543 to 0.244	0.613

Chapter 4

4 General Conclusion

The apicultural sector is integral to the agricultural landscape and the Canadian economy as well as that of other countries. It plays a critical role in the pollination of a diverse array of crops that significantly contribute to global food security (Klein et al., 2007). Despite the importance of these pollinators, beekeepers are currently facing a lack of options for effectively managing pests and pathogens that threaten honey bee colony health. Traditional interventions, predominantly antibiotics and chemical pesticides, not only exhibit diminishing efficacy but also raise concerns regarding environmental contamination, disruption of indigenous microbes and the emergence of resistant strains, thereby undermining the sustainability of apicultural practices (Goulson et al., 2015; Piva et al., 2020). However, probiotics, by virtue of modulating the microbiome and bolstering the immune responses of honey bees (Daisley et al., 2019; Vásquez et al., 2012), offer a novel paradigm for enhancing colony resilience against multifaceted threats. In this thesis, the potential of such microbial interventions was explored, first, by discussing the interactions between *Paenibacillus larvae* and other honey bee-adapted microorganisms (Chapter Two) and, second by testing if the delivery of specific strains of lactobacilli can mitigate *P. larvae* infections and *Varroa destructor* infestations in honey bee colonies (Chapter Three).

In Chapter Two, the complex interplay is explored between honey bees, the pathogen *P. larvae* responsible for American foulbrood (AFB), and the wider microbial ecosystem surrounding them. Despite a century of focused research into the pathogenesis of AFB, a critical gap persists in our understanding, especially concerning the role of microbial ecology. I argue that the intricate dynamics within the bee microbiota, as well as the interactions between bees and their environment, are crucial in shifting from asymptomatic to symptomatic disease states. This underscores the necessity of adopting a broader ecological lens in approaching disease management strategies, moving beyond the conventional aim of simply eradicating the *P. larvae* pathogen (Daisley et al., 2023).

A key argument put forward in the synthetic review of this thesis is of the viability of probiotics as a sustainable avenue to bolster honey bee health and fortify their resistance against AFB. Chronic antibiotic use comes with a host of adverse effects, as stated herein (Daisley et al., 2020b), so if probiotic applications can be as effective without these adverse effects it would significantly impact hive management. The need to integrate the manipulation of the honey bee microbiota into apicultural practices is discussed as a method not only to counteract *P. larvae* infections effectively but also to herald innovative, ecologically sensitive disease control measures.

In conclusion, this thesis advocates for a paradigm shift towards holistic management strategies that account for the complex interactions among microbial communities, host immunity, and environmental variables in combating AFB. This view point stresses the imperative for ongoing research to refine the application of probiotics, understand their mechanisms of action comprehensively, and evaluate their long-term benefits on bee health and productivity. By fostering an enriched understanding of the microbial foundations of AFB and exploring microbiome-centric solutions, the thesis lays out a path towards a sustainable future for beekeeping—a future where the health of honey bee colonies is not only preserved but also enhanced, thereby ensuring their irreplaceable role in global pollination and food security is sustained (Khalifa et al., 2021).

Chapter Three deciphers the intricacies of honey bee health, particularly focusing on the mitigation of *P. larvae* infections and *Varroa destructor* infestations through probiotic applications. The venture was underpinned by a hypothesis that introducing beneficial microbes into bee colonies could offer a sustainable alternative to traditional pest and disease management strategies, which are often reliant on chemical interventions that pose risks of resistance development and ecological disruption. The experiment involving the three-strain lactobacilli consortium (LX3) unveiled a promising avenue for employing probiotics against *Varroa* mites, a pervasive threat to bee populations globally.

Administration of the probiotic strains via protein patties, particularly, emerged as an effective delivery method, significantly reducing mite levels across diverse environmental settings, including urban, agricultural, and forage-rich locales. This

outcome not only attests to the potential of specific probiotic formulations in pest control but also to the pivotal role of delivery mechanisms in optimizing their effectiveness. The observed ineffectiveness of spray methods, in contrast, highlights the nuanced nature of probiotic application and the necessity for tailored approaches based on the target pest or pathogen and the ecological context.

An intriguing aspect of this study was the observation that uninoculated protein patties could inadvertently facilitate mite proliferation. This underscores the complexity of hive management, where interventions intended to bolster bee health may have unintended consequences. It emphasizes the need for a holistic understanding of hive dynamics, where nutritional supplements are carefully balanced with probiotic strategies to ensure that the former does not undermine the effectiveness of the latter.

Despite the success against *Varroa* mites, the LX3 consortium did not exhibit a similar impact on *P. larvae* loads within the colonies studied here. This disparity points to the specificity of probiotic action and suggests that the mechanisms by which probiotics influence bee health are varied and pathogen-dependent as well as potentially geographically different. It also signals the potential limitations of current delivery methods in reaching the niches within the hive where *P. larvae* proliferate. Furthermore, the finding raises questions about the interaction between probiotics and the bee gut microbiome, particularly in the context of AFB pathogenesis. The role of the microbiota in either facilitating or hindering disease progression offers a fertile ground for future research, with implications for developing microbiome-based strategies for disease control.

The insights gleaned from this research carry profound implications for sustainable beekeeping practices. They suggest that the strategic use of probiotics, especially when delivered in a manner that aligns with the bees' natural behaviors and environmental conditions, can enhance resilience against specific threats like *Varroa* mites without the downsides associated with chemical treatments. However, the complexity of microbial interactions within the hive and the varied responses of different pathogens to probiotic

treatments underscore the necessity for a nuanced, ecologically informed approach to bee health management.

Looking forward, the path to effective, sustainable management of bee health lies in a deeper understanding of the microbial ecologies of bee colonies. Future studies should aim to unravel the complex web of interactions between introduced probiotics, the native bee microbiome, pathogens, and environmental factors. Specifically, research should focus on optimizing probiotic formulations and delivery methods for a broad spectrum of pathogens, understanding the impact of nutritional supplements on microbial dynamics within the hive, and exploring the potential of microbiome engineering as a holistic strategy for disease and pest control in honey bee populations.

In conclusion, this thesis contributes to a nuanced understanding of the potential and challenges of using probiotics to enhance honey bee health. While the findings reveal promising avenues for sustainable pest and disease management, they also highlight the complexities inherent in microbial interventions within bee colonies. As we advance, it is imperative that bee health research continues to adopt a holistic perspective, integrating insights from microbiology, ecology, and beekeeping practice to develop strategies that are not only effective but also sustainable and aligned with the ecological realities of bee populations.

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Appendices

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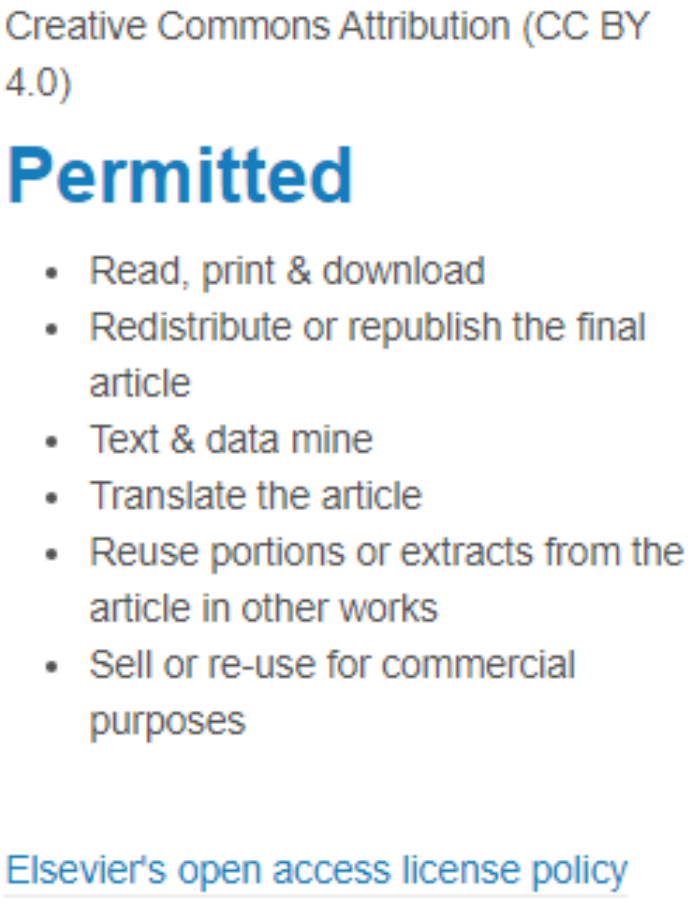

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