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Exploring the potential of the insect gut-brain axis to modulate the hygienic behaviour of Western honey bees (Apis mellifera)

Sophie M. Killam, *Western University*

Supervisor: Thompson, Graham J., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

The Western honey bee, *Apis mellifera*, is vulnerable to disease, especially disease afflicting the brood. To counter this, bees exhibit hygienic behaviour: nurse bees detect olfactory cues indicative of disease and remove affected pupae. Selecting for this trait is challenging for beekeepers, who may instead resort to quick-fix antibiotics. In this thesis, I explore the potential of beneficial bacteria ("probiotics") to promote hygienic behaviour. I fed bees two species of lactic acid-producing bacteria that may help synthesize olfactory-associated neurotransmitters, then tested for changes in hygiene and microbial diversity. I detected small increases in hygiene and small changes to microbiota composition in the short term, but these treatment effects were generally small and variable. This research nonetheless provides a foundation for a field study with more power to detect small but potentially significant differences through the bee gut-brain axis, which could ultimately serve the beekeeping community by providing recommendations for best practices.

Keywords

Probiotics, social behaviour, microbiota, gut-brain axis, hygienic behaviour, disease-resistance mechanisms, Western honey bee

Summary for Lay Audience

Honey bees live in large colonies of closely related individuals, making them vulnerable to the spread of disease. Many of these diseases target the brood, which are tightly packed in cells and can readily spread infection. The brood is cared for by 15-20 day-old worker bees ('nurses'). To prevent the spread of infection, nurses detect scents emitted from dead or diseased brood and then remove the brood from the colony. This behaviour, known as hygiene, is invaluable to beekeepers, but it can be difficult to breed for due to its complex genetic basis. Consequently, beekeepers often use antibiotics, despite side effects like antibiotic resistance and disruption to the gut microbiota. In my project, I draw from the growing field of probiotics to determine if certain bacteria can promote hygiene within colonies. I supplemented hives with two species of bacteria that may help produce the brain's chemical messengers (neurotransmitters). Specifically, neurotransmitters potentially relevant to olfactory (scent) disease detection. Then, I tested for effects on the expression of hygienic behaviour. I also sequenced the guts of nurse bees to determine which bacterial species were present before and after probiotic treatment. Despite a lack of statistically significant treatment effects, I did see small changes in the expression of hygienic behaviour, as well as some changes in the composition of the gut bacterial community, suggestive of a possible acute treatment effect. Lastly, I interpret my behavioural and gut microbial community results in the context of how they might affect beekeeping practices.

Co-Authorship Statement

Chapter 2: I jointly conceived and wrote this chapter with my supervisor, Dr. Graham Thompson, with support and input from Dr. Brendan Daisley, Dr. Morgan Kleiber, and Julia Lacika. Figure 1 was adapted from one made by J. Lacika. This chapter has been submitted as a mini-review to the international, peer-reviewed journal *Frontiers in Bee Science* (April 23, 2023; Manuscript ID: 1422265). Images were made in BioRender. A preliminary citation follows:

Killam, S. M., Daisley, B. A., Kleiber, M. L. Lacika, J. F. Thompson, G. J. (2024) *A case for microbial therapeutics to bolster hive health and performance of honey bees* [Manuscript submitted for publication]. Department of Biology, Western University.

Chapter 3: I performed all fieldwork, assembled and analyzed all data and created all figures. Dr. Kait Al provided guidance and expertise regarding the molecular sequence analysis. Specifically, Dr. Al helped me to array the indexed primers necessary for multiplex sequencing on an Illumina platform, which was then performed by David Carter at the London Regional Genomics Centre. Dr. Kait Al also assisted with DNA quality control, choice of sequencing kits, and de-multiplexing the sequence reads after sequencing. I analyzed the sequencing results with input and code from Dr. Kait Al. If this chapter is published, I will be the first author, followed by Drs. Kait Al, Brendan Daisley, and Graham Thompson, with an acknowledgement of the support and services rendered by Robarts Research Institute.

This thesis was jointly conceived, and the writing was edited with my supervisor, Dr. Graham Thompson.

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List of Abbreviations

5-HT

Serotonin

AFB

American Foulbrood

DA

Dopamine

DNA

Deoxyribonucleic acid

dsRNA

double-stranded ribonucleic acid

FKB

Freeze-kill brood assay

GABA

Gamma-aminobutyric acid

LAB

Lactic acid-producing bacteria

OA

Octopamine

PKB

Pin-killed brood assay

RNA

Ribonucleic acid

RNAi

Ribonucleic acid interference

Glossary

16S Ribosomal RNA Gene

The 16S ribosomal RNA (rRNA) gene is DNA that codes for the RNA component of the 30S subunit of the bacterial ribosome which can be used to determine bacterial taxonomy in microbial samples

Altruism/Altruistic

A behaviour that benefits the receiver at the cost of the actor

Apiary

An area in which bees are kept; also known as a bee yard

BioPatty

A standard pollen patty typically used in beekeeping, with the addition of probiotic strains of bacteria

Caste

In social insects, groups of individuals that belong to the same species but have a different appearance, usually related to their role in the colony

Dysbiosis

An imbalance in the gut bacterial composition, either a loss of beneficial bacteria, overall loss of bacterial abundance, or presence of pathogenic bacteria

Eusociality

The highest level of social organization, typically characterized by cooperative brood care, overlapping generations of adults within the same colony, and reproductive division of labour

Gut-brain Axis

A bi-directional pathway within the body that links the metabolic function of microbes within the gastrointestinal tract to the central nervous system

Haplodiploidy

A sex-determination system in which males develop from unfertilized eggs and are haploid (having a single set of chromosomes), while females develop from fertilized eggs and are diploid (having two sets of paired chromosomes)

Holobiont

A multicellular host organism and all its associated symbionts, such as bacteria, archaea, viruses, and fungi

Hymenoptera/Hymenopterans

A large order of insects that includes the bees, typically having two sets of wings and females having a stinger, with all members of the order being haplodiploid

Inclusive Fitness Theory

The idea that an organism's contribution to the next generation is derived not only directly, from reproduction, but indirectly, from the success and survival of relatives

Kin Selection Theory

Individuals with altruistic traits can forego their own opportunities for reproduction in favour of increasing their relative's reproductive success.

Lateral Horn

A region of the insect brain responsible for integrating olfactory input, coordinating behaviour with odour information

Microbiota

The collection of microorganisms that exist in and on all multicellular organisms, which may be mutualistic, commensal, or pathogenic

Mushroom Bodies

Regions in the insect brain that are responsible for multisensory integration, learning and memory

Pollen Patty

A protein supplement or substitute used by beekeepers to help feed colonies, typically in the spring and fall when natural forage might be lacking

Probiotics

Live microorganisms that can be administered as a supplement to confer health benefits to a host

Queenright

A description of a colony that has a laying queen in the hive; opposite of queenless

Response Threshold

The level of stimulus required to elicit certain behaviours, varies between individuals

Social Amplification

The process whereby experimental effects or behaviours can be reflected up from the level of the individual in social-living animals to influence the collective hive

Social Immunity

Collective immune defenses exhibited by social species to combat their increased risk of infection and disease transmission

Symbiosis/Symbiotic

An association between members of two different species that can be mutualistic (both individuals benefit), commensal (one benefits and the other neither benefits nor is harmed), or parasitic (one organism benefits and the other is harmed)

Temporal Polyethism

A method of labour division in many eusocial species in which the task an adult performs is dependent on its age

Chapter 1

1 General Introduction

Hymenoptera are a large and diverse order of insects that contain the ants, bees, and wasps. Many of these species live in highly integrated colonies, whereby individuals specialize in performing certain roles that, together, form a functional society (Andersson, 1984). Among eusocial Hymenoptera, honey bees are of particular interest to human culture because of their role in agriculture as pollinators and, of course, in the production of edible honey (Agriculture and Agri-food Canada, 2022). One species, *Apis mellifera*, is widely employed in the agri-food sector and is therefore widely studied for its health, immunity and performance. One growing area of research is to test if the deliberate application of bee-friendly bacteria (probiotics) can support the health of human-managed colonies (Abdi et al., 2023). In this thesis, I add to this growing base of knowledge by, first, writing a synthetic review of the literature that explores this idea in relation to bee nest cleaning (i.e., hygienic) behaviour and second, performing an empirical field and lab study that tests specific predictions made in my review. In the review, I introduce a novel concept in bee microbial therapeutics that I call 'social amplification' whereby the effect of probiotics on a subset of treated bees can influence others through social interaction and thereby 'amplify' the effect throughout the colony. In my model, I invoke the honey bee gut-brain axis as a potential mechanism through which the effect is realized. In my empirical chapter, I perform an in-apiary study that tests how two species of lactic acid-producing bacteria may potentially influence the hygienic capabilities of bee colonies at our campus apiary. Finally, I close with practical applications for this study and potential routes forward for researchers and beekeepers alike.

1.1 The evolution and diversity of eusocial insects

Eusociality is a phenomenon typically observed in insects such as ants, bees, wasps, and termites (Andersson, 1984), that is often characterized by a reproductive division of

labour, where group members are divided into reproductive castes and non-reproductive castes, the latter of which do not reproduce but nonetheless contribute to the reproductive effort by caring for the brood and performing other non-reproductive tasks (Nowak et al., 2010). On the surface, eusociality decreases the direct fitness of the non-reproductive caste (i.e., they have few or no offspring), but inclusive fitness theory argues that the total fitness of these reproductively altruistic individuals is - not intuitively - increased via routes of kinship within the colony that may, in some cases, provide reproductive helpers with extra indirect fitness (i.e., production of non-descendent kin) that more than compensates for the altruistic sacrifice. Further, in the social Hymenoptera, the relatedness asymmetries that enable the indirect effect may be associated with a haplodiploid mechanism of sex determination (Rautiala et al., 2019). Under this system, fertilized eggs develop into diploid females, whereas unfertilized eggs develop into haploid males (Olejarz et al., 2015). It is established from analysis of pedigrees that, when queen mothers are singly mated, full sisters can be related by as much as 75% (half their genome is 100% identical through a single gene copy – i.e., haploid – paternity and the remaining half is 50% identical through normal diploid recombination; ½ + ¼ = ¾ or 75% related). Haplodiploidy and other relatedness asymmetries are considered to have favoured the evolution of eusociality and divisions of labour in Hymenoptera, however, this is not an all-encompassing explanation for eusociality as a whole (Nowak et al., 2010).

In honey bee colonies, the labour is split not only between reproductive and non-reproductive task specialists but can be further split into sub-tasks among the non-reproductives themselves. One version of this sub-specialization, and the one used by honey bees, is known as “temporal polyethism”. Here, the likelihood of tasks performed is predicted by the age of the worker, with younger workers tending to work within the colony and older workers tending to perform out-of-hive tasks such as foraging (Beshers et al., 2001). This pattern can be plastic, however, and the performance of a task can fluctuate based on the current needs of the colony, with workers accelerating, delaying, or reversing development into a different task specialty (Huang and Robinson, 1996). After emergence, young bees typically perform in-hive tasks such as cell cleaning, hygiene, and brood care, then graduate to hive maintenance and guarding behaviours before

leaving the nest to forage (Dolezal and Toth, 2014). Due to this complex age-based behavioural repertoire, honey bees have become a model for the study of social behaviour (Menzel, 2012).

1.2 Honey bees' economic and ecological value in beekeeping

In addition to their importance as a model of social organization, honey bees are also important for pollination and play a vital role in the agriculture-based economy. Wild pollinators are declining globally due to anthropogenic factors, which could cause deficits in crop pollination (Dicks et al., 2021). This may place more demand on commercial pollination from managed honey bee colonies. The total value of commercial pollination to the Canadian economy exceeded $3.18 billion in 2021 (Agriculture and Agri-food Canada, 2022). In addition to pollination services, domesticated honey bees also produce many goods that beekeepers can harvest and sell, namely, honey. In 2021, honey production in Canada neared 900,000 lbs and was valued at $2.78 million (Agriculture and Agri-food Canada, 2022). Managed honey bees are also important pollinators in natural areas, especially where native pollinators are in decline due to anthropogenic factors such as habitat loss (Hung et al., 2018).

Domesticated honey bee colonies are tightly regulated. In Ontario, apiculture is regulated under the Ontario Bees Act, and agencies such as the Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA) and the Ontario Beekeepers Association (OBA) develop policies, inspect hives and yards, and generate reports on the state of beekeeping. The Canadian Association of Professional Apiculturists (CAPA) also produces many documents on apiculture in Canada, including the Canadian Best Management Practices for Honey Bee Health. These reports focus primarily on disease mitigation in Canada, including *Nosema* (caused by two related species of microsporidian parasites)*, Varroa* mites, American and European Foulbrood (bacterial brood infections), Small Hive Beetle, and viral infections (Eccles et al., 2016).

1.3 Challenges associated with beekeeping and pollination services

One of the biggest challenges that beekeepers must contend with is contagious disease. Honey bees, due to their social living structure (eusociality), are highly vulnerable to the spread of diseases and parasites that can decimate colonies (Evans and Spivak, 2010). As presented throughout this thesis, honey bees have evolved a mechanism known as hygienic behaviour to mitigate the risk of disease transmission (Wilson-Rich et al., 2008). This heritable trait can be difficult to select for despite well-thought-out selective breeding programs (Niño and Cameron Jasper, 2015); therefore, many beekeepers use antibiotics and other chemicals on their hives, which can be immediately effective, but have unintended downstream effects such as accumulation within honey (Lima et al., 2020), disturbances to the gut (Daisley et al., 2020), and antibiotic resistance (Ludvigsen et al. 2018). Further, antibiotic medications are falling out of favour against trends in organic farming and are highly restricted or even banned in some districts - notably, much of Europe (Croppi et al., 2021).

1.3.1 Practical solutions for promoting colony health and immunity

One alternative to antibiotic use is RNA interference (RNAi): a process wherein double-stranded RNA (dsRNA) molecules can trigger the degradation of other RNA molecules. RNAi can be used to impair the replication of RNA viruses in bees (Maori et al. 2009; Smeele et al. 2023), and dsRNA can even be transmitted to parasites such as *Varroa* and *Nosema* and can silence essential genes for parasite function (Garbian et al. 2012; Huang et al. 2023). There is also progress being made in honey bee 'vaccines': positive results in larval survivorship against foulbrood have been found in colonies with immune-primed queens that can apparently transfer antimicrobials to their daughter offspring and render them less vulnerable to American foulbrood infections (Dickel et al. 2022). However, this process can be labour-intensive and requires extensive queen-handling and in-lab manipulation. Alternatively, some natural therapies, such as essential oils, can be used against honey bee diseases such as foulbrood, chalkbrood, and parasites (Hýbl et al. 2021; Tutun et al. 2018), which is both convenient and safe for bees.

One approach that potentially eschews the need for selective breeding, antibiotics, or bioengineering, and which may complement natural remedies such as plant oils, is administering beneficial bacteria to support the function of native bee gut microbes. This function is already tightly co-evolved between microbe and host (Guerrero et al. 2013) and includes a role for symbiotic bacteria in host immunity, metabolism and behaviour (Raymann and Moran 2018). The relatively new idea of leveraging the already tight symbioses between gut microbes and bees is the basis of the microbial therapeutic approach that has recently surged in the literature (Alberoni et al., 2016; Alonso-Salces et al., 2017; Vásquez et al., 2012). Beneficial bacteria, or "probiotics" as a commonly used term, have massive potential to support the health and vitality of hives, potentially helping to slow or reverse rates of colony collapse (Abdi et al., 2023).

1.4 Hypothesis, objectives, and goals of the thesis

The goal of this thesis is to use honey bees to examine whether there is a functional relationship between the gut and brain that can alter behaviour. In the second chapter, I present a review of the current literature on probiotics for honey bees and the association between the microbiota and neurotransmitter concentrations in honey bee brains. My goal for this chapter is to review these topics, showing how the microbiota can influence behaviour via the gut-brain axis, and then present the hypothesis and main ideas that I test in Chapter 3. In the third chapter, I conduct an empirical study on the ideas presented in Chapter 2 to investigate how the gut-brain axis of Western honey bees could affect olfactory thresholds that modulate hygienic behaviour.

I hypothesize that the application of probiotic strains that aid in the synthesis of olfactory-associated neurotransmitters will impact the threshold response of nurse bees to cues emitted from dead or diseased brood. I predict that these probiotics will lower the threshold response, thereby increasing the prevalence of hygienic behaviour within the treated colonies. To test this, I had two main objectives: 1) To determine whether specific probiotic treatment can alter the gut microbiota of worker bees in an apiary, and 2) To determine whether probiotic treatment can impact the prevalence of hygienic behaviour within an apiary. I used one species of probiotic that is a core gut community member,

Bifidobacterium asteroides (Kwong and Moran, 2016), and one species that is less common in honey bee guts but can be acquired from the environment, *Lactiplantibacillus plantarum*, which may have probiotic effects (Iorizzo et al., 2021).

My experiment consisted of both field and lab components. I carried out a field experiment with nine hives of Western honey bees, which were given two rounds of probiotic treatment across a three-week study. I applied treatment in the form of a pollen patty supplemented with probiotics (hereafter referred to as a BioPatty, in contrast to the standard pollen patty which lacks bacterial supplementation). Hygiene was tested thrice, once before treatment, after the first treatment, and finally, after the second treatment. In the lab, I investigated treatment effects on the gut using 16S ribosomal RNA gene sequencing to see if the treatment persisted in the gut (Objective 1). The results from the hygiene assays were analyzed to test for differences between treatment groups (Objective 2) and then ultimately to compare with the results from sequencing to reveal links between the gut and brain (behaviour) of the organism. The results of this project could provide the first evidence of a functional brain-gut axis relevant to disease management. My work could also promote the discussion of practical alternatives to antibiotics in domestic honey bees.

1.5 References

Abdi, K., Ben Said, M., Crotti, E., Masmoudi, A. S., & Cherif, A. (2023). The promise of probiotics in honeybee health and disease management. *Archives of Microbiology*, *205*(2), 73. https://doi.org/10.1007/s00203-023-03416-z

Agriculture and Agri-Food Canada. (2021). *Statistical Overview of the Canadian Honey and Bee Industry and the Economic Contribution of Honey Bee Pollination*. https://agriculture.canada.ca/en/sector/horticulture/reports/statistical-overview-canadian-honey-and-bee-industry-2021

Alberoni, D., Gaggìa, F., Baffoni, L., & Di Gioia, D. (2016). Beneficial microorganisms for honey bees: Problems and progresses. *Applied Microbiology and Biotechnology*, *100*(22), 9469–9482. https://doi.org/10.1007/s00253-016-7870-4

Alonso-Salces, R. M., Cugnata, N. M., Guaspari, E., Pellegrini, M. C., Aubone, I., De Piano, F. G., Antunez, K., & Fuselli, S. R. (2017). Natural strategies for the control of *Paenibacillus larvae*, the causative agent of American foulbrood in honey bees: A review. *Apidologie*, *48*(3), 387–400. https://doi.org/10.1007/s13592-016-0483-1

Andersson, M. (1984). The evolution of eusociality. *Annual Review of Ecology and Systematics*, *15*, 165–189.

Beshers, S. N., Huang, Z. Y., Oono, Y., & Robinson, G. E. (2001). Social inhibition and the regulation of temporal polyethism in honey bees. *Journal of Theoretical Biology*, *213*(3), 461–479. https://doi.org/10.1006/jtbi.2001.2427

Croppi, S., Yu, L., Robinette, C. S., Hassler, E. E., Newmark, A. J., Scott, A., Cazier, J., Song, J., & Formato, G. (2021). Impact of legislation on antibiotic use and awareness of beekeepers. *Journal of Apicultural Science*, *65*(2), 265–277. https://doi.org/10.2478/jas-2021-0022

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Gibbons, S., Chernyshova, A. M., Al, K. F., Faragalla, K. M., Burton, J. P., Thompson, G. J., & Reid, G. (2020). *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Communications Biology*, *3*(1), Article 1. https://doi.org/10.1038/s42003-020-01259-8

Dickel, F., Bos, N. M. P., Hughes, H., Martín-Hernández, R., Higes, M., Kleiser, A., & Freitak, D. (2022). The oral vaccination with *Paenibacillus larvae* bacterin can decrease susceptibility to American Foulbrood infection in honey bees—A safety and efficacy study. *Frontiers in Veterinary Science*, *9*, 946237. https://doi.org/10.3389/fvets.2022.946237

Dicks, L. V., Breeze, T. D., Ngo, H. T., Senapathi, D., An, J., Aizen, M. A., Basu, P., Buchori, D., Galetto, L., Garibaldi, L. A., Gemmill-Herren, B., Howlett, B. G., Imperatriz-Fonseca, V. L., Johnson, S. D., Kovács-Hostyánszki, A., Kwon, Y. J., Lattorff, H. M. G., Lungharwo, T., Seymour, C. L., … Potts, S. G. (2021). A global-scale expert assessment of drivers and risks associated with pollinator decline. *Nature Ecology & Evolution*, *5*(10), 1453–1461. https://doi.org/10.1038/s41559-021-01534-9

Dolezal, A. G., & Toth, A. L. (2014). Honey bee sociogenomics: A genome-scale perspective on bee social behavior and health. *Apidologie*, *45*(3), 375–395. https://doi.org/10.1007/s13592-013-0251-4

Eccles, L., Kempers, M., Gonzales Mijares, R., Thurston, D., & Borges, D. (2016). *Canadian Best Management Practices for Honey Bee Health – CAPA*. https://capabees.com/canadian-best-management-practices-for-honey-bee-health/

Evans, J. D., & Spivak, M. (2010). Socialized medicine: Individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, *103*, S62–S72. https://doi.org/10.1016/j.jip.2009.06.019

Garbian, Y., Maori, E., Kalev, H., Shafir, S., & Sela, I. (2012). Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. *PLoS Pathogens*, *8*(12), e1003035. https://doi.org/10.1371/journal.ppat.1003035

Guerrero, R., Margulis, L., & Berlanga, M. (2013). Symbiogenesis: The holobiont as a unit of evolution. *International Microbiology: The Official Journal of the Spanish Society for Microbiology*, *16*(3), 133–143. https://doi.org/10.2436/20.1501.01.188

Huang, Q., Lariviere, P. J., Powell, J. E., & Moran, N. A. (2023). Engineered gut symbiont inhibits microsporidian parasite and improves honey bee survival. *Proceedings of the National Academy of Sciences*, *120*(25), e2220922120. https://doi.org/10.1073/pnas.2220922120

Huang, Z.-Y., & Robinson, G. E. (1996). Regulation of honey bee division of labor by colony age demography. *Behavioral Ecology and Sociobiology*, *39*(3), 147–158. https://doi.org/10.1007/s002650050276

Hung, K.-L. J., Kingston, J. M., Albrecht, M., Holway, D. A., & Kohn, J. R. (2018). The worldwide importance of honey bees as pollinators in natural habitats. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1870), 20172140. https://doi.org/10.1098/rspb.2017.2140

Hýbl, M., Bohatá, A., Rádsetoulalová, I., Kopecký, M., Hoštičková, I., Vaníčková, A., & Mráz, P. (2021). Evaluating the efficacy of 30 different essential oils against *Varroa destructor* and honey bee workers (*Apis mellifera*). *Insects*, *12*(11). https://doi.org/10.3390/insects12111045

Iorizzo, M., Testa, B., Ganassi, S., Lombardi, S. J., Ianiro, M., Letizia, F., Succi, M., Tremonte, P., Vergalito, F., Cozzolino, A., Sorrentino, E., Petrarca, S., De Cristofaro, A., & Coppola, R. (2021). Probiotic properties and potentiality of *Lactiplantibacillus plantarum* strains for the biological control of chalkbrood disease. *Journal of Fungi*, *7*(5). https://doi.org/10.3390/jof7050379

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature Reviews Microbiology*, *14*(6). https://doi.org/10.1038/nrmicro.2016.43

Lima, C. M. G., Nora, F. M. D., Seraglio, S. K. T., Silva, J. M., da Marzoque, H. J., Santana, R. F., Verruck, S., & Scussel, V. M. (2020). Antibiotic residues in honey: A public health issue. *Research, Society and Development*, *9*(11). https://doi.org/10.33448/rsd-v9i11.9604

Ludvigsen, J., Amdam, G. V., Rudi, K., & L'Abée-Lund, T. M. (2018). Detection and characterization of streptomycin resistance (strA-strB) in a honeybee gut symbiont (*Snodgrassella alvi*) and the associated risk of antibiotic resistance transfer. *Microbial Ecology*, *76*(3), 588–591. https://doi.org/10.1007/s00248-018-1171-7

Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., & Sela, I. (2009). IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Molecular Biology*, *18*(1), 55–60. https://doi.org/10.1111/j.1365-2583.2009.00847.x

Menzel, R. (2012). The honeybee as a model for understanding the basis of cognition. *Nature Reviews Neuroscience*, *13*(11). https://doi.org/10.1038/nrn3357

Niño, E. L., & Cameron Jasper, W. (2015). Improving the future of honey bee breeding programs by employing recent scientific advances. *Current Opinion in Insect Science*, *10*, 163–169. https://doi.org/10.1016/j.cois.2015.05.005

Nowak, M. A., Tarnita, C. E., & Wilson, E. O. (2010). The evolution of eusociality. *Nature*, *466*(7310), 1057–1062. https://doi.org/10.1038/nature09205

Olejarz, J. W., Allen, B., Veller, C., & Nowak, M. A. (2015). The evolution of non-reproductive workers in insect colonies with haplodiploid genetics. *eLife*, *4*, e08918. https://doi.org/10.7554/eLife.08918

Rautiala, P., Helanterä, H., & Puurtinen, M. (2019). Extended haplodiploidy hypothesis. *Evolution Letters*, *3*(3), 263–270. https://doi.org/10.1002/evl3.119

Raymann, K., & Moran, N. A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current Opinion in Insect Science*, *26*, 97–104. https://doi.org/10.1016/j.cois.2018.02.012

Smeele, Z. E., Baty, J. W., & Lester, P. J. (2023). Effects of deformed wing virus-targeting dsRNA on viral loads in bees parasitised and non-parasitised by *Varroa destructor*. *Viruses*, *15*(11). https://doi.org/10.3390/v15112259

Tutun, H., Koç, N., & Kart, A. (2018). Plant essential oils used against some bee diseases. *Turkish Journal of Agriculture - Food Science and Technology*, *6*(1), Article 1. https://doi.org/10.24925/turjaf.v6i1.34-45.1502

Vásquez, A., Forsgren, E., Fries, I., Paxton, R. J., Flaberg, E., Szekely, L., & Olofsson, T. C. (2012). Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. *PLoS ONE*, *7*(3), e33188. https://doi.org/10.1371/journal.pone.0033188

Wilson-Rich, N., Dres, S. T., & Starks, P. T. (2008). The ontogeny of immunity: Development of innate immune strength in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, *54*(10), 1392–1399. https://doi.org/10.1016/j.jinsphys.2008.07.016

Chapter 2

2 Synthetic Review: A case for microbial therapeutics to bolster hive health and performance of honey bees

The holobiont theory of evolution explains how individuals are deeply symbiotic with their gut microbes, such that microbes are adapted to influence host metabolism, immunity and behaviour, as signalled from the gut to the brain. For eusocial taxa like the Western honey bee (*Apis mellifera*), this gut-brain axis may scale up from the individual to affect entire colonies. Here, I examine how microbial supplementation of honey bee feeds could manipulate the gut-brain axis to affect hygienic and other social behaviours relevant to beekeeping, such as foraging, recruitment (dance language) and defense. To illustrate this concept, I focus on various lactic acid-producing bacteria that can synthesize neurotransmitters such as octopamine, dopamine, serotonin and γ-aminobutyric acid, which can influence an individual bee's behavioural cycles and responsiveness to environmental cues. If the behaviour of a worker bee can be deliberately manipulated, and this effect multiplied across many workers, microbial neurotherapeutics could conceivably render colonies more behaviourally responsive to symptoms of disease, more motivated to forage or possibly less aggressive towards beekeepers. Drawing from the scientific literature, I infer how microbial supplements, such as neurostimulatory or neurosuppressive probiotics, could be applied or even engineered to co-opt the gut-brain axis to bolster hive health or improve performance. The mechanistic link between the gut microbiota and the collective social behaviour of hives remains an understudied aspect of honey bee social biology with relevance to apiculture.

2.1 Introduction

The evolutionary association between multicellular hosts and their unicellular gut microbes represents a symbiosis that supports the host’s immune, metabolic and digestive systems (Guerrero et al., 2013; Rosenberg et al., 2010). Dysbiotic shifts in the gut

microbiota, typically characterized by a relative decrease in symbionts and an overgrowth of pathobionts, can detrimentally impact the host's well-being (Carding et al., 2015). One mechanism that mediates the relationship between host and microbe is the gut-brain axis, which links the metabolic function of microbes within the gastrointestinal tract (i.e., the gut) to the central nervous system (i.e., the brain) and thus to the performance and behaviour of the organism (Mayer and Tillisch, 2011).

Studies on the gut-brain axis have primarily focused on vertebrates, but it is now established that this mechanism can influence the health and behaviour of invertebrates, including insects (Dus et al., 2015; Liberti and Engel, 2020). For social insects, where behavioural responses are coordinated among large numbers of individuals, any effects of the gut-brain axis should be amplified to influence the collective behaviour of entire colonies (Sarkar et al., 2020). This prospect of 'social amplification' presents an opportunity to directly manipulate the gut-brain axis of some critical subset of individuals within a colony, with the change-of-behaviour effect then ramifying throughout a larger group.

In the highly social honey bee *Apis mellifera,* there is massive potential for the social amplification of gut-brain axis effects (Figure 1). In a leading study, Liberti et al. (2022) demonstrated that workers with experimentally homogenized gut microbiomes interacted more frequently in a controlled setting and that specific metabolites associated with those microbes could statistically predict the number of interactions. This association between gut microbe composition and the nature of head-to-head interactions suggests that the gut-brain axis of honey bees is functional and potentially mutable as an apicultural tool. However, few studies have examined how supplementation of colonies with bacteria known to have neurodevelopmental effects might influence aspects of beekeeping.

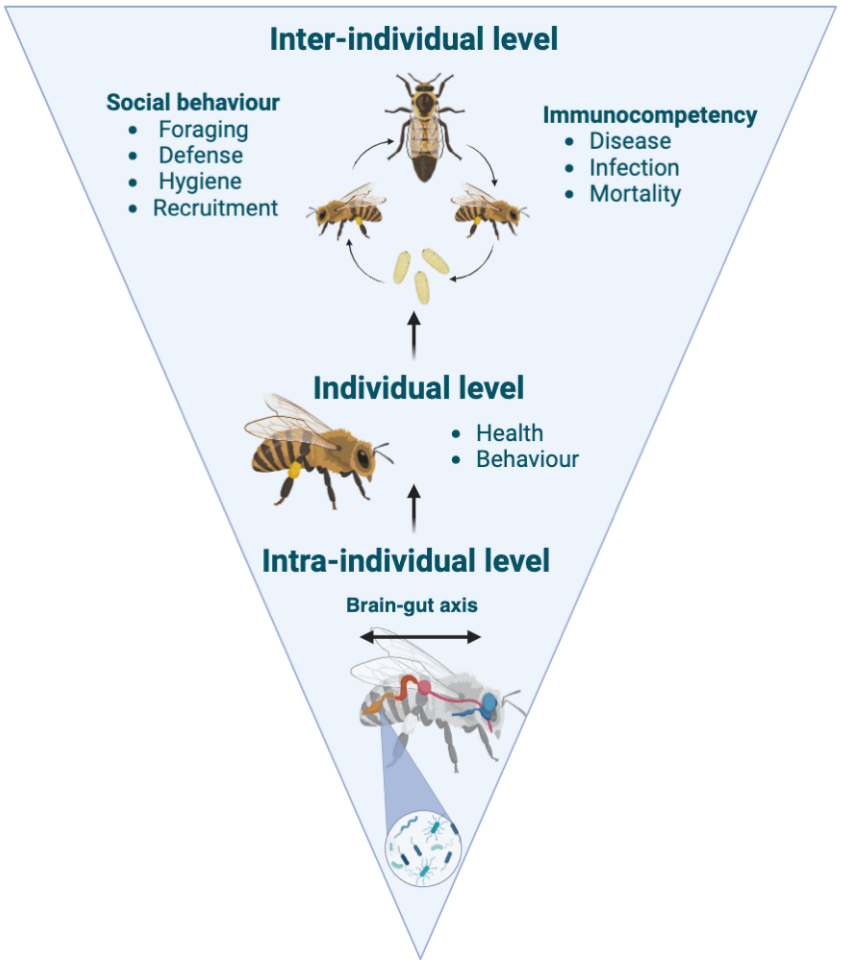

Figure 1. Social amplification from the individual to the colony level.

The gut-brain axis, a bidirectional path of communication between the gut and brain (depicted in the lowest panel), is known to affect the health and behaviour of individual insects, including honey bees. Because honey bees live in highly interconnected societies where individuality is subsumed into a larger integrated whole (the colony), the gut-brain axis may influence behaviour above the level of individuals to affect the whole colony's collective behaviour, an effect known as *social amplification*.

One stereotypic behaviour that seems potentially amenable to microbial therapeutic manipulation is hygienic behaviour – the systemic tendency to detect and dispose of diseased larvae and pupae from the hive, particularly as they are likely to cause infection (Spivak and Gilliam, 2015; Sprau et al., 2023). Honey bees, like other social insects, live in densely populated colonies of closely related individuals, rendering them vulnerable to the spread of contagion. As such, they have evolved forms of social immunity to combat this risk (Cremer et al., 2007). The hygienic response to infection, expressed by nurse-age workers (approximately 15-20 days old), is likely triggered by an odour-sensitive threshold (Masterman et al., 2000) that is mediated, in part, by genetically variable loci (Oxley et al., 2010). Selecting for hygienic strains is possible (Erez et al., 2022), but, in practice, bee breeding can be a slow or ineffective process, requiring considerable financial considerations and expertise in bee husbandry (Niño and Cameron Jasper, 2015). Further, the expression of hygiene varies beyond genetics as a function of season and environmental factors such as floral availability, weather, nectar flows, and amount of brood and bees in the colony (Spivak and Reuter, 1998; Uzunov et al., 2014).

As an effort to complement the bee's natural tendency to keep their colonies disease-free, many beekeepers (outside of Europe) use antibiotics, which can be immediately effective against certain pathogens, but these medicated treatments are tightly regulated due to concerns about residual accumulation in honey, as well as other off-target side effects (Lima et al., 2020), including disruption of the natural bee gut microbiota which, paradoxically, can leave colonies more vulnerable to subsequent infection (Daisley et al., 2020b; Raymann et al., 2017). Alternative disease management interventions in beekeeping include essential oils (Hýbl et al., 2021), RNA interference technologies (Garbian et al., 2012) or variations of transgenerational immune priming (Dickel et al., 2022). These techniques are, however, not yet well-tested or established. One remaining approach that complements or even circumvents some of these remedies involves administering living bacteria to hives in support of native bee gut microbes (Motta et al., 2022).

In this essay, I explore the potential to co-opt the gut-brain axis of managed honey bees to modify hygiene and potentially other environmentally cued social responses that are

relevant to beekeeping. I provide perspective on the deliberate enrichment of bee guts with bacterial strains to lower the individual response threshold to disease cues, which is an approach that, with development and testing, could enhance the colony-wide hygienic response (Figure 2). Although this approach has not been conclusively tested, manipulating the gut-brain axis could offer a new strategy for managing perennial bacterial diseases such as American or European foulbrood, and potentially any type of pest or pathogen that is naturally removed by hygiene.

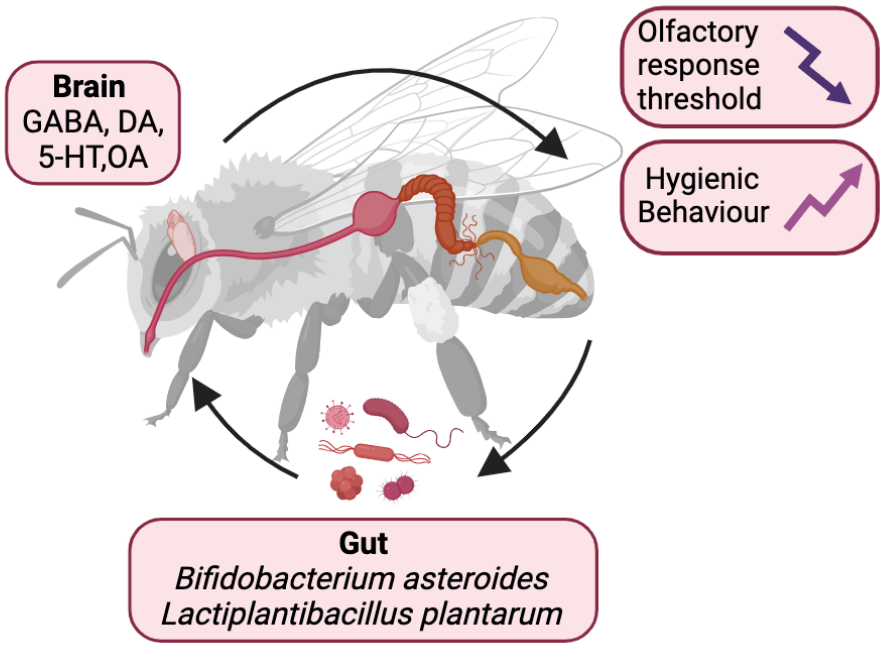

Figure 2. Depiction of gut-brain axis affecting the olfactory-mediated hygienic threshold response of a worker honey bee.

Supplementing the gut microbiota with bacteria that have neuro-stimulatory effects (e.g. *Bifidobacterium asteroides*, *Lactiplantibacillus plantarum*) may aid in the synthesis of neurotransmitters [e.g. γ-aminobutyric acid (GABA), serotonin (5-HT), dopamine (DA), octopamine (OA)] that lower the olfactory-mediated hygienic response threshold. This is the main idea that I am testing in my thesis.

2.2 Gut microbiota and the potential for effects on neurotransmission

The microbiota of the Western honey bee is dominated by several species of *Lactobacillus* and *Bombilactobacillus*, as well as *Gilliamella apicola, Snodgrassella alvi* and *Bifidobacterium asteroides*, all of which are consistently found in the hindgut of adult workers (Raymann and Moran, 2018). Other commonly detected bacteria found in association with honey bees include *Frischella perrara*, *Bartonella apis*, *Bombella apis*, *Apilactobacillus kunkeei*, and several species of *Fructobacillus* (Bonilla-Rosso and Engel, 2018). Within colonies, the microbiota is quite homogenous and primarily transmitted through social interactions such as trophallaxis, encountering hive materials, and fecal routes (Powell et al., 2014); however, there is some variation in gut microbe composition among kin (Vernier et al., 2020), castes (Kapheim et al., 2015) and geographical areas (Jones et al., 2018).

The microbiota is thought to affect many systems within the host; Alberoni et al. (2016) summarize some of these effects, which include nutrient uptake, the production of fatty acids, amino acids and other metabolites, and protection of the host from pathogens and parasites, either by stimulating immune function or by directly inhibiting pathogen growth. Recent research has exploited this co-evolved relationship between microbiota and host – the holobiont – to demonstrate that strategic manipulation of the worker gut microbiome can help bees recover from dysbiosis following antibiotic use and a chronic AFB outbreak (Daisley et al., 2020b) and even bolster bee immunity to protect against further gut-borne disease (Daisley et al., 2020a; Raymann and Moran, 2018). Despite the prospect of microbial therapeutics in the practice of apiculture, the idea of using gut microbe manipulations as a beekeeping tool has received relatively little research attention (Chmiel et al., 2021) and this despite the availability of some reportedly bee-friendly 'probiotic' products (Damico et al., 2023).

As an extension of the holobiont, the microbiota of individuals could scale up to affect the collective behaviour of whole social groups (Sarkar et al., 2020; Jones et al., 2018). As one example, consider that worker bees have evolved an olfactory-cued sensory

threshold that triggers a hygienic response; once the scent of disease becomes sufficiently intense, it can elicit a hygienic response from a proportion of the worker bees, whereby the most sensitive bees react first (Beshers and Fewell, 2001; Oldroyd and Thompson, 2006). The stronger the scent, the larger the proportion of workers that will be triggered and thus respond. What if the threshold itself could be lowered, such that a greater proportion of bees are early responders?

At a mechanistic level, the olfactory stimuli are detected by a worker's antennae, and neurotransmitters such as octopamine, γ-aminobutyric acid (GABA), serotonin or dopamine relay that signal to the mushroom bodies and lateral horns of the bee brain for processing (Paoli and Galizia, 2021). In mammals, the vagus nerve, a major component of the parasympathetic nervous system, mediates the communication between the gut and brain by sensing microbe-produced metabolites and signalling to the central nervous system (Bonaz et al., 2018). In insects, the analogous conduit is the antennal nerve (Schoofs et al., 2014), which appears to function similarly, relaying gut sensory information to the brain, as evidenced by changes to feeding and presumably other types of environmentally responsive behaviours (Miroschnikow et al., 2020; Salim et al., 2021).

Certain gut-borne bacteria can produce neurotransmitters (Zhang et al., 2022) or stimulate the host's innate production of these neurotransmitters via the production of their precursors (Chen et al., 2021; Table 1). Lactic acid-producing bacteria (LAB), including species within the genera *Lactobacillus* and *Bifidobacterium,* coevolved with bees over millions of years and are abundant in bee guts (Vásquez et al., 2012). LAB can synthesize GABA, at least in mammalian hosts, via the glutamic acid decarboxylase system (Cui et al., 2020). LAB can also modulate levels of serotonin production by regulating its precursor tryptophan (Zhang et al., 2020). Moreover, LAB are associated with the production of dopamine in vitro (Özoğul et al., 2012) via the conversion of its precursor (levodopa) from the amino acid l-tyrosine (Sarkar et al., 2020). In the gut of the roundworm *Caenorhabditis elegans*, bacteria may produce octopamine indirectly by producing its amino acid precursor, tyramine (O'Donnell et al., 2020).

Table 1. Bacterial interactions via the production, degradation or modulation of honey bee neurotransmitters.

Neuro-transmitter	Bacterium	Mechanism	Reference
Octopamine	Various gut community members	Tyrosine synthesis, precursor to octopamine	O'Donnell et al. 2020
GABA	*Lactobacillus* spp., *Bifidobacterium* spp.	Glutamic acid decarboxylase system	Cui et al. 2020
Serotonin	*Lactobacillus* spp. *Lactiplantibacillus plantarum*	Interference with tryptophan, precursor to serotonin	Özoğul et al. 2012 Zhang et al., 2020
Dopamine	*Lactobacillus* spp.	Synthesized from amino acids in vitro	Özoğul et al. 2012
	Lactic acid-producing bacteria	Synthesize the precursor levodopa, which can pass through the blood-brain barrier	Sarkar et al. 2020

While a host may obtain small amounts of these neurotransmitters or their precursors from the environment or from its own diet (Chen et al., 2021), the bacterial communities in the gut are the predominant source of these metabolites (Cui et al., 2020; Table 1). This functional linkage between the bacteria in the gut and the production of neurotransmitters or their precursors suggests that LAB may be harnessed within an apicultural context to increase the neurotransmission of disease-associated olfactory cues in worker bees. If a critical number of workers could be rendered more sensitive, a probiotic supplement that specifically lowered the response threshold to disease or that affected other threshold-gated behaviours could be designed for the beekeeping community.

2.2.1 Octopamine

The biogenic amine octopamine may have a practical link to the hygienic response of workers. The concentration of octopamine in the worker's brain tends to increase with age, which, in turn, affects age-based behavioural plasticity and the duties performed by workers within colonies (Schulz et al., 2002). Spivak et al. (2003) observed differences in the expression of octopamine in the brains of nurse bees from hygienic and non-hygienic lines, suggesting that this neurotransmitter is functionally associated with sensitivity to cues from diseased brood. This relationship between octopamine and the age- or genotype-associated expression of hygiene suggests that increasing octopamine concentration would be a viable target for microbial therapeutics research. The age-based changes in octopamine levels further suggest that bees of a certain age may be the best candidates to respond. The association between concentrations of octopamine and the hygienic response warrants future research.

2.2.2 Gamma-aminobutyric acid (GABA)

The amino acid neurotransmitter GABA is taxonomically widespread and thus plays a fundamental role in signal processing (Mustard et al., 2020). For honey bees, GABAergic neurons are present in all principal olfactory centres, such as the mushroom bodies and lateral horns (Sandoz, 2011), as well as other areas of the brain (Bicker, 1999). It is associated with learning and memory of the worker caste (El Hassani et al., 2005), as

well as locomotion and motor control (Mustard et al., 2020). Conversely, injection with GABA receptor antagonists can reduce bee mobility and impair their ability to right themselves after falling (Mustard et al., 2020). Injection with GABA receptor antagonists can also hinder olfactory neurons and diminish a bee's ability to discriminate between different odours (Stopfer et al., 1997). As hygiene is a motor behaviour that is olfactory-mediated (Masterman et al., 2000), GABA may pose an interesting candidate for modulation of the hygienic behaviour of nurse bees. If hives can be supplemented with LAB that produce GABA, for example, *Lactiplantibacillus plantarum* (Cui et al., 2020), then this effect on hygiene may be deliberately amplified within the colony.

2.2.3 Serotonin and dopamine

Serotonin is a biogenic amine that affects the senses of honey bees, but here, the effect appears to reduce sensitivity to olfactory cues. Injection of serotonin into bees decreases the spontaneous action of some neurons and impairs memory (Erber et al., 1993). Zhang et al. (2022) found that enriching the native gut microbiota with *Gilliamella apicola* and *Lactobacillus* spp. reduced serotonin levels in the brains compared to gnotobiotic (i.e., gut-sterilized) bees. Like serotonin, dopamine can dampen responsiveness to stimuli (Mercer and Menzel, 1982) and affect locomotion and motor behaviour in honey bees (Mustard et al., 2010). Zhang et al. (2022) demonstrated that dopamine levels can be decreased by gut microbes; they mono-colonized bees in-lab with different strains of native bacteria (*Gilliamella apicola*, *Lactobacillus* or *Bombilactobacillus*), and then compared dopamine levels to gnotobiotic bees. The findings suggest an optimal dopamine concentration that can affect behaviour, and that the desired effect may be less, not more, of the neurotransmitter. Combinatorial enrichment of bee guts with a mix of probiotic strains that simultaneously increase octopamine and GABA while decreasing dopamine and serotonin may therefore be desirable. These complex manipulations of the bee gut microbiome could come from competition with the production of other neurotransmitters by the probiotics used to supplement the hive or by interference with the production of the precursors to these neurotransmitters (O'Donnell et al., 2020).

2.3 Testing probiotic effects on hygienic behaviour

Hygiene is a complex behaviour. Nurses share and delegate hygiene-associated tasks, specializing in areas such as uncapping the brood cell or removing diseased offspring (Barrs et al., 2021). Originally, hygiene assays specifically targeted the detection and removal of chalkbrood (*Ascosphaera apis*) and foulbrood (*Paenobacillus larvae*) by inoculating small sections of brood with these pathogens (Leclercq et al., 2018), but our current understanding of hygiene has broadened to include behavioural responses against other microbial sources of infection (Valizadeh et al., 2020) or against infestation by ectoparasites (e.g., *Varroa* mite-sensitive hygiene; Mondet et al., 2015). Olfaction may play a lesser role in *Varroa*-sensitive hygiene (Sprau et al., 2023; Tsuruda et al., 2012), but Pitek et al. (submitted manuscript) demonstrated that supplementing with a three-strain LAB consortium reduces *Varroa* load in managed populations of honey bees in Ontario, showing that probiotics may also affect these forms of hygiene.

I predict that administering probiotics aiding in the synthesis of olfactory-associated neurotransmitters, such as lactic acid-producing species, will modulate any genetic effects on hygiene and associated sensitivity to disease cues. This modulation may lower the hygienic threshold response of nurse bees to, in effect, render bees more hygienic. Given that LAB can help synthesize key neurotransmitters or their precursors (Table 1), I propose supplementing hives with two LAB species: *Bifidobacterium asteroides*, a core gut community member, and *Lactiplantibacillus plantarum* which is not a core member of the microbiota but can be found in honey bee guts. I suggest exploring the abilities of these and other candidate strains to affect the concentration of octopamine, GABA, serotonin, dopamine and, possibly, hygienic behaviour. Ideally, future studies would demonstrate that the specific bacteria administered can colonize, even transiently, the guts of treated bees, correlate with the concentration of specific neurotransmitters or their precursors in bee brains, and ultimately affect the hygienic response. Together, these three test criteria would help to link treatment to a change in behaviour via the gut-brain axis.

The most common field assay for measuring hygiene is the freeze-kill brood (FKB) assay, which involves experimentally killing a small portion of brood with liquid nitrogen, then counting the proportion of the moribund brood removed over a set period (usually 24-48 hours; Spivak and Downey, 1998). The pin-killed brood (PKB) assay, which involves individually piercing pupae with a needle and determining the proportion of brood removed after 24-48 hours (Newton and Ostasiewski, 1986), is a variation of this assay, and yet others are possible (Leclercq et al., 2018). To investigate changes to the microbiota following treatment, researchers can employ 16S ribosomal RNA gene sequencing of the V3-V4 region to evaluate microbial community structure (as in Daisley et al., 2023). From this, the diversity within and between samples can be assessed for differences due to treatment. Various options are available to test the impact of bacterial supplements on the brain, such as high-performance liquid chromatography (or liquid chromatography-mass spectrometry) to determine neurotransmitter concentrations or histochemical staining to view the distribution of neurotransmitters in the brain. Figure 3 displays an experimental throughput for this type of analysis.

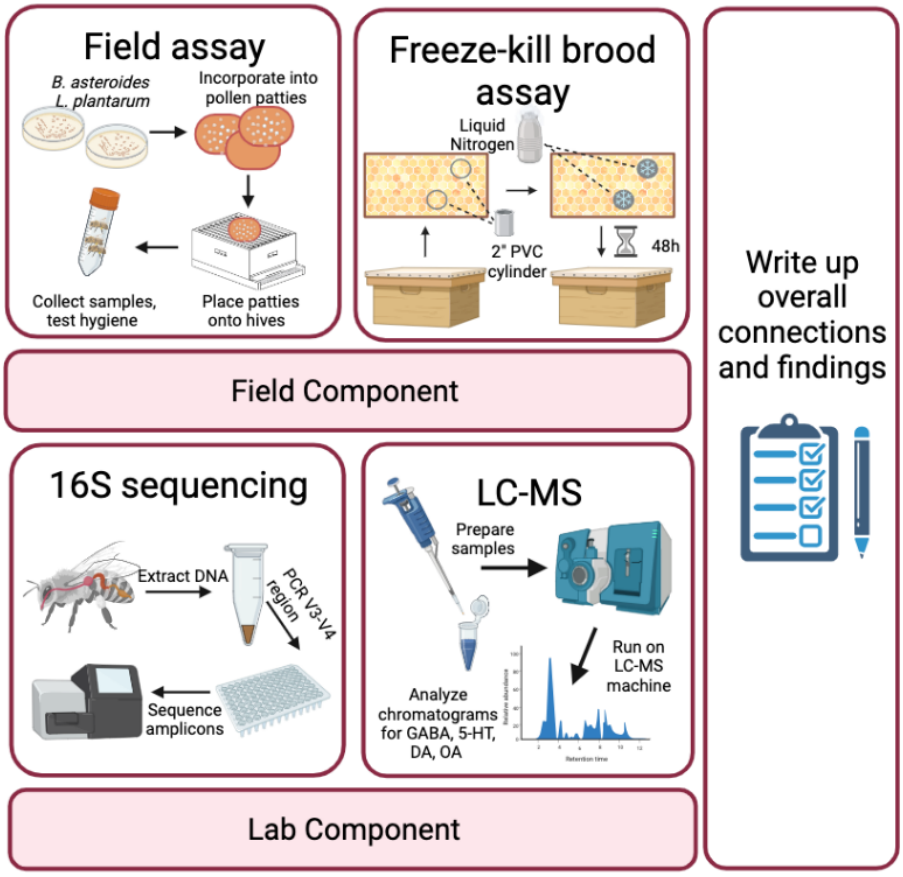

Figure 3. Proposed experimental design for probiotic administration influencing honey bee hygiene.

Testing the hypothesis that certain probiotics may alter hygienic behaviour via the gut-brain axis requires experimental considerations in both a controlled laboratory setting as well as within the field. Incorporating *Bifidobacterium asteroides* and *Lactiplantibacillus plantarum* into “pollen patties", placed into hives, presumably will affect hygiene as determined by a freeze-kill brood assay. In conjunction with the field assays, these effects can be confirmed via 16S ribosomal RNA sequencing and liquid chromatography-mass spectrometry to quantify GABA, 5-HT, DA, and OA in gut and brain tissue.

2.4 The gut-brain axis as a mechanism to modulate social behaviours

In addition to hygiene, the concepts proposed here could be extended to other honey bee behaviours, namely foraging, recruitment and defense. Recent work has demonstrated that variations in the microbiota of bees can influence foraging behaviour (Vernier et al., 2023). As in hygiene, foraging is intricately linked to olfaction (de Brito Sanchez, 2011; Paoli and Galizia, 2021); octopamine and GABA both contribute to the foraging process (Chatterjee et al., 2021; Giray et al., 2007). Octopamine influences response thresholds to sucrose (Page and Erber, 2002), potentially increasing foraging efforts, as well as influencing food preference during foraging (Giray et al., 2007). GABA receptors are more abundant in the brains of bees scouting for new food sources, and GABAergic neuron activity increases when foragers are orienting themselves to food sources or the hive (Kiya and Kubo, 2010). Overall, changes in GABA and its precursor, glutamate, signalling in the brain appear to modulate scouting behaviour in foragers (Chatterjee et al., 2021), although there is more to discover from this connection. Artificial application of octopamine has been shown to induce early foraging in honey bees (Schulz et al., 2002), highlighting the importance of this neurotransmitter in foraging (Table 2). Researchers can perform field assays to estimate total foraging behaviour and determine if the probiotic application has an effect.

Table 2. Four neurotransmitters and their association to hygiene, foraging, defense and recruitment behaviour of worker honey bees.

Neuro-transmitter	Hygiene	Foraging	Defense	Recruitment
Octopamine	Enhanced sensitivity to olfactory cues (Erber et al. 1993)	Induces early foraging (Schulz et al. 2002)	Linked to dominance and aggression (Hunt 2007)	Time spent following directions to food sources (Linn et al. 2020)
	Division of labour (Schulz et al. 2002)	Influences foraging preferences (Giray et al. 2007) Impacts sucrose response thresholds (Page and Erber 2002)		
GABA	Locomotion and motor control (Mustard et al. 2020)	Searching frequency for new food sources (Chatterjee et al. 2021)	Peaks during the age when worker bees guard (Hunt 2007)	
	Odour processing, learning and memory (El Hassani et al. 2005)			
Serotonin	Antennal and proboscis responsiveness to stimuli (Erber et al. 1993)			
Dopamine	Motor control and coordination (Sarkar et al. 2020)			

As a distinct but related behaviour to foraging, honey bees recruit others to food sources using intricate dances, conveying information on the distance, direction and value of the food (Wenner et al., 1967). Octopamine and dopamine can help determine how long a bee follows the dance instructions and the frequency with which a bee will ultimately be recruited (Table 2, Linn et al., 2020). Waggle dance activity can be recorded by using an observation hive (Biesmeijer and Seeley, 2005) or video recording software. Octopamine and GABA are also thought to influence defensive behaviour within colonies (Hunt, 2007, Table 2), suggesting that *Bifidobacterium* spp. and *Lactobacillus* spp. may affect defensive behaviour via the gut-brain axis. Characteristics of the microbiota may also influence social recognition used in defense. In-lab assays by Vernier et al. (2020) showed that bees would accept intruders from another colony if they were colonized by the same microbes but would reject intruders with dissimilar microbial communities. Field assays for defense involve using a patch of material to aggravate guard bees and counting the number of stings it receives.

All core bacterial species found in the honey bee gut can be cultivated and manipulated in the laboratory (Zheng et al., 2018), making the honey bee a functional system for studying microbial effects on health and behaviour. Bees can be raised with germ-free guts in the lab (Powell et al., 2014), which allows for experimental colonization with strains of interest. In addition to laboratory experiments, field experiments can also be conducted, as proposed here, by applying probiotics directly to hives via probiotic-infused pollen patties (Corby-Harris et al., 2016) or probiotic sprays (Daisley et al., 2023). Strains of interest can be chosen based on the desired downstream behavioural effect and the neuromodulator that bacterial species may help produce, then grown in-lab according to the needs of that species. Further avenues of investigation could involve freeze-drying beneficial bacteria, increasing colony-forming unit counts and bacterial survivorship in different media for application to hives.

2.5 Conclusion

The potential of honey bee probiotics is promising, with numerous studies exploring the relationship between gut bacteria and brain neurotransmitters. While the impact on

colony health and behaviour is an emerging field, the role of healthy gut microbiota in supporting organism health and combating environmental and pathogenic stress is recognized. Mounting evidence suggests there is a link between neurotransmitters, microbiota composition, and worker bee hygienic behaviour. However, future studies are required to determine if extensions of this concept can be applied to experimentally modulate foraging, defense, and recruitment behaviours. These research initiatives offer promising avenues to improve the health, survival, and productivity of managed honey bees while advancing our understanding of the gut-brain axis at both the individual and colony levels.

2.6 References

Alberoni, D., Gaggìa, F., Baffoni, L., Di Gioia, D. (2016). Beneficial microorganisms for honey bees: Problems and progresses. *Applied Microbiology and Biotechnology*, *100*(22), 9469–9482. https://doi.org/10.1007/s00253-016-7870-4

Barrs, K.R., Ani, M.O., Eversman, K.K., Rowell, J.T., Wagoner, K.M., Rueppell, O. (2021). Time-accuracy trade-off and task partitioning of hygienic behavior among honey bee (*Apis mellifera*) workers. *Behavioral Ecology and Sociobiology*, *75*, 12. https://doi.org/10.1007/s00265-020-02940-y

Beshers, S.N., Fewell, J.H. (2001). Models of division of labor in social insects. *Annual Reviews of Entomology*, *46*, 413–440. https://doi.org/10.1146/annurev.ento.46.1.413

Bicker, G. (1999). Histochemistry of classical neurotransmitters in antennal lobes and mushroom bodies of the honeybee. *Microscopy Research and Techniques*, *45*, 174–183. https://doi.org/10.1002/(SICI)1097-0029(19990501)45:3<174::AID-JEMT5>3.0.CO;2-U

Biesmeijer, J.C., Seeley, T.D. (2005). The use of waggle dance information by honey bees throughout their foraging careers. *Behavioral Ecology and Sociobiology*, *59*, 133–142. https://doi.org/10.1007/s00265-005-0019-6

Bonaz, B., Bazin, T., Pellissier, S. (2018). The vagus nerve at the interface of the microbiota-brain-gut axis. *Frontiers in Neuroscience*, *12*. https://doi.org/10.3389/fnins.2018.00049

Bonilla-Rosso, G., Engel, P. (2018). Functional roles and metabolic niches in the honey bee gut microbiota. *Current Opinion in Microbiology*, *43*, 69–76. https://doi.org/10.1016/j.mib.2017.12.009

Carding, S., Verbeke, K., Vipond, D.T., Corfe, B.M., Owen, L.J. (2015). Dysbiosis of the gut microbiota in disease. *Microbial Ecology in Health and Disease*, *26*, 26191. https://doi.org/10.3402/mehd.v26.26191

Chatterjee, A., Bais, D., Brockmann, A., Ramesh, D. (2021). Search behavior of individual foragers involves neurotransmitter systems characteristic for social scouting. *Frontiers in Insect Science*, *1*. https://doi.org/10.3389/finsc.2021.664978

Chen, Y., Xu, J., Chen, Y. (2021). Regulation of neurotransmitters by the gut microbiota and effects on cognition in neurological disorders. *Nutrients*, *13*, Article 6. https://doi.org/10.3390/nu13062099

Chmiel, J.A., Pitek, A.P., Burton, J.P., Thompson, G.J., Reid, G. (2021). Meta-analysis on the effect of bacterial interventions on honey bee productivity and the treatment of infection. *Apidologie*, *52*, 960–972. https://doi.org/10.1007/s13592-021-00879-1

Corby-Harris, V., Snyder, L., Meador, C.A.D., Naldo, R., Mott, B., Anderson, K.E. (2016). *Parasaccharibacter apium*, gen. nov., sp. nov., improves honey bee (Hymenoptera: Apidae) resistance to *Nosema*. *Journal of Economic Entomology*, *109*(2), 537–543. https://doi.org/10.1093/jee/tow012

Cremer, S., Armitage, S.A.O., Schmid-Hempel, P. (2007). Social immunity. *Current Biology*, *17*(16). 693–702. https://doi.org/10.1016/j.cub.2007.06.008

Cui, Y., Miao, K., Niyaphorn, S., Qu, X. (2020). Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. *International Journal of Molecular Sciences*, *21*, Article 3. https://doi.org/10.3390/ijms21030995

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Al, K. F., Chernyshova, A. M., Faragalla, K. M., Burton, J. P., Thompson, G. J., & Reid, G. (2020a). Novel probiotic approach to counter *Paenibacillus larvae* infection in honey bees. *The ISME Journal*, *14*(2), 476–491. https://doi.org/10.1038/s41396-019-0541-6

Daisley, B.A., Pitek, A.P., Chmiel, J.A., Gibbons, S., Chernyshova, A.M., Al, K.F., Faragalla, K.M., Burton, J.P., Thompson, G.J., Reid, G. (2020b). *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Communications Biology*, *3*, Article 1. https://doi.org/10.1038/s42003-020-01259-8

Daisley, B.A., Pitek, A.P., Torres, C., Lowery, R., Adair, B.A., Al, K.F., Niño, B., Burton, J.P., Allen-Vercoe, E., Thompson, G.J., Reid, G., Niño, E. (2023). Delivery mechanism can enhance probiotic activity against honey bee pathogens. *The ISME Journal*, *17*, Article 9. https://doi.org/10.1038/s41396-023-01422-z

Damico, M.E., Beasley, B., Greenstein, D., Raymann, K. (2023). Testing the effectiveness of a commercially sold probiotic on restoring the gut microbiota of honey bees: A field study. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-023-10203-1

de Brito Sanchez, M.G. (2011). Taste perception in honey bees. *Chemical Senses*, *36*, 675–692. https://doi.org/10.1093/chemse/bjr040

Dickel, F., Bos, N.M.P., Hughes, H., Martín-Hernández, R., Higes, M., Kleiser, A., Freitak, D. (2022). The oral vaccination with *Paenibacillus larvae* bacterin can decrease susceptibility to American Foulbrood infection in honey bees—A safety and efficacy study. *Frontiers in Veterinary Science*, *9*. https://doi.org/10.3389/fvets.2022.946237

Dus, M., Lai, J.S.Y., Gunapala, K.M., Min, S., Tayler, T.D., Hergarden, A.C., Geraud, E., Joseph, C.M., Suh, G.S.B. (2015). Nutrient sensor in the brain directs the action of the brain-gut axis in drosophila. *Neuron*, *87*, 139–151. https://doi.org/10.1016/j.neuron.2015.05.032

El Hassani, A.K., Dacher, M., Gauthier, M., Armengaud, C. (2005). Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*). *Pharmacology Biochemistry and Behavior*, *82*, 30–39. https://doi.org/10.1016/j.pbb.2005.07.008

Erber, J., Kloppenburg, P., Scheidler, A. (1993). Neuromodulation by serotonin and octopamine in the honeybee: Behaviour, neuroanatomy and electrophysiology. *Experientia*, *49*, 1073–1083. https://doi.org/10.1007/BF01929916

Erez, T., Bonda, E., Kahanov, P., Rueppell, O., Wagoner, K., Chejanovsky, N., Soroker, V. (2022). Multiple benefits of breeding honey bees for hygienic behavior. *Journal of Invertebrate Pathology*, *193*, 107788. https://doi.org/10.1016/j.jip.2022.107788

Garbian, Y., Maori, E., Kalev, H., Shafir, S., Sela, I. (2012). Bidirectional transfer of RNAi between honey bee and *Varroa* destructor: *Varroa* gene silencing reduces *Varroa* population. *PLoS Pathogens*, *8*, e1003035. https://doi.org/10.1371/journal.ppat.1003035

Giray, T., Galindo, A., Oskay, D. (2007). Octopamine influences honey bee foraging preference. *Journal of Insect Physiology*, *53*, 691–698. https://doi.org/10.1016/j.jinsphys.2007.03.016

Guerrero, R., Margulis, L., Berlanga, M. (2013). Symbiogenesis: The holobiont as a unit of evolution. *International Microbiology: The Official Journal of the Spanish Society for Microbiology*, *16*, 133–143. https://doi.org/10.2436/20.1501.01.188

Hunt, G.J. (2007). Flight and fight: A comparative view of the neurophysiology and genetics of honey bee defensive behavior. *Journal of Insect Physiology*, *53*, 399–410. https://doi.org/10.1016/j.jinsphys.2007.01.010

Hýbl, M., Bohatá, A., Rádsetoulalová, I., Kopecký, M., Hoštičková, I., Vaníčková, A., Mráz, P. (2021). Evaluating the efficacy of 30 different essential oils against *Varroa destructor* and honey bee workers (*Apis mellifera*). *Insects*, *12*(11). https://doi.org/10.3390/insects12111045

Jones, J.C., Fruciano, C., Marchant, J., Hildebrand, F., Forslund, S., Bork, P., Engel, P., Hughes, I. (2018). The gut microbiome is associated with behavioural task in honey bees. *Insectes Sociaux*, *65*, 419–429. https://doi.org/10.1007/s00040-018-0624-9

Kapheim, K.M., Rao, V.D., Yeoman, C.J., Wilson, B.A., White, B.A., Goldenfeld, N., Robinson, G.E. (2015). Caste-specific differences in hindgut microbial communities of honey bees (*Apis mellifera*). *PLoS ONE*, *10*, e0123911. https://doi.org/10.1371/journal.pone.0123911

Kiya, T., Kubo, T. (2010). Analysis of GABAergic and non-GABAergic neuron activity in the optic lobes of the forager and re-orienting worker honeybee (*Apis mellifera* L.). *PLoS ONE*, *5*, e8833. https://doi.org/10.1371/journal.pone.0008833

Leclercq, G., Francis, F., Gengler, N., Blacquière, T. (2018). Bioassays to quantify hygienic behavior in honey bee (*Apis mellifera* L.) colonies: A review. *Journal of Apicultural Research*, *57*, 1–11. https://doi.org/10.1080/00218839.2018.1494916

Liberti, J., Engel, P. (2020). The gut microbiota—brain axis of insects. *Current Opinion in Insect Science*, *39*, 6–13. https://doi.org/10.1016/j.cois.2020.01.004

Liberti, J., Kay, T., Quinn, A., Kesner, L., Frank, E.T., Cabirol, A., Richardson, T.O., Engel, P., Keller, L. (2022). The gut microbiota affects the social network of honeybees. *Nature Ecology and Evolution*, *6*(10). https://doi.org/10.1038/s41559-022-01840-w

Lima, C.M.G., Nora, F.M.D., Seraglio, S.K.T., Silva, J.M., da Marzoque, H.J., Santana, R.F., Verruck, S., Scussel, V.M. (2020). Antibiotic residues in honey: A public health issue. *Research, Society and Development*, *9*, Article 11. https://doi.org/10.33448/rsd-v9i11.9604

Linn, M., Glaser, S.M., Peng, T., Grüter, C. (2020). Octopamine and dopamine mediate waggle dance following and information use in honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1936). https://doi.org/10.1098/rspb.2020.1950

Masterman, R., Smith, B.H., Spivak, M. (2000). Brood odor discrimination abilities in hygienic honey bees *(Apis mellifera* L.*)* using proboscis extension reflex conditioning. *Journal of Insect Behavior*, *13*, 87–101. https://doi.org/10.1023/A:1007767626594

Mayer, E.A., Tillisch, K. (2011). The brain-gut axis in abdominal pain syndromes. *Annual Review of Medicine*, *62*, 10.1146/annurev-med-012309–103958. https://doi.org/10.1146/annurev-med-012309-103958

Mercer, A.R., Menzel, R. (1982). The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee *Apis mellifera*. *Journal of Comparative Physiology*, *145*, 363–368. https://doi.org/10.1007/BF00619340

Miroschnikow, A., Schlegel, P., Pankratz, M.J. (2020). Making feeding decisions in the *Drosophila* nervous system. *Current Biology*, *30*, R831–R840. https://doi.org/10.1016/j.cub.2020.06.036

Mondet, F., Alaux, C., Severac, D., Rohmer, M., Mercer, A.R., Le Conte, Y. (2015). Antennae hold a key to Varroa-sensitive hygiene behaviour in honey bees. *Scientific Reports*, *5*(10454). https://doi.org/10.1038/srep10454

Motta, E.V.S., Powell, J.E., Leonard, S.P., Moran, N.A. (2022). Prospects for probiotics in social bees. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *377*(1853), 20210156. https://doi.org/10.1098/rstb.2021.0156

Mustard, J.A., Jones, L., Wright, G.A. (2020). GABA signaling affects motor function in the honey bee. *Journal of Insect Physiology*, *120*(103989). https://doi.org/10.1016/j.jinsphys.2019.103989

Mustard, J.A., Pham, P.M., Smith, B.H. (2010). Modulation of motor behavior by dopamine and the D1-like dopamine receptor AmDOP2 in the honey bee. *Journal of Insect Physiology*, *56*, 422–430. https://doi.org/10.1016/j.jinsphys.2009.11.018

Newton, D.C., Ostasiewski, N.J.J. (1986). A simplified bioassay for behavioral resistance to American foulbrood in honey bees. *The American Bee Journal*, *126*, 278–281.

Niño, E.L., Cameron Jasper, W. (2015). Improving the future of honey bee breeding programs by employing recent scientific advances. *Current Opinion in Insect Science*, *10*, 163–169. https://doi.org/10.1016/j.cois.2015.05.005

O'Donnell, M.P., Fox, B.W., Chao, P.H., Schroeder, F.C., Sengupta, P. (2020). A neurotransmitter produced by gut bacteria modulates host sensory behaviour. *Nature*, *583*, 415–420. https://doi.org/10.1038/s41586-020-2395-5

Oldroyd, B.P., Thompson, G.J. (2006). Behavioural genetics of the honey bee *Apis mellifera*. *Advances in Insect Physiology*, *33*, 1–49. https://doi.org/10.1016/S0065-2806(06)33001-9

Oxley, P.R., Spivak, M., Oldroyd, B.P. (2010). Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (*Apis mellifera*). *Molecular Ecology*, *19*, 1452–1461. https://doi.org/10.1111/j.1365-294X.2010.04569.x

Özoğul, F., Kuley, E., Özoğul, Y., Özoğul, İ. (2012). The function of lactic acid bacteria on biogenic amines production by food-borne pathogens in arginine decarboxylase broth. *Food Science and Technology Research*, *18*, 795–804. https://doi.org/10.3136/fstr.18.795

Page, R.E., Erber, J. (2002). Levels of behavioral organization and the evolution of division of labor. *Naturwissenschaften*, *89*(3), 91–106. https://doi.org/10.1007/s00114-002-0299-x

Paoli, M., Galizia, G.C. (2021). Olfactory coding in honeybees. *Cell and Tissue Research*, *383*, 35–58. https://doi.org/10.1007/s00441-020-03385-5

Powell, J.E., Martinson, V.G., Urban-Mead, K., Moran, N.A. (2014). Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera. Applied and Environmental Microbiology*, *80*, 7378–7387. https://doi.org/10.1128/AEM.01861-14

Raymann, K., Moran, N.A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current Opinion in Insect Science*, *26*, 97–104. https://doi.org/10.1016/j.cois.2018.02.012

Raymann, K., Shaffer, Z., Moran, N.A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biology*, *15*, e2001861. https://doi.org/10.1371/journal.pbio.2001861

Rosenberg, E., Sharon, G., Atad, I., Zilber-Rosenberg, I. (2010). The evolution of animals and plants via symbiosis with microorganisms. *Environmental Microbiology Report*, *2*, 500–506. https://doi.org/10.1111/j.1758-2229.2010.00177.x

Salim, S., Banu, A., Alwa, A., Gowda, S.B.M., Mohammad, F. (2021). The gut-microbiota-brain axis in autism: What *Drosophila* models can offer? *Journal of Neurodevelopmental Disorders*, *13*(37). https://doi.org/10.1186/s11689-021-09378-x

Sandoz, J.C. (2011). Behavioral and neurophysiological study of olfactory perception and learning in honeybees. *Fronteirs in Systems Neuroscience*, *5*, 98. https://doi.org/10.3389/fnsys.2011.00098

Sarkar, A., Harty, S., Johnson, K.V.A., Moeller, A.H., Carmody, R.N., Lehto, S.M., Erdman, S.E., Dunbar, R.I.M., Burnet, P.W.J. (2020). The role of the microbiome in the neurobiology of social behaviour. *Biological Reviews*, *95*, 1131–1166. https://doi.org/10.1111/brv.12603

Schoofs, A., Hückesfeld, S., Surendran, S., Pankratz, M.J. (2014). Serotonergic pathways in the *Drosophila* larval enteric nervous system. *Journal of Insect Physiology*, *69*, 118–125. https://doi.org/10.1016/j.jinsphys.2014.05.022

Schulz, D.J., Barron, A.B., Robinson, G.E. (2002). A role for octopamine in honey bee division of labor. *Brain, Behavior and Evolution*, *60*, 350–359. https://doi.org/10.1159/000067788

Spivak, M., Downey, D.L. (1998). Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *91*, 64–70. https://doi.org/10.1093/jee/91.1.64

Spivak, M., Gilliam, M. (2015). Hygienic behaviour of honey bees and its application for control of brood diseases and Varroa: Part II. Studies on hygienic behaviour since the Rothenbuhler era. *Bee World*, *79*, 169–186. https://doi.org/10.1080/0005772X.1998.11099408

Spivak, M., Masterman, R., Ross, R., Mesce, K.A. (2003). Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. *Journal of Neurobiology*, *55*, 341–354. https://doi.org/10.1002/neu.10219

Spivak, M., Reuter, G.S. (1998). Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie*, *29*, 291–302. https://doi.org/10.1051/apido:19980308

Sprau, L., Traynor, K., Rosenkranz, P. (2023). Honey bees *(Apis mellifera)* preselected for *Varroa* sensitive hygiene discriminate between live and dead *Varroa destructor* and inanimate objects. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-37356-x

Stopfer, M., Bhagavan, S., Smith, B.H., Laurent, G. (1997). Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature*, *390*(6655). https://doi.org/10.1038/36335

Tsuruda, J.M., Harris, J.W., Bourgeois, L., Danka, R.G., Hunt, G.J. (2012). High-resolution linkage analyses to identify genes that influence *Varroa* sensitive hygiene behavior in honey bees. *PLoS ONE*, *7*, e48276. https://doi.org/10.1371/journal.pone.0048276

Uzunov, A., Costa, C., Panasiuk, B., Meixner, M., Kryger, P., Hatjina, F., Bouga, M., Andonov, S., Bienkowska, M., Conte, Y. L., Wilde, J., Gerula, D., Kiprijanovska, H., Filipi, J., Petrov, P., Ruottinen, L., Pechhacker, H., Berg, S., Dyrba, W., … Büchler, R. (2014). Swarming, defensive and hygienic behaviour in honey bee colonies of

different genetic origin in a pan-European experiment. *Journal of Apicultural Research*, *53*(2), 248–260. https://doi.org/10.3896/IBRA.1.53.2.06

Valizadeh, P., Guzman-Novoa, E., Goodwin, P.H. (2020). Effect of immune inducers on *Nosema ceranae* multiplication and their impact on honey bee (*Apis mellifera* L.) survivorship and behaviors. *Insects*, *11*(9). https://doi.org/10.3390/insects11090572

Vásquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L., Olofsson, T.C. (2012). Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. *PLoS ONE*, *7*(3), e33188. https://doi.org/10.1371/journal.pone.0033188

Vernier, C.L., Chin, I.M., Adu-Oppong, B., Krupp, J.J., Levine, J., Dantas, G., Ben-Shahar, Y. (2020). The gut microbiome defines social group membership in honey bee colonies. *Science Advances*, *6*, eabd3431. https://doi.org/10.1126/sciadv.abd3431

Vernier, C. L., Nguyen, L. A., Gernat, T., Ahmed, A. C., Chen, Z., & Robinson, G. E. (2024). Gut microbiota contribute to variations in honey bee foraging intensity. *The ISME Journal*, *18*(1), wrae030. https://doi.org/10.1093/ismejo/wrae030

Wenner, A.M., Wells, P.H., Rohlf, F.J. (1967). An analysis of the waggle dance and recruitment in honey bees. *Physiological Zoology*, *40*, 317–344. https://doi.org/10.1086/physzool.40.4.30158452

Zhang, Z., Mu, X., Cao, Q., Shi, Y., Hu, X., Zheng, H. (2020). *Honeybee gut microbiota modulates host behaviors and neurological processes* (2020.12.19.423587). bioRxiv. https://doi.org/10.1101/2020.12.19.423587

Zhang, Z., Mu, X., Shi, Y., Zheng, H. (2022). Distinct roles of honeybee gut bacteria on host metabolism and neurological processes. *Microbiology Spectrum*, *10*, e02438-21. https://doi.org/10.1128/spectrum.02438-21

Zheng, H., Steele, M.I., Leonard, S.P., Motta, E.V.S., Moran, N.A. (2018). Honey bees as models for gut microbiota research. *Lab Animal*, *47*(11). https://doi.org/10.1038/s41684-018-0173-x

Chapter 3

3 Testing for an effect of probiotic application on hygienic behaviour

3.1 Introduction

In this chapter, I performed an empirical study to test the effect of probiotic treatment on the expression of hygienic behaviour in a managed apiary. This field study served as a complement to existing laboratory-based studies showing that the microbiota of individuals can affect their social behaviour (Liberti et al., 2022; Vernier et al., 2020). My goal for this research was to advance the current understanding of the gut-brain axis, a bi-directional pathway of communication within the body that is mediated by gut microbes, from its current basis of the individual to the collective, or hive. This study will serve as preliminary research to inform recommendations for practical applications of probiotics, such as in commercial or hobbyist beekeeping practices.

3.1.1 Social demographics of honey bee disease

Honey bees live in densely populated hives of close kin, making them demographically vulnerable to the spread of parasites and communicable diseases, of which there are many afflicting honey bee colonies (Evans and Spivak, 2010; Genersch, 2010). This vulnerability can extend to neighbouring hives when they are densely packed into single areas, which is typical of apiaries, leading to population-wide infections. Cross-contamination between colonies can therefore be unwittingly accelerated by the beekeepers themselves through hive placement and the movement of bees and equipment between colonies (Fries and Camazine, 2001). The social demographics of the hive are therefore an important aspect of bee biology that should factor into integrated strategies of hive management and disease control (Evans and Spivak, 2010). In social insects, diseases can be spread not only within the original colony but to new daughter colonies via swarming (vertical transmission) and to other hives by drifting or robbing (horizontal transmission, Fries and Camazine, 2001).

At the colony level, it is the adult worker caste that is most active and interacting, and thus a potential vector for disease transmission. Some of the worst infections that afflict bee colonies, however, are borne in the brood (Food and Agriculture Organization of the United Nations, 2020). Brood-borne diseases include chalkbrood (caused by the fungus *Ascosphaera apis)*, American foulbrood (caused by the bacterium *Paenibacillus larvae*), and the ectoparasitic mite *Varroa destructor*, which preferentially develops within drone (male) brood cells. These diseases, as well as anthropogenic factors, are currently associated with the highest risk for colony loss in Canada and elsewhere (Hristov et al., 2020). The occurrence and degree of destruction caused by diseases can also depend on environmental conditions: temperature, relative humidity, and food availability (Momot & Rothenbuhler, 1971; Uzunov et al., 2014); and the bees themselves, their genetic resistance to disease and their ability to perform effective hygiene behaviour (Wilson-Rich et al., 2008).

Best management advises an array of strategies and guidelines to be used to minimize the risk of disease and colony loss (Stanimirović et al., 2019). In addition, it is the bees themselves that have evolved defenses that render some stocks more genetically resistant to infection than others (Cremer et al., 2007). One conspicuous behavioural trait that honey bees and other social insects have evolved is a tendency to detect and remove disease from each other or from their hive environment, which is best known as hygienic behaviour (Wilson-Rich et al., 2008; reviewed in Leclercq et al., 2017). First described by Rothenbuhler (1964), the behaviour is performed by nurse bees, who care for the colony's brood; nurses typically feed, inspect and cap brood cells. Hygiene is heritable and appears to be regulated by a few loci that regulate its expression in response to environmental cues (Bigio et al., 2013).

Nurses carrying the hygienic trait detect dead or diseased larvae via olfactory cues, then uncap the wax-covered brood cell and remove the diseased offspring (Rothenbuhler, 1964). Measuring hygiene is an important tool for beekeepers; it allows them to determine the performance of each colony and select breeder colonies that they can selectively propagate. However, it can be difficult and costly to breed for hygiene, which has only limited response to artificial selection (Leclercq et al., 2017). Specifically,

hygiene is linked to six to seven quantitative trait loci that partially explain the observed phenotypic variance within a test population of hives (Lapidge et al., 2002; Oxley et al., 2010).

3.1.2 Standardized assays to measure hygienic behaviour

Field assays for hygiene involve experimentally destroying a small portion of the brood, and then counting the proportion of diseased brood removed after a certain amount of time (Spivak & Downey, 1998). The most common assay used to measure hygiene is the freeze-kill brood (FKB) assay, which involves selecting an area of same-aged, capped pupae and freezing them with liquid nitrogen, then tracking the proportion of the deceased pupae that are removed after 24-48 hours. Because the youngest brood (egg, larva, and prepupa, approximately 1-11 days old) and oldest brood (developing body colour, 15-21 days old) may react differently to freezing, it is common for hygiene assays to focus on brood of intermediate age (roughly 12-14 days old) as evidenced by a developmental marker (white to pink eye colour; Pérez-Sato et al., 2009; Spivak & Downey, 1998).

Other hygiene assays are also possible, such as the pin-killed brood assay. This assay involves piercing each pupa through its cell capping using an insect pin (Newton and Ostasiewski, 1986). The pin-killed brood assay is quick and does not damage the comb, yet the diameter of the pin used to kill the pupae can drastically affect experimental results, leading to a lack of accuracy and reproducibility (Leclercq et al., 2018). Other assays involved inoculating brood with pathogens to determine their removal rate (reviewed in Spivak and Gilliam, 1998), but this type of assay is no longer popular due to the potential for the spread of disease and colony loss, although researchers can capitalize on natural infections to perform experiments (Daisley et al., 2020a; Leclercq et al., 2018).

3.1.3 Probiotics as a micro-therapeutic tool to promote gut health and hygiene

Because of the importance of hygiene as well as the difficulty in selecting for hygienic bees, many beekeepers treat their hives with antibiotics, which is linked with antibiotic

resistance (Ludvigsen et al., 2018; Evans, 2003) and perturbations to the natural gut community (Daisley et al. 2020b; Raymann et al., 2017; Bulson et al., 2021). Alternative solutions that balance and support the natural gut microbiome are therefore needed. Probiotics offer one potential avenue for beekeepers to help bees maintain healthy and hygienic hives without the side effects of antibiotics. In essence, probiotics are beneficial microorganisms that can be administered to support an organism's health, particularly as it affects the gastrointestinal system (Williams, 2010).

Most probiotics are intended for human use, whether as a capsule or in food such as yogurt, but recent work is extending the concept to insect hosts (Savio et al., 2022), especially economically or agriculturally important species such as the honey bee. In honey bees, research shows that select bacterial strains, when strategically administered, can bolster the immune system and increase survivability against pathogens (Borges et al., 2021; Daisley et al., 2020a). The potential for probiotics in beekeeping is therefore promising and may help protect bees against other forms of environmental stress, such as exposure to environmental contaminants and pesticides (Chmiel et al., 2020). Lactic acid-producing bacteria (LAB, including *Lactobacillus* and *Bifidobacterium* species) are commonly cited as potential bee-friendly probiotics, and most research to date has been focused on testing strains from these genera as candidates for bacterial disease control in hives (Evivie et al., 2017; Rodríguez et al., 2023).

The probiotics used in the present study are two species of LAB: *Lactiplantibacillus plantarum* and *Bifidobacterium asteroides*. *L. plantarum* is a hardy species found in fermented foods, plants, and the human gastrointestinal tract, with proven health benefits for humans (Nordström et al., 2021). This species is also thought to influence honey bee health - for example, Iorizzo et al. (2021) found that *L. plantarum* possessed antifungal properties that can aid in the biological control of chalkbrood (*Ascosphaera apis*) in hives. Additionally, a three-strain consortium of LAB including *L. plantarum, A. kunkeei, and L. rhamnosus* was found to reduce American foulbrood pathogen load in hives and increase survivability during a foulbrood outbreak (Daisley et al., 2020a, see also Arredondo et al., 2018). Bifidobacteria are found in a range of animal guts and are commonly used as probiotics; *B. asteroides*, specifically, is a core species in honey bee

guts and is considered to aid in immunity and resistance to environmental stress (Pino et al., 2022). This species can impact bee development by stimulating hormone production (Kešnerová et al., 2017), and is therefore related to age-based task allocation in workers.

3.1.4 The honey bee gut microbiota

The honey bee microbiota consists of five core species that are ubiquitous in adult workers: *Snodgrassella alvi, Gilliamella apicola, Bifidobacterium asteroides, Lactobacillus* and *Bombilactobacillus* (Kwong and Moran, 2016). Other species, such as *Frischella perrara* and *Bartonella apis,* are also present in many adult honey bee guts (Raymann & Moran, 2018). The greatest bacterial abundance and diversity is found in the hindgut, consisting of the ileum and rectum, but species may also be found in the midgut and foregut (Martinson et al., 2012). The microbiota of bees is transmitted primarily through social interactions within the hive (Powell et al., 2014), and as such, individuals within a given hive are expected to have very similar microbiomes, with variation in relative abundances expected between behavioural tasks and castes (Jones et al. 2018; Kapheim et al. 2015). The abundance and diversity of the microbial community are thought to influence the host organism's immunity, development, nutrition, digestion, and brain function (Alberoni et al., 2016; Kešnerová et al., 2017; Zhang et al., 2020, 2022).

Characterization of the microbiota through 16S ribosomal RNA sequencing is a popular method in microbiome studies (Daisley and Reid, 2021). This gene is a popular target for these studies as it is found in all species of bacteria and it contains variable and conserved regions, allowing for both the distinction of phylogenetic relationships and the ability to create primers to target the sequences (Větrovský & Baldrian, 2013). One limitation of 16S rRNA sequencing is the number of 16S gene copy numbers in bacteria, which can vary from 1 to 15 copies depending on the species (Stoddard et al., 2015). For the core species found in honey bee guts, the 16S copy number ranges from 2-5. The two species used to supplement hives in this study, *Bifidobacterium asteroides* and *Lactiplantibacillus plantarum*, have 2 and 5 copies of the rRNA operon, respectively (retrieved from the Ribosomal RNA Database, Stoddard et al., 2015).

In this thesis, I am testing for a functional gut-brain axis in Western honey bees (*Apis mellifera*) by experimentally manipulating the gut microbiome through the application of probiotics and then testing for subsequent changes in behaviour. Specifically, I look at hygienic behaviour, an economically and commercially valuable trait with vital implications for colony health and beekeeping. For this study, my main objectives are to: 1) determine whether probiotic treatment by *L. plantarum* and *B. asteroides* can alter the gut microbiota of worker bees in an apiary, and 2) determine whether probiotic treatment can impact the prevalence of hygienic behaviour within an apiary. My goal for this project is to provide evidence of a functional and manipulatable gut-brain axis that can affect apicultural-relevant traits, such that this study could serve as a foundation for future advances in beekeeping.

3.2 Methods

3.2.1 Colony preparation and maintenance

In the spring of 2023, I established a set of four queenright colonies of Western honey bees (*Apis mellifera*) housed in standard ten-frame Langstroth hives at Western University's Teaching, Research and Community Apiary. I monitored each colony's growth in response to warm weather and availability of forage and, in May of that year, added a single brood box to each, which enabled further growth to an estimated 20,000 workers per colony. After further growth, I then split each colony into three. Four of these therefore retained their original queen, while the others were re-queened with virgins of Carolinian stock (*Apis mellifera carnica*; sourced from KF Bees Honey Pty Ltd, St. Thomas, Ontario). Three of the 12 colonies were excluded from the experiment prior to the start of it due to poor queen acceptance. In early June, a medium honey 'super' (a shallower bee box designed for collecting honey) was placed on each experimental hive above a queen excluder to give the bees even more room to expand. At the beginning of the experiment in late June, all hives were at approximately equal strength: between four to six frames of brood and four to six frames of honey in each brood box and a honey super on top.

3.2.2 Bacterial culturing and patty making

I chose two species of lactic acid-producing bacteria, *Lactiplantibacillus plantarum* (strain Lp 39) and *Bifidobacterium asteroides* (strain HB-1052), as the strains for my experiment. I cultured both strains on de Man, Rogosa and Sharpe (MRS) media, comprising 12.5 g MRS broth and 3 g agar, supplemented with 2 g of D-fructose and 0.2 g of L-cysteine as per previous protocols in my lab. To prepare the media, the dry ingredients were mixed with 200mL ddH_2O, stirred, and autoclaved on the Liquid 20 cycle, followed by a 20-minute cooling period. I streaked three Petri dishes per strain and incubated them for 48 hours at 37°C. *Lactiplantibacillus plantarum* grows readily under aerobic conditions but for *Bifidobacterium asteroides,* I used the AnaeroGen™ Compact Atmosphere Generating System (Thermo Fisher Scientific) to generate the anaerobic

atmosphere that this strain prefers (Pino et al., 2022). After 48 hours I picked single colonies and transferred them to 50mL Falcon tubes that each contained approximately 40mL of MRS liquid broth supplemented with D-fructose and L-cysteine. I then allowed the strains to grow for an additional 24 hours before measuring absorbance at 600nm on a Nanodrop (Nour et al. 2014) and, from this information, estimated the number of colony-forming units (CFUs) according to the McFarland standard.

Separately, I prepared each BioPatty in triplicate with 97.1 g of soy flour, 252.3 g of granulated sucrose, 52 g of debittered brewer's yeast, combined with a syrup solution (150 mL ddH_2O and 300 g of sucrose; initially heated until clear, Daisley et al., 2020a). For culture media containing at least 1x10^9 CFU/mL, I centrifuged each Falcon tube for 10 minutes at 4°C, discarded the media and resuspended the pellet by vortexing in 3mL of 0.01 M phosphate-buffered saline (PBS). I added the bacteria in PBS to the cooled sucrose solution and incorporated the dry ingredients to form a dough. For control patties, I followed the same procedure, except I added only 3 mL of PBS without bacteria. Finally, I wrapped 250 g of dough in wax paper to form a patty and delivered them to hives within two hours. After seven days, the percent of the patty consumed was calculated by collecting any remaining patty after one week, weighing it, determining the difference between the remaining weight and the original weight, and then dividing that by the original weight.

3.2.3 Treating colonies and assessing the hygienic response

Using a total of nine colonies, I established three treatment groups designated by the live culture fed to them in a pollen patty medium. Specifically, I established three colonies each that were fed with patties containing *Bifidobacterium asteroides* or *Lactiplantibacillus plantarum*, as well as a no-treatment control group that was fed patties containing no assigned microbial culture (Figure 4). Before applying any treatment, I first estimated the baseline hygiene score for each colony by measuring the proportion of early-stage pupae (as inferred from white to pink eye colour; Wang et al., 2015) removed from two separate circular sections of brood comb (2-inch diameter) that had been killed with liquid nitrogen. Here, I used the standard freeze-kill brood assay

(Leclercq et al., 2018), to estimate each colony's natural tendency to remove dead and decaying pupae from brood cell chambers.

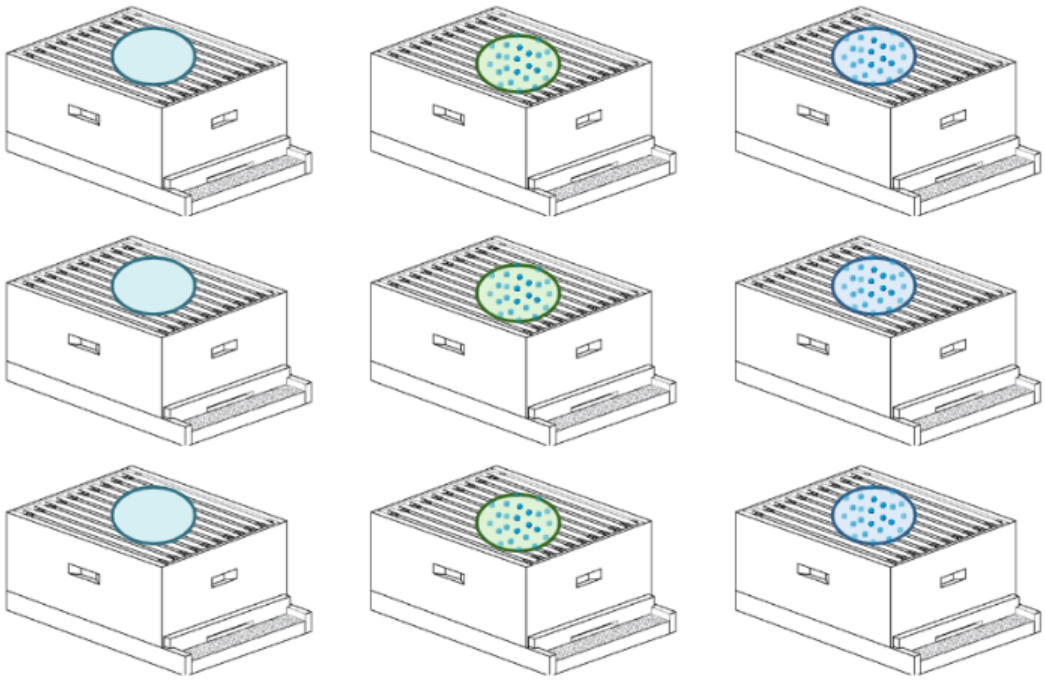

Figure 4. Experimental Design.

I used nine colonies of Western honey bees divided into three treatment groups: vehicle control (no probiotics), *Lactiplantibacillus plantarum,* and *Bifidobacterium asteroides.* Treatment was applied via pollen patties which were applied in weeks 1 and 2. Each colony was assayed thrice to determine hygiene.

I identified an area of same-aged brood and pressed a 2-inch diameter piece of PVC (a 'collar') into the middle of the section and filled it with 60mL of liquid nitrogen (Figure 5). I then repeated this with a second collar (Appendix A). Once the liquid nitrogen had dissipated (roughly 5 minutes), I removed the collars and photographed each frame before returning them to the hive. After approximately 48 hours, I removed the frames and photographed them again. I then used ImageJ v1.53t to count how many frozen cells had been uncapped and the pupa removed, how many had been uncapped and pupa partially removed, and how many were left capped (with no removal).

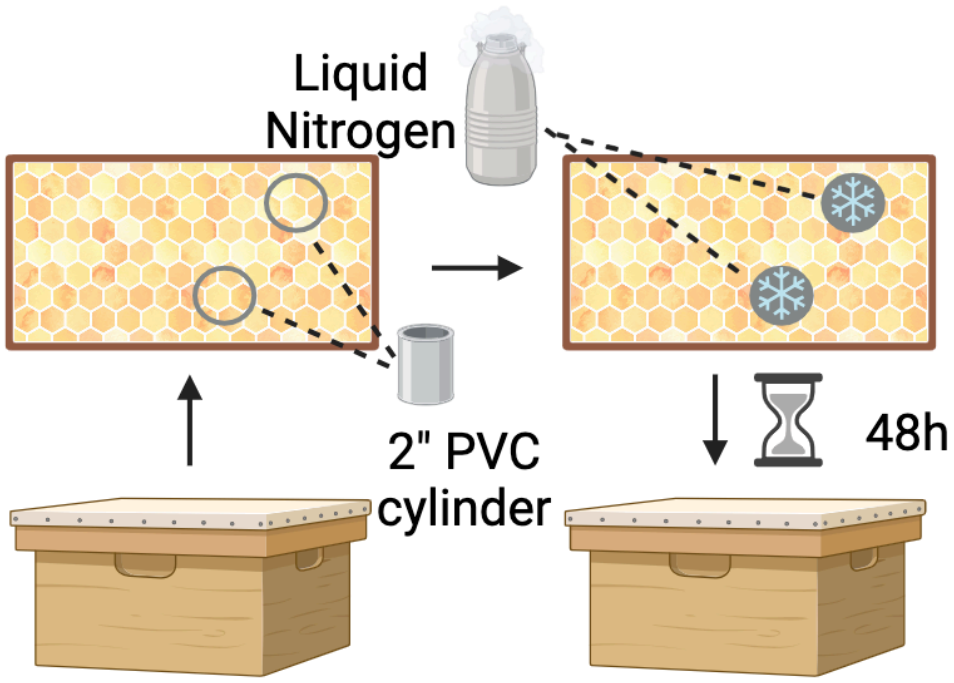

Figure 5. Freeze-kill brood assay.

To determine hygienic behaviour, I chose a frame of capped brood and pressed two 2-inch pieces of PVC pipe ('collars') into two areas of same-aged brood, then filled them with liquid nitrogen. I then removed the collar, photographed the frame, and replaced it in the hive. I returned roughly 48 hours later to determine the portion of the brood removed. I employed the formula (number of cells frozen–number of non-empty cells after the test/(number of cells frozen)) for both collared areas for two measures of hygiene for each colony as a proportion out of 1.

I based my estimates of hygiene on the proportion of freeze-killed brood removed after approximately 48 hours. After this period, I administered the designated treatment to each colony and re-tested the hygienic response after five days, again using the freeze-kill brood assay. I again treated colonies and re-estimated the hygienic response for a final time after another five days. The complete timeline for this experiment is shown in Figure 6. To control for variation in baseline scores between individual colonies, I compared the change in hygiene score after one or two treatments to the colony's baseline, measured before any application of treatment.

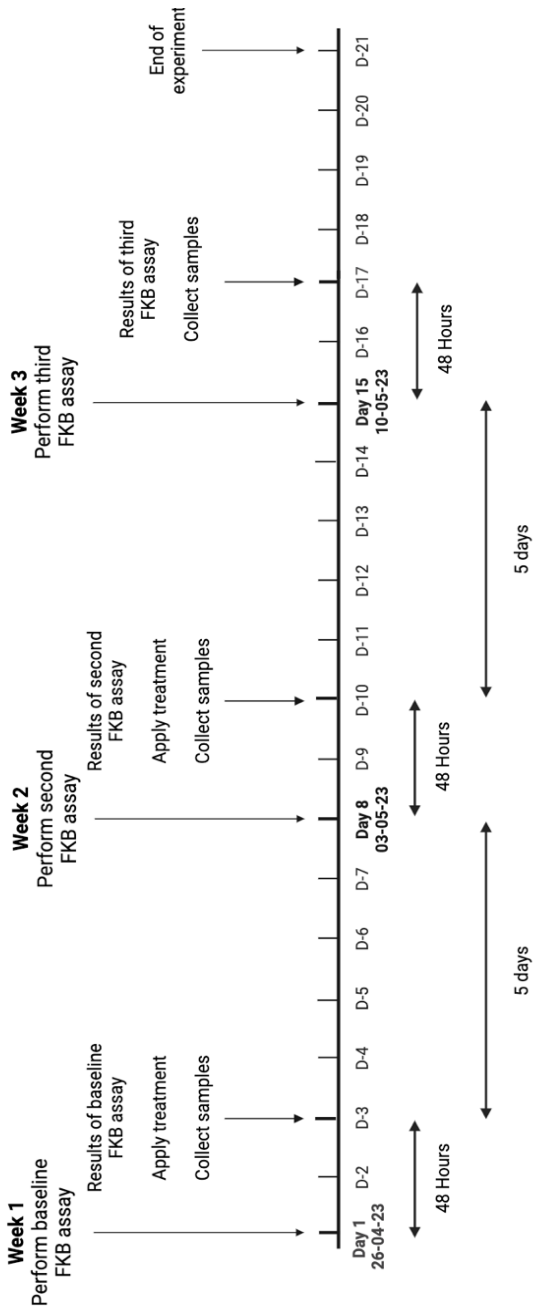

Figure 6. Timeline of experiment.

The total length of the experiment was three weeks, with hygiene testing occurring seven days apart on days 1, 8, and 15. Results of hygiene testing, patty application, and sample collection (~50 bees per Falcon tube) for gut analysis occurred ~48 hours after each freeze-kill brood (FKB) assay. Patties were consumed over five days before testing occurred again. In this five-day incubation period, samples were processed and photographs of assayed frames were analyzed.

3.2.4 Gut dissections and DNA extraction

Upon returning to photograph the frames, I collected samples of nurse bees directly from the assayed frame into Falcon tubes. These tubes were placed on dry ice and transported back to the laboratory, where they were stored at -80 C. I dissected partially thawed bees on ice by removing whole guts (hindgut, midgut, and crop) and pooled them in triplicate into single Eppendorf tubes with three replicates per hive. In total, I dissected nine bees per hive (three sets of three) for a total of 27 gut samples per week over three weeks. I extracted DNA from pooled samples using the QIAamp PowerFecal Pro kit (Qiagen, Germantown, Maryland) and stored it at -20°C until sequencing.

3.2.5 Indexing and 16S rRNA gene sequencing

Sample preparation was done at Lawson Health Research Institute at St Joseph's Health Care London (London, Ontario) following the protocol described in Daisley et al. (2020a). To amplify the V3-V4 region of the bacterial 16S ribosomal RNA gene, I used the established Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') primer set (Herlemann et al., 2011). Three controls were included with the samples: a negative control (the elution buffer from the last step of the DNA extraction); a PCR control (without any DNA template in the PCR mix); and a positive control (DNA extract from a pure culture of *Staphylococcus aureus* strain Newman). The PCR reaction mixture contained 10 µL at 3.2µM of each primer combination (9x9, 18 primers total for 81 unique sample barcodes) which were arrayed in a 96 well plate, 2 µL of template DNA, and 20 µL of GoTaq Hot start colourless MasterMix (Promega, Madison, Wisconsin, USA). The plate was then sealed with a foil cover and placed in a thermocycler (Bio-Techne, Minneapolis), programmed for an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for one minute, 50°C for one minute, 72°C for one minute, and a final extension phase at 72°C for five minutes. To test the concentration and integrity of my amplified DNA product, samples were sent to the London Regional Genomics Centre (London, Ontario), where they checked the concentrations and conducted quality control analysis using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, California). From this, the amplicon

was determined to be approximately 620bp in length, therefore sequencing proceeded with a 600 cycle (paired-end, 2 x 314 base pairs) MiSeq sequencing kit (Illumina Inc., San Diego, California) to sequence the V3-V4 region of the 16S gene with the addition of 5% phi X-174, which is used as a positive control in Illumina sequencing runs.

3.2.6 Bioinformatics and statistical analyses

To test for differences in the hygienic response as a function of treatment over weeks I used a linear-mixed effect model (LMM) in RStudio (v. 4.3.2), using the packages lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), tidyverse (Wickham et al., 2019), rstatix (Kassambara, 2023), and core R packages. As fixed effects, I used treatment and week as well as an interaction term. As a subject variable, I had hive, and collar as a within-subject variable. Assumptions were tested on the residuals after fitting the model to confirm the proper model was fitted. The data visually did not deviate from normality on a Q-Q Plot in R, a density plot showed that the data followed an approximately normal distribution, the variance was visually homogenous, and residuals showed a linear relationship with fitted values, therefore the model seemed to be appropriate. After the LMM, I used paired t-tests to assess the significance of any pairwise comparison between weeks within single treatment groups, as performed in IBM SPSS Statistics (v. 29).

I demultiplexed raw sequence reads from fastq files using Cutadapt (Martin, 2011) and quality-filtered using the DADA2 pipeline in R (Callahan et al., 2016). I assigned taxonomy using the SILVA v138 training set (Quast et al., 2012), then pruned sequence variants (SVs) such that the final dataset used in downstream analyses retained samples with >1000 reads, SVs present at >1% relative abundance in any sample, SVs with >0.01% abundance in every sample, and SVs with >100 total reads across samples (Appendix B). The code was adapted from code available at https://github.com/kait-al/Microbiome_SOPs/tree/main. I used the general linear model framework of the R package MaAslin2 (Microbiome Multivariable Association with Linear Models 2; Mallick et al. 2021) to analyze microbial abundance data. I used Week 1 (baseline) as a reference against which to compare changes in Week 2 and Week 3 according to treatment groups. P-values were subjected to multiple hypothesis testing correction using

the Benjamini-Hochberg method with a false discovery rate of 0.25 (Benjamini and Hochberg, 1995). Supporting R packages used included CoDaSeq (Gloor et al., 2016), compositions (van den Boogaart et al., 2023), zCompositions (Palarea-Albaladejo & Martín-Fernández, 2015), dplyr (Wickham et al., 2023), vegan (Oksanen et al., 2022), and core R packages.

Additionally, I used Aitchison distances, a measure of difference in compositional data defined as the Euclidean distance after centred log ratio (CLR) transformation (Gloor et al. 2017), to perform a principal component analysis (PCA) in R (v. 4.3.2) to look at whether week and treatment showed an obvious difference in the composition of their microbiome samples (beta diversity) according to the variation in the data. I calculated Shannon's Diversity Index in R (v. 4.3.2) using packages phyloseq (McMurdie & Holmes, 2013) and dplyr (Wickham et al., 2023) to look at within-sample diversity, and subsequently compared Shannon's Indices between time points and treatment groups using a LMM in R v 4.3.2 to look at week, treatment, and their interaction effect on diversity. As above, assumptions were tested on the residuals after fitting the model to confirm the proper model was fitted. The data did not deviate from normality on a Q-Q Plot, a density plot showed that the data followed an approximately normal distribution, the variance was visually homogenous, and residuals showed a linear relationship with fitted values, therefore the model seemed appropriate. Code for both linear mixed models is available at https://github.com/SocialBiologyGroupWesternU/Killam-MSc-2024-R-markdown.

3.3 Results

3.3.1 Patty consumption

Nearly all hives consumed their entire patty within the weeklong span it was in the hive (Figure 7). Only a single hive (Hive 1: Treatment Group *Bifidobacterium*) did not consume 100% of the patty in either of the two applications. Five out of the nine hives consumed 100% of the patty for both applications, while eight of the nine hives consumed 100% of the patty in at least one application.

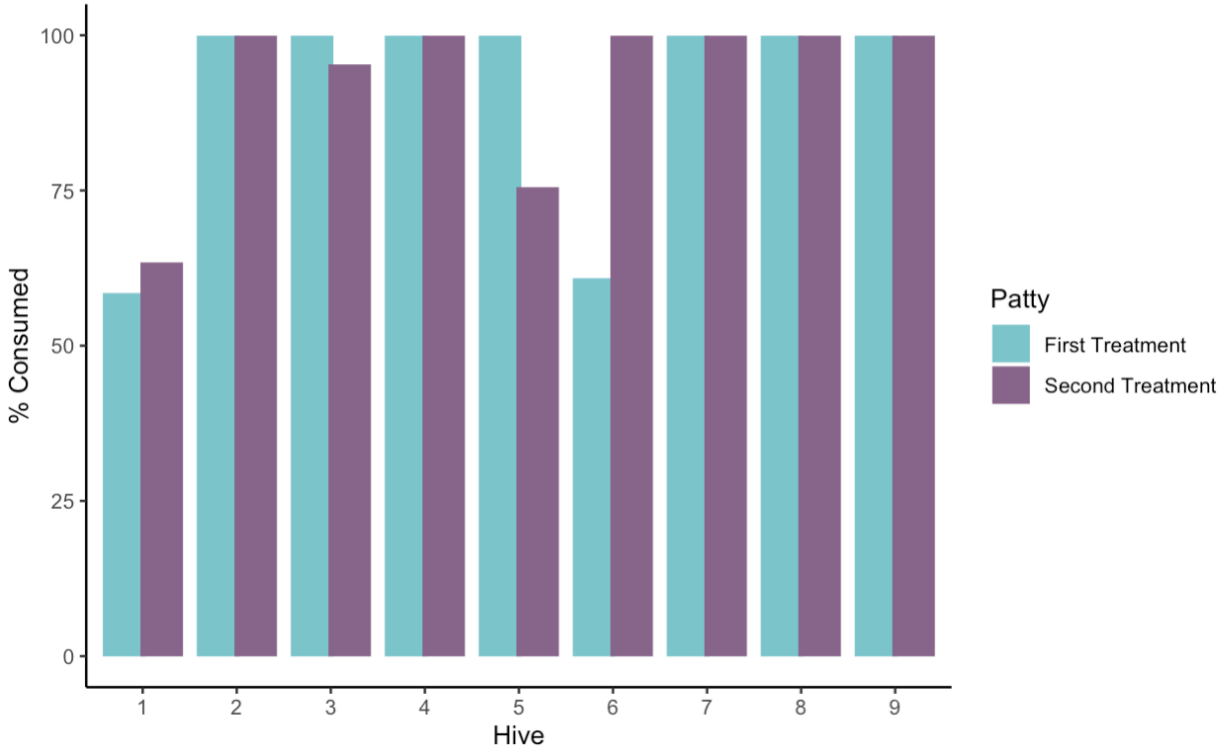

Figure 7. Patty consumption of individual hives.

Patties were applied to each hive twice at 250 grams per patty, and the percent of the patty consumed was calculated as a measure of whether hives were consuming the treatment.

3.3.2 Hygienic behaviour

Hygiene scores varied from 16% to 100%, depending on the colony, and varied between each week (Table 3, Table 4). My starting population was therefore not particularly hygienic. This variation in score persisted across the length of the experiment, with no significant trend up or down over 15 days according to treatment or week (LMM Treatment: $F = 0.0138$, $df_{numerator} = 2$, $df_{denominator} = 15$, $P = 0.9863$; LMM Week: $F = 1.0914$, $df_{numerator} = 2$, $df_{denominator} = 30$, $P = 0.3487$; LMM Treatment:Week interaction: $F = 1.0880$, $df_{numerator} = 4$, $df_{denominator} = 30$, $P = 0.3802$; Table 5; Figure 8). The amount of variance explained in the entire model was 49.2%, while the fixed effects explained 6.4% (conditional R^2: 0.492; marginal R^2: 0.064).

Table 3. Raw hygiene scores across weeks as a proportion out of 1.

I calculated scores from counts of brood cells in ImageJ using the formula (number of cells frozen–number of non-empty cells after the test/(number of cells frozen)).

Hive	Treatment	Week 1 Collar 1	Week 1 Collar 2	Week 2 Collar 1	Week 2 Collar 2	Week 3 Collar 1	Week 3 Collar 2
1	*Bifidobacterium*	0.6145	0.4066	0.6867	0.381	0.3721	0.3659
4	*Bifidobacterium*	0.7123	0.4545	0.5584	0.625	1	0.7925
7	*Bifidobacterium*	0.6216	0.6548	0.8987	0.4839	0.8028	0.7821
3	*Lactiplantibacillus*	0.9254	0.7059	0.8919	0.9846	0.8519	0.7582
6	*Lactiplantibacillus*	0.3208	0.382	0.4588	0.3371	0.6901	0.1948
9	*Lactiplantibacillus*	0.6282	0.5301	0.6471	0.6966	0.4217	0.5542
2	Vehicle	0.9302	0.7826	0.8333	0.7995	0.7011	0.7791
5	Vehicle	0.3174	0.4923	0.7229	0.5814	0.7442	0.8293
8	Vehicle	0.4253	0.6588	0.1935	0.1579	0.5192	0.7966

Table 4. Change in average hygiene scores (averaged between collar 1 and collar 2) as compared to baseline, Week 1.

Red indicates a negative change in score while green indicates a positive change in score.

Hive	Treatment	Week 1 to 2	Week 1 to 3
1	*Bifidobacterium*	0.0233	-0.1416
4	*Bifidobacterium*	0.0083	0.3129
7	*Bifidobacterium*	0.0531	0.1543
3	*Lactiplantibacillus*	0.1226	-0.106
6	*Lactiplantibacillus*	0.0466	0.0911
9	*Lactiplantibacillus*	0.0927	-0.0912
2	Vehicle	-0.04	-0.1163
5	Vehicle	0.2473	0.3819
8	Vehicle	-0.3664	0.1158

Table 5. Results of a linear mixed model in R using fixed effects Treatment (*Bifidobacterium, Lactiplantibacillus,* and Vehicle) and Week (1, 2, and 3), Hive as the subject variable and Collar as the within-subject variable.

The reference levels used for the lmer() function in R were Week 1 and Treatment Vehicle. The coefficient represents the strength and direction of association with the response variable (Hygiene). Smaller t-values are indicative of the similarity between groups, while the confidence interval shows 95% confidence that the population mean for hygiene scores falls between the calculated interval.

Fixed effects	Coefficient	Standard error	t-value	Signifi-cance	Confidence interval 2.5%, 97.5%
Intercept	0.6011	0.0901	6.669	<0.001	[0.436, 0.767]
Bifidobacterium	-0.0237	0.1275	-0.186	0.854	[-0.258, 0.210]
Lactiplantibacillus	-0.0190	0.1275	-0.149	0.882	[-0.253, 0.215]
Week 2	-0.0530	0.0939	-0.565	0.576	[-0.226, 0.119]
Week 3	0.1272	0.0939	1.355	0.186	[-0.045, 0.300]
Bifidobacterium:Week 2	0.0813	0.1327	0.612	0.545	[-0.163, 0.325]
Lactiplantibacillus:Week 2	0.1403	0.1327	1.057	0.299	[-0.104, 0.384]
Bifidobacterium:Week 3	-0.0186	0.1327	-0.140	0.889	[-0.263, 0.225]
Lactiplantibacillus:Week 3	-0.1307	0.1327	-0.985	0.333	[-0.375, 0.113]

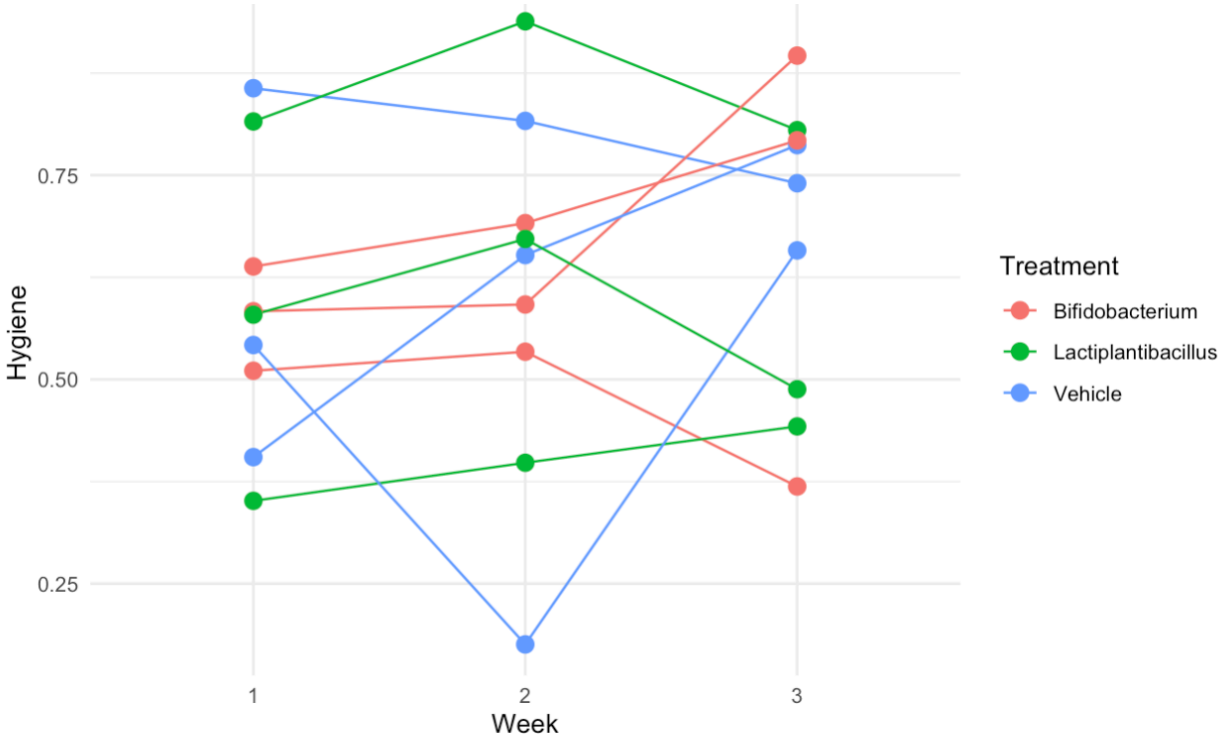

Figure 8. Hygiene was variable in my study population.

This graph shows the relationship between each hive (lines) and each treatment group (colours) across Weeks 1, 2, and 3. Trends in increasing or decreasing hygiene did not appear to be conserved among treatment groups. Hygiene was averaged across the two collars.

Despite the variable scores, single treatments did become marginally more hygienic after one week (Figure 9). Specifically, hives treated with *Lactiplantibacillus* increased their hygienic response following a single round of treatment (paired t-test, t= 4.274, df=2, one-sided $P = 0.025$, Table 4). Likewise, hives treated with *Bifidobacterium* increased their hygienic response slightly after one round of treatment, but this effect was not significant (paired t-test, t= 2.219, df=2, one-sided $P =0.078$, Table 4). In neither case, however, did the effect persist beyond the first week, either between week 2 and week 3 (*Bifidobacterium*: paired t-test, t= 0.613, df=2, one-sided $P =0.301$; *Lactiplantibacillus*: paired t-test, t= -1.352, df=2, one-sided $P =0.155$), or between week 1 and week 3 (*Bifidobacterium*: paired t-test, t= 0.819, df=2, one-sided $P = 0.249$; *Lactiplantibacillus*: paired t-test, t= -0.064, df=2, one-sided $P =0.477$), despite the addition of a second BioPatty. In the vehicle treatment group, as predicted, none of these three direct comparisons showed a significant change in hygiene (week 1 and 2: paired t-test, t= -0.284, df=2, one-sided $P =0.402$; week 2 and 3: paired t-test, t= 1.105, df=2, one-sided p=0.192; week 1 and 3: paired t-test, t= 0.882, df=2, one-sided $P =0.235$).

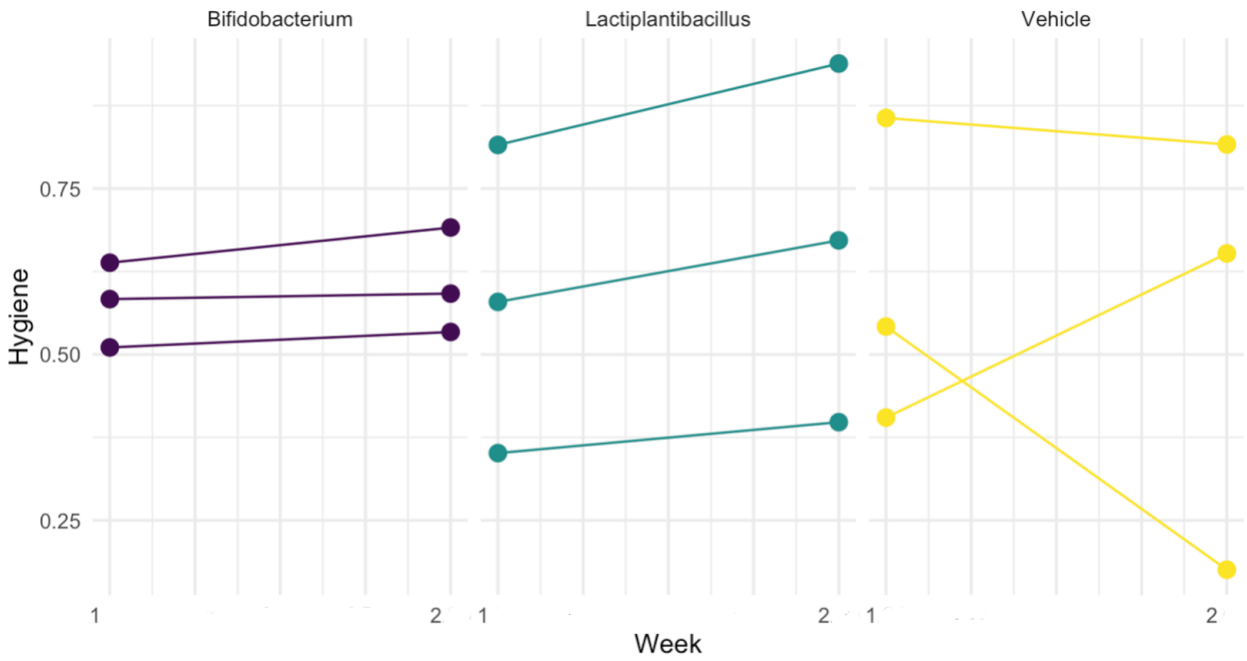

Figure 9. Acute effects of hygiene.

This graph shows the relationship between hives (lines) in each of the three treatment groups between their baseline scores and their scores after a single treatment. We see a slight increase in both probiotic-treated groups, but this effect did not yield overall significance in the linear-mixed model.

3.3.3 16S rRNA Sequencing

Preliminary results from a principal component analysis (PCA) suggested that neither the week nor treatment group appeared to greatly impact the composition of the microbiota, as there was little separation on the plot (Figure 10). Additionally, principal components 1 and 2 accounted for just 14.1% of the variation in the data.

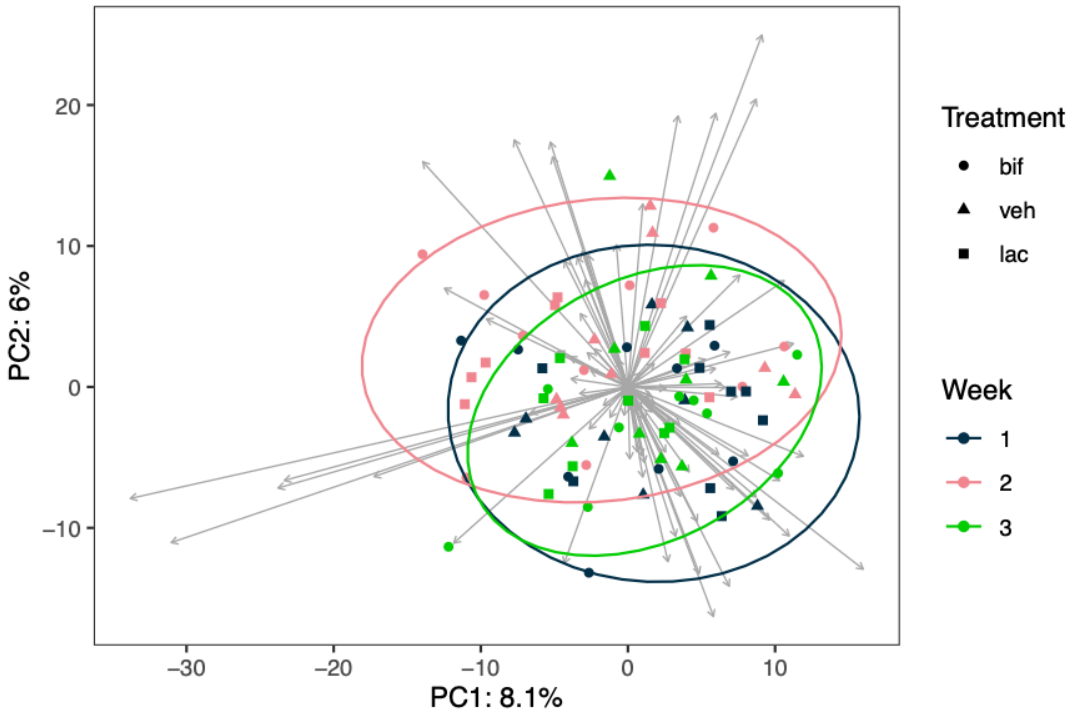

Figure 10. Comparison of the microbiota of nurse bees.

A principal component analysis (PCA) of gut microbiota samples from nurse bees using CLR-transformed Aitchison distances for beta diversity. Distances between the points account for differences in the compositions of the sample microbiota. About 14% of the variance in gut microbe composition is explained by principal components 1 and 2. Week is represented by the colour of the data points, with the ellipses representing the 95% confidence interval for sample weeks. The shape of the data points corresponds to the treatment group, where bif, veh, and lac stand for *Bifidobacterium*, Vehicle Control, and *Lactiplantibacillus*, respectively. Grey arrows represent the strength of association between each sequence variant identified in the gut. Axes are the first and second principal components accounting for variation in the data. Overall, there is little separation between weeks or treatments, suggesting very little variation within the data that could correspond to treatment.

Measures of alpha diversity using Shannon's Index did not differ by treatment, week, or treatment:week (LMM Treatment: $F = 1.8401$, $df_{numerator} = 2$, $df_{denominator} = 24$, $P = 0.1804$; LMM Week: $F = 1.4867$, $df_{numerator} = 2$, $df_{denominator} = 48$, $P = 0.2364$; LMM Treatment:Week: $F = 2.0063$, $df_{numerator} = 4$, $df_{denominator} = 48$, $P = 0.1087$; Table 6; Figure 11). The amount of variance explained in the entire model was 29.4%, while the fixed effects explained 15.1% (conditional R^2: 0.294; marginal R^2: 0.151). My molecular taxonomic analysis of the honey bee worker gut microbiota confirmed the presence of core gut community members such as *Lactobacillus, Bombilactobacillus*, *Bifidobacterium, Gilliamella,* and *Snodgrassella*. However, the relative abundance of the ten most abundant genera did not vary with treatment (Figure 12).

Table 6. Results of a linear mixed model for Shannon's Index.

Fixed effects used were Treatment (*Bifidobacterium, Lactiplantibacillus,* and Vehicle) and Week (1, 2, and 3). The subject factor was Hive, and the within-subject factor was Replicates. The reference levels used for the lmer() function in R were Week 1 and Treatment Vehicle. The coefficient represents the strength and direction of association with the response variable (Shannon's Index of alpha diversity). Smaller t-values are indicative of the similarity between groups, while the confidence interval shows 95% confidence that the population mean for Shannon's index falls between the calculated interval.

Fixed effects	Co-efficient	Standard error	t-value	Signifi-cance	Confidence interval 2.5%, 97.5%
Intercept	3.443	0.079	43.749	<0.001	[3.296, 3.590]
Bifidobacterium	-0.091	0.108	-0.841	0.403	[-0.293, 0.112]
Lactiplantibacillus	-0.106	0.108	-0.977	0.332	[-0.308, 0.097]
Week 2	0.008	0.099	0.085	0.933	[-0.179, 0.195]
Week 3	-0.004	0.099	-0.036	0.972	[-0.191, 0.183]
Bifidobacterium:Week 2	0.078	0.138	0.565	0.575	[-0.181, 0.338]
Lactiplantibacillus:Week 2	0.030	0.138	0.220	0.827	[-0.229, 0.291]
Bifidobacterium:Week 3	-0.211	0.138	-1.534	0.132	[-0.471, 0.048]
Lactiplantibacillus:Week 3	0.070	0.138	0.506	0.615	[-0.189, 0.330]

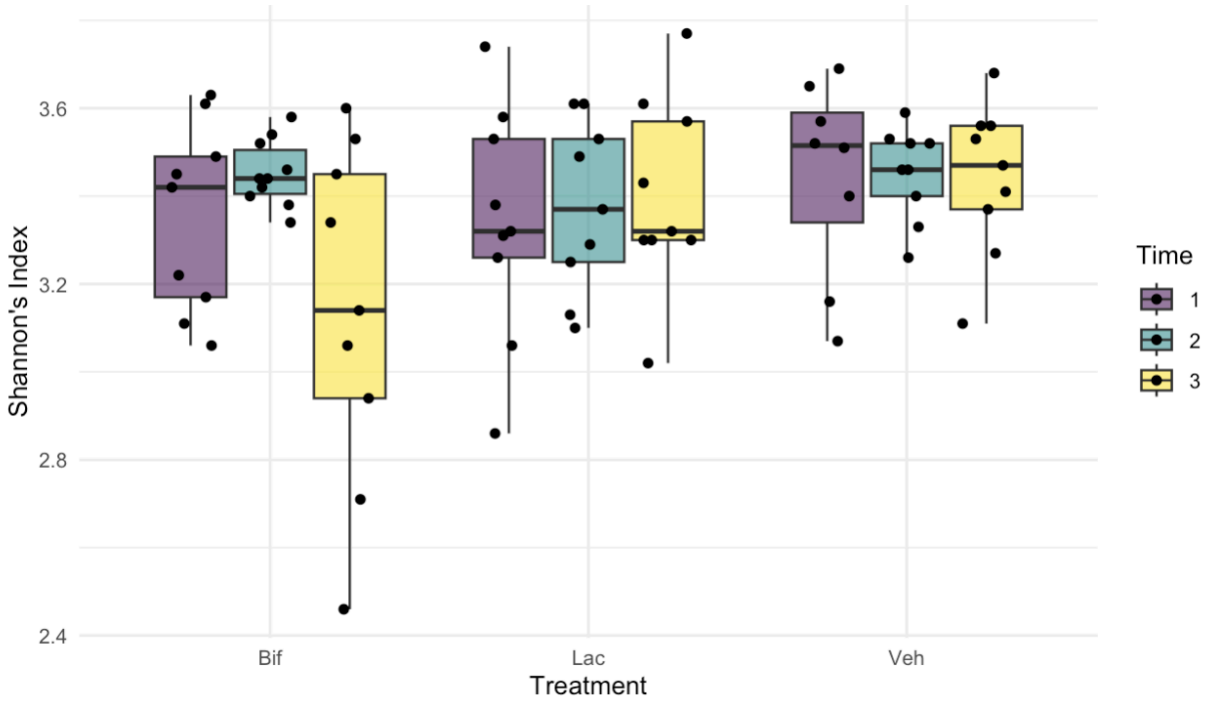

Figure 11. Alpha diversity of gut samples of nurse bees.

Shannon's Index of alpha diversity was compared between time points within treatment groups using a linear mixed model assessing Treatment, Week, and their interaction. Treatments are grouped by time points along the X axis: *Bifidobacterium* (Bif), *Lactiplantibacillus* (Lac), and Vehicle control (Veh).

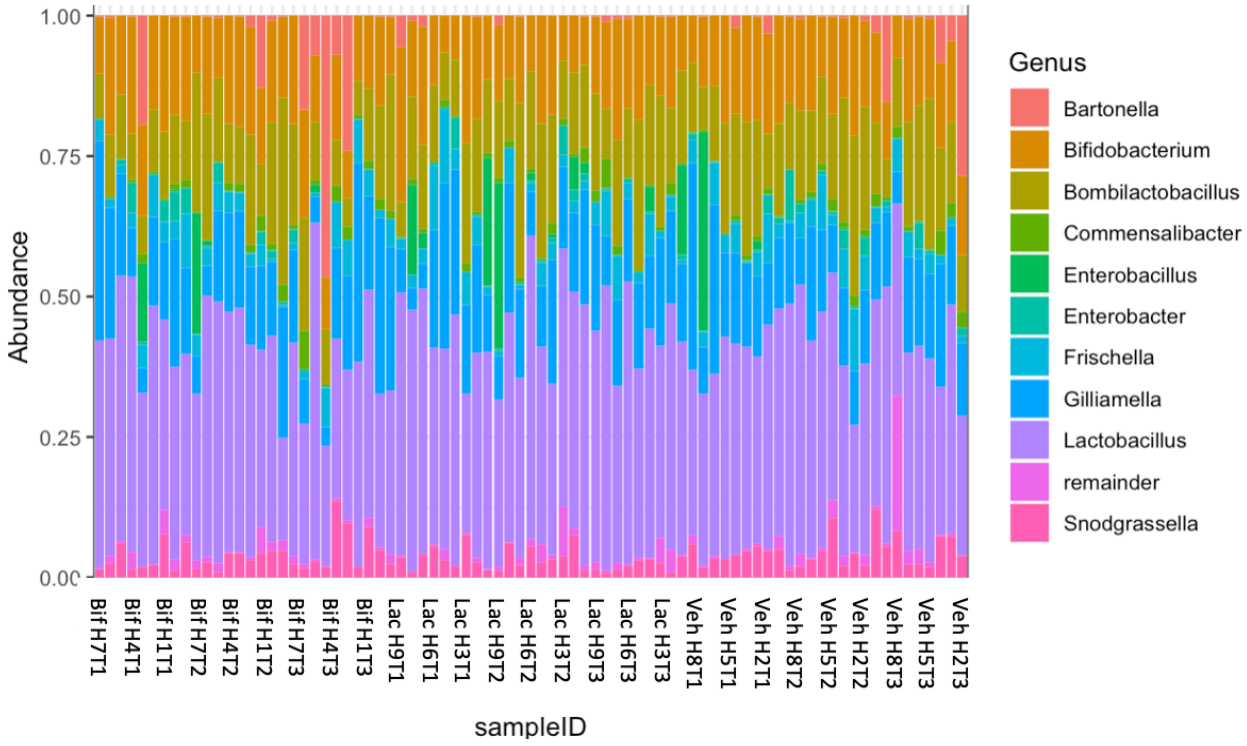

Figure 12. Relative abundance of bacterial genera from honey bee guts.
This barplot shows the proportions of the ten most abundant genera of my samples, with the remaining members of the microbiota combined into a remainder. Each column represents an individual sample, with three samples from each hive, however, the legend at the bottom gives just the hive and timepoint of its three replicates for visual simplicity. Samples are grouped according to treatment (*Bifidobacterium asteroides, Lactiplantibacillus plantarum*, or vehicle control; three colonies each shown at three time points: Week 1, Week 2, and Week 3, but show no obvious groupings attributable to treatment.

Bombilactobacillus was found in increased quantity in Week 3 (MaAslin2, *P*=0.0005, Figure 13 panel A) and Week 2 (MaAslin2, *P* =0.0031; Figure 13 panel B), while *Lactobacillus* spp. levels were found to be significantly increased in the *Bifidobacterium*-treated hives (MaAslin2, *P* =0.0011, *P* =0028; Figure 13 panels C and D), and *Commensalibacter* was increased during Week 3 (MaAslin2, *P* =0.0040, Figure 13 panel E). Conversely, *Gilliamella* levels were significantly decreased during Week 2 (MaAslin2, *P* =0.0040; Figure 13 panel D). More information on these associations is found in Table 6.

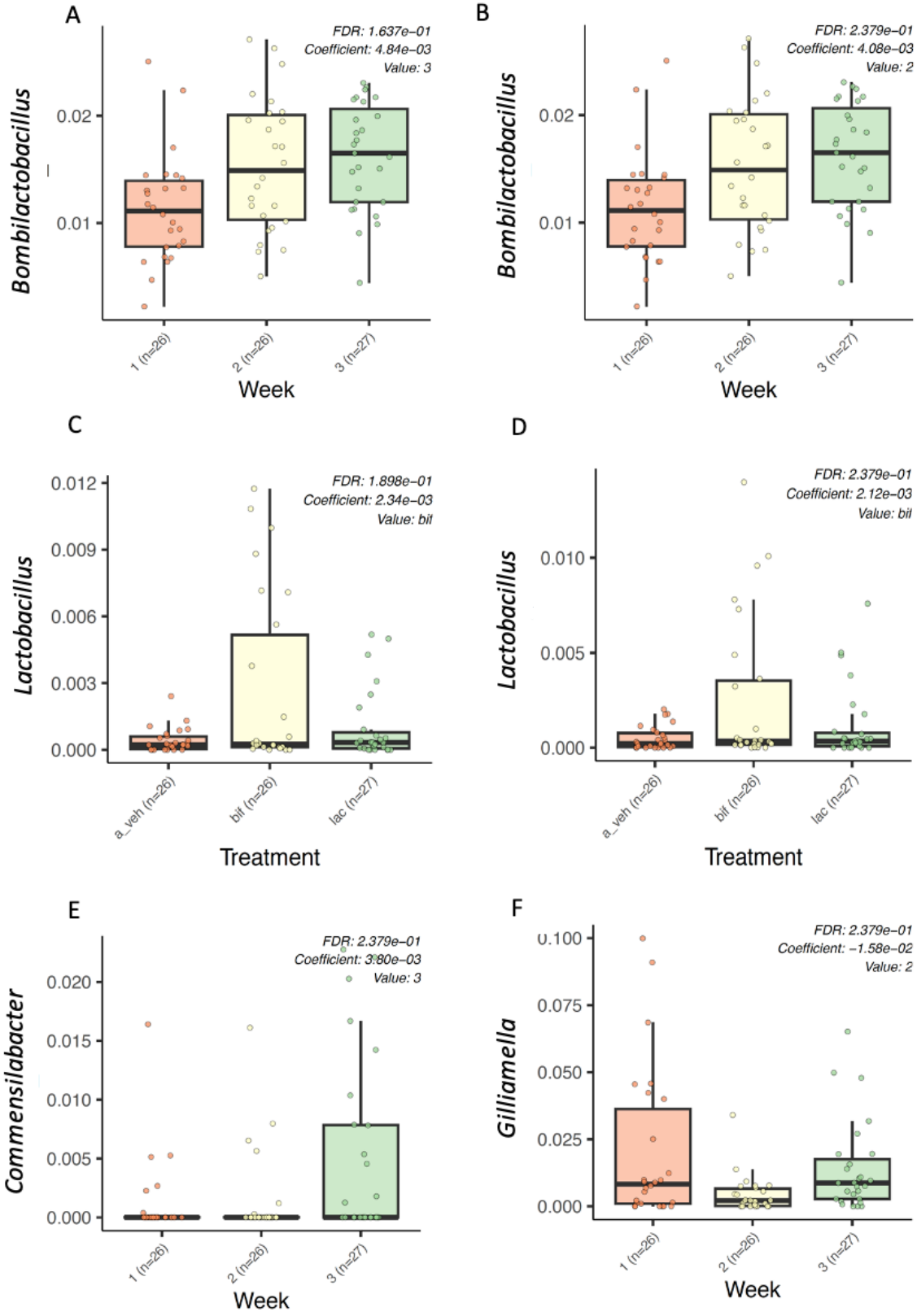
A
FDR: 1.637e−01
Coefficient: 4.84e−03
Value: 3
Bombilactobacillus
0.02
0.01
1 (n=26)
2 (n=26)
3 (n=27)
Week
B
FDR: 2.379e−01
Coefficient: 4.08e−03
Value: 2
Bombilactobacillus
0.02
0.01
1 (n=26)
2 (n=26)
3 (n=27)
Week
C
FDR: 1.898e−01
Coefficient: 2.34e−03
Value: bif
Lactobacillus
0.012
0.009
0.006
0.003
0.000
a_veh (n=26)
bif (n=26)
lac (n=27)
Treatment
D
FDR: 2.379e−01
Coefficient: 2.12e−03
Value: bif
Lactobacillus
0.010
0.005
0.000
a_veh (n=26)
bif (n=26)
lac (n=27)
Treatment
E
FDR: 2.379e−01
Coefficient: 3.80e−03
Value: 3
Commensilabacter
0.020
0.015
0.010
0.005
0.000
1 (n=26)
2 (n=26)
3 (n=27)
Week
F
FDR: 2.379e−01
Coefficient: −1.58e−02
Value: 2
Gilliamella
0.100
0.075
0.050
0.025
0.000
1 (n=26)
2 (n=26)
3 (n=27)
Week

Figure 13. Genera found in increased or decreased abundance in the guts of nurse bees.

These graphs were generated from MaAslin2 'significant results' output, and show each significant value of Treatment or Week, where appropriate. The coefficient is the effect size of the model, while FDR is the false discovery rate with Benjamini-Hochberg correction. A) There was a significant increase in levels of *Bombilactobacillus* at week 3 B) *Bombilactobacillus* was found in greater quantity in week 2 C) *Lactobacillus* spp. was significantly increased in the *Bifidobacterium* treatment group D) *Lactobacillus* spp. was significantly increased in the *Bifidobacterium* treatment group E) *Commensalibacter* was significantly higher during week 3 F) *Gilliamella* was decreased during week 2.

Table 7. Significant associations with specific factors and the microbiota.

This table is adapted from the MaAslin2 "significant results" output and shows which genera were found in significant association with which variables and the level of that variable. The coefficient is the model effect size, while the Q-value is the corrected P-value using the Benjamini-Hochberg procedure with FDR<0.25.

Genera	Variable	Coefficient	Standard Error	P-value	Q-value
Bombilactobacillus	Week 3	0.0048	0.0013	0.0005	0.1637
Lactobacillus spp.	*Bifidobacterium*	0.0023	0.0007	0.0011	0.1898
Bombilactobacillus	Week 2	0.0041	0.0013	0.0031	0.2379
Gilliamella	Week 2	-0.0158	0.0052	0.0036	0.2379
Commensalibacter	Week 3	0.0038	0.0013	0.0040	0.2379
Lactobacillus spp.	*Bifidobacterium*	0.0021	0.0007	0.0028	0.2379

3.4 Discussion

In this thesis, I used the insect gut-brain axis framework (Liberti and Engel, 2020) to study whether deliberately chosen strains of probiotic bacteria could influence the hygienic behaviour of living colonies of honey bees. I supplemented nine colonies of Western honey bees (*Apis mellifera*) with either *Bifidobacterium asteroides* or *Lactiplantibacillus plantarum* and tested for any changes to baseline hygiene ability. Hygienic behaviour is an important mechanism for disease resistance in a highly social insect that lives in close quarters with kin (Cremer et al., 2007), and as such, is also an economically valuable trait in commercial beekeeping (Erez et al., 2022). My findings yielded several important results. First, I observed that edible 'BioPatty' is a useful practical vehicle for the delivery of regulated doses of bacterial strains to hives – namely, bees readily consume the medium. This general result is consistent with previous work from my research group that used the BioPatty (Daisley et al., 2020a, 2023) but this is the first demonstration using *B. asteroides* or *L. plantarum* at all or on their own.

Second, my observations of hygienic behaviour revealed that hygiene is potentially affected by gut-brain axis manipulations, with a single application of probiotics being associated with the most promising acute effect on hygiene, but this effect is not statistically significant in a linear-mixed model analysis and does not persist upon a second dose of bacteria. Finally, from 16S ribosomal RNA sequencing analysis, I found that neither *B. asteroides* nor *L. plantarum* colonized the gut as a nutritional supplement, although both were found in the gut in standard abundances, but there were small changes in the overall gut microbial composition according to both treatment group and experimental week. It is not yet clear if the acute, albeit non-significant, changes in hygiene I observed are functionally associated with these relatively minor changes that I observed in gut microbiome diversity, but that unknown is now the focus of future work. I also confirmed the presence of all core species of bacteria ubiquitously found in adult honey bees (Kwong and Moran, 2016), confirming both the health of the hives and the validity of the 16S results. Overall, my results are novel and preliminary, but form the basis for a larger field study to confirm the small changes in hygiene behaviour and gut microbiota that I found.

3.4.1 Hygiene may have been acutely affected but this effect was not sustained

Hygiene scores are expected to vary widely and naturally by hive (Spivak, 1996), and scores greater than 95% are generally considered properly hygienic by commercial beekeeping standards (Spivak & Downey, 1998). Only two collars met this criterion: one from the *B. asteroides* group, and one from the *L. plantarum* group (Table 3). After a single treatment, hives in the two probiotic treatment groups saw increases in hygiene scores that were relatively consistent, while after two treatments both groups saw much greater increases in variation both positively and negatively (Table 4). High variation was seen throughout the vehicle control treatment group, showing that Hive (i.e. genotype) remains an important factor that likely influences hygiene (Spivak and Reuter, 1998; Uzunov et al., 2014), especially in my colonies in the absence of bacterial treatment. Other factors not directly under my control may likewise have contributed to the relatively high level of behavioural variance associated with the untreated group.

My results indicate that pollen patties may potentially increase variation in colony hygiene. That is, in the absence of bacterial supplements, bees were most variable in their expression of hygiene (Figure 8). When bacteria were mixed into the patties in live culture to create BioPatties, however, the variation in hygiene dampened slightly and steady patterns were seen such that, after a single treatment, there was a subtle but insignificant increase in hygienic scores (Figure 9). This pattern was similar between my two treatment groups despite different baselines, which thus both contrast with the variable no-treatment (vehicle) control. The trend in hygiene scores in the two treatment groups is not maintained after a second application of probiotics (Figure 8; Table 4), suggesting that there is a trade-off between the potential beneficial effects of the bacteria and potential disruption to the microbiota by repeated application of pollen substitutes.

Pollen patties are commonly used as a supplemental food source in beekeeping, often being fed to colonies several times a season. Although applied in good faith, it is becoming better understood that protein supplements in the form of pollen patties can have detrimental effects on colonies. For example, colonies given pollen patty vehicles

showed an unexpected increase in the pathogen load of *Paenibacillus larvae,* the causative agent of American foulbrood disease (Daisley et al., 2020a), and increased levels of black queen cell virus compared to bees with natural forage (DeGrandi-Hoffman et al., 2016). My preliminary results suggest a potential additional side-effect of pollen patties: an increase in sporadic expression of their natural hygienic ability, but this effect needs to be confirmed in larger field studies.

I suggest that one possible mechanism for greater pathogen loads upon substitute pollen feeding is that patties may somehow disrupt the natural social behaviours of the colony, including hygiene, possibly via dysbiosis of the natural gut microbiota. This returns to the idea of social amplification, that the gut-brain axis can confer both individual and colony-level effects (Figure 1). My study suggests that perturbations to the hive's social behaviour due to pollen substitutes could be somewhat dampened by the addition of probiotics. In their study, Daisley et al. (2020a) found that while pollen patties alone increased *P. larvae* load in hives, delivering strains of lactobacilli in the pollen patties decreased pathogen load and increased survival. The realization of this potential mechanism is one of the most biologically significant results of my thesis and could potentially be of widespread interest to the beekeeping community with further testing and validation.

Despite a relatively consistent increase in hygiene after a single BioPatty treatment, a second application increased hygienic variability to a degree similar to the pollen patty alone. This suggests a trade-off between the beneficial effects of *B. asteroides* and *L. plantarum* and the potentially detrimental effects of the pollen patty vehicles: a single application maintains the positive hygienic effects of the probiotics but subsequent applications reveal the negative effects on hygiene from the pollen patties. Therefore, if I had to make a general, preliminary recommendation regarding the application, it would be to supply only patties infused with probiotics and do so only once.

The Ontario Beekeeper's Association (OBA)'s recommendation is to, in the spring, patty-feed any colonies that are to be used for queen-rearing or commercial pollination, as well as those that will be split to make new colonies (Ontario Beekeeper's Association,

n.d.). However, they do not provide information on the number of patties to be used. In the Fall, the OBA does not recommend pollen patty feeding. In contrast to the guide from the OBA, the Canadian Association of Professional Apiculturists (CAPA) recommends supplementing all hives with pollen patties in the spring and continuing to feed until the first nectar flow; however, they are careful to point out that in certain areas, such as Ontario, this may not be necessary and could even be detrimental (Eccles et al., 2016). All in all, it seems that recommendations regarding pollen patty feeding are conservative, especially with regard to Ontario, but my research may help to round out such recommendations so that pollen patties can be used for supplemental feeding when necessary without the risk of detrimental effects such as disruption to the gut.

Currently, there are probiotics on the market for honey bees, such as Super DFM-Honey™, Fat Bee, and SCD Probiotics, which are supplied as a powder or liquid, but the effectiveness of these products is mostly unknown and even disputed. For example, Damico et al. (2023) tested Super DFM-Honey Bee™'s claim that it can replenish the microbiota of bees that has been lost to agricultural practices and environmental conditions. They found that, after routine treatment with the antibiotic oxytetracycline, the microbiota of bees given Super DFM was not anymore like that of control hives than was the microbiota of bees treated with antibiotics but not given Super DFM, suggesting no effect of the probiotic. Additionally, bees treated with Super DFM actually had fewer bacteria in their gut after treatment (Damico et al., 2023). This finding is in line with other studies that have found no impact on the microbiota of commercially sold probiotics (Anderson et al., 2024; Motta et al., 2022). Chmiel et al. (2021) review the potential for such probiotic treatments and highlight the need for reporting the exact strains and doses contained in the products, rigorous testing in the lab and field to quantify effects using larger sample sizes, published reports of any stated or implied effects, and clear instructions about mode and timing of delivery. The results of my study also suggest that further studies need to be done on larger sample sizes to account for variation among hives and environmental sources of variation.

Of the two probiotics used in this study, *B. asteroides* and *L. plantarum,* only *B. asteroides* is a core species in the honey bee gut (Bottacini et al., 2012). Contrary to my

initial prediction, hygiene scores increased more in the *L. plantarum*-treated hives than in the *B. asteroides*-treated ones. This finding goes against the standard assumption that abundant native gut community members have superior probiotic effects to less established strains (Damico et al., 2023). Core strains are thought to have coevolved for a specific purpose or property expressed within their host (Koskella and Bergelson, 2020), which gives rise to the idea that their supplementation to above-normal levels, as attempted here, may have probiotic effects. However, strains chosen for bacterial supplementation of hives need not be core species to be effective (Chmiel et al., 2021). For example, *L. plantarum* is widespread in the environment and can be found in plants and honey bee guts, and when supplied to bee colonies in a prescribed manner, this species is thought to have antibacterial and antifungal properties (Daisley et al., 2020a; Iorizzo et al., 2021; Nordström et al., 2021). The effects of less abundant strains may therefore even surpass the benefits of the core bacteria under certain conditions. The choice and utility of strains for probiotic effect is an area of active research (Anderson et al., 2024; Motta et al., 2022).

The results of my linear mixed model found that the amount of variance explained by the random effects in the model was 49.2% (Table 5). In this case, the random effect was hive nested with collar. The fixed effects, week and treatment, accounted for 6.4% of the variance in the data. This suggests that lactic acid-producing bacteria (treatment) and season (week) can have marginal effects on hygiene, but the biggest factor remains the genetics of the individuals in the colony. This is in accordance with other studies that have looked at the heritability of hygienic behaviour (Boutin et al., 2015; Masterman et al., 2000; Rothenbuhler, 1964). Although the benefits of selective breeding are undeniable (Erez et al., 2022), more work needs to be done on the possibility of boosting hygiene more quickly than breeding alone.

3.4.2 Treatment did not persist in the gut

Despite supplementing the hives with live cultures of *Bifidobacterium asteroides* and *Lactiplantibacillus plantarum,* I did not see any changes in composition specific to these two strains (Figure 10). Additionally, principal components 1 and 2 in my principal

component analysis accounted for very little variance in the data relative to similar studies (Daisley et al., 2020a; Kwong et al., 2017). Hives were supplemented at a concentration of 1x10^9 colony-forming units per gram, which is in line with previous work by Daisley et al. (2020a), in which the three supplemented strains of lactobacilli were found in greater abundance after treatment. One explanation for this is that the targeted nurse bees did not feed on the patty, although nurses typically feed the most on pollen compared to other task groups (Crailsheim et al., 1992), as the foragers continue to search for food outside the hive. Due to the sharing of resources within the hive (Boomsma and Gawne, 2018), it was reasonable to presume that most nurse bees would encounter the patty or the bacteria indirectly during the treatment; however, this was not guaranteed. Additionally, while honey bees do not store pollen patties in cells as they do natural pollen (Oliver, 2021), they may not have consumed it before the bacteria died, as little information exists on bacterial survivability in patties.

Another potential explanation is that the nurses did interact with the patty and receive the benefits of the probiotics, but these strains simply passed through the gut before sampling and sequencing occurred, thus conferring transient, short-lived benefits. This explanation is supported by not only the acute effects I observed on hygienic behaviour but also the fine-scale changes to the microbiota discovered via 16S rRNA gene sequencing, as the application of pollen patties is sometimes thought to disrupt the gut (Daisley et al., 2020a; DeGrandi-Hoffman et al., 2016). The 16S rRNA gene sequencing is validated through my discovery of all expected community members (Figure 12): *Lactobacillus* spp., *Bombilactobacillus* spp., *Bifidobacterium asteroides, Snodgrassella alvi,* and *Gilliamella apicola,* which represent the core community ubiquitous to all adult workers (Kwong and Moran, 2016), as well as other members that are commonly found in smaller abundance such as *Frischella perrara, Bartonella apis, Bombella apis,* and *Commensalibacter* sp. (Smutin et al., 2022).

Fine-scale changes observed in the composition of the gut included significant increases in *Bombilactobacillus* and *Commensalibacter* in Week 3 (Figure 13; Table 7). Additionally, I saw significant increases in the abundance of *Lactobacillus* spp. in *Bifidobacterium* treated hives and noted an increase in *Bombilactobacillus* and a decrease

in *Gilliamella* abundance in Week 2 (Figure 13; Table 7). The shifts in microbiota corresponding to weeks are likely attributable to changes in foraging during the experiment, such as repeated application of pollen substitutes in lieu of natural foraging, as well as changes in available natural forage during the experimental weeks. The increase in species of *Lactobacillus* in the *Bifidobacterium* treatment group suggests the possibility of strain competition within the gut (Kern et al., 2021). The native strain of *B. asteroides* to these honeybees' gut may have differed from the strain provided via the pollen patties, which created competition for resources within the host that may have lowered the abundance of both strains, allowing *Lactobacillus* to increase in abundance. As both *Bifidobacterium* and *Lactobacillus* reside primarily in the distal end of the hindgut (Kwong and Moran, 2016), I would expect them to occupy different niches that allow for coexistence (Kern et al., 2021), thus providing evidence that competition may have occurred within *Bifidobacterium* strains and had downstream effects.

In my sequencing analysis, one potential source of variance is differences in 16S ribosomal RNA gene copy number. However, overall, the copy number for the 16S gene does not vary widely in the common honey bee symbionts, ranging only between 2 and 5 copy numbers (Stoddard et al., 2015). Additionally, the accuracy of calculating and accounting for 16S copy numbers is debated, with some studies recommending against it (Louca et al., 2018). Other studies have found that for beta diversity metrics, such as Aitchison distance used in this thesis and generated principal component analyses, effect sizes and therefore bias are small (Gao and Wu, 2023), and thus copy number correction provides limited benefits.

I also analyzed Shannon's Index of alpha diversity in both treatment groups and weeks, but my linear mixed model did not show significant effects for either of these two variables (Table 6). Although we do see differences in the variation of indices in the samples, there is no visible trend (Figure 11). This is in accordance with another study that found no difference in alpha diversity between probiotic-treated groups and vehicle control groups, although differences in alpha diversity were seen following antibiotic treatments (Daisley et al., 2020b).

Overall, our treatment seems to have introduced some small changes in the gut, but this effect was not consistent with our applied treatment. Despite this, one thing to note is that all changes in the microbiota corresponded to species that are commonly present in the microbiota naturally (reviewed in Smutin et al., 2022). This could suggest that if the hives were actively in a state of dysbiosis due to a pathogen or parasite, BioPatties could help replenish the natural microbiota in addition to decreasing pathogen load as other studies have found (Daisley et al., 2020a). This provides evidence for probiotic-infused patties as not only a mechanism to support hygiene and other social behaviours but to support the overall health of hives particularly as it may relate to the natural gut composition.

3.4.3 Future directions and limitations

This study represents a preliminary field trial which is intended to give rise to a larger body of research on the impacts of probiotics on hygienic behaviour. The results that I have shown here are promising for their acute effects on hygiene, yet this study was limited in the number of hives that I was able to use in the summer of 2023. The primary recommendation from this research going forward is thus to repeat the experiment with a greater number of colonies. Other studies have performed hygienic experiments with n=19 to 72 hives (Bigio et al., 2013; Spivak and Reuter, 1998), yet these experiments looked only at hygiene, and environmental factors that could affect it rather than the internal state of the bees' microbiome and did not follow up with subsequent lab assays. The field of probiotics for honey bees is growing, and a particular emphasis has been placed on the importance of field studies for these beneficial microbes (Chmiel et al., 2021).

Other options for future studies include looking at neurotransmitter concentrations in the brain via RNA sequencing (Naeger and Robinson, 2016), quantitative real-time PCR (Powell et al., 2014), or liquid chromatography-mass spectrometry (Zhang et al., 2022), as presented in Chapter Two. Researchers could also use histochemical staining to look at the distribution of key neurotransmitters in the bees' brains (Bicker, 1999). This would allow for a complete look at the gut-brain axis, not just using behaviour as a proxy to

assess the brain but to physically confirm potential treatment effects from neurotransmitter titers. Additionally, these techniques could be used to assess a range of social behaviours, from hygiene to foraging (Chatterjee et al., 2021), defense (Hunt, 2007), and recruitment (Linn et al., 2020).

Another limitation of this work was the number of bacterial strains that it was feasible to test. I used previous studies as a basis on which to select my strains (Cui et al., 2020; Daisley et al., 2020a; Vásquez et al., 2012); however, other strains of bacteria could have similar probiotic effects in hives. Therefore going forward it may also be advisable to test other strains in addition to re-testing *Lactiplantibacillus plantarum* and *Bifidobacterium asteroides* with a larger sample size. One strain of interest is *Apilactibacillus kunkeei,* which is a strain that is native to the honey bee crop (Tamarit et al., 2015), and is thought to have beneficial effects on bees (Arredondo et al., 2018). Lastly, attempting to quantify ideal dosages and then standardize dosage, timing, and duration across probiotic studies would be an important step in creating scientifically sound probiotic treatments with applications for beekeeping (Alberoni et al., 2016).

3.5 References

Alberoni, D., Gaggìa, F., Baffoni, L., & Di Gioia, D. (2016). Beneficial microorganisms for honey bees: Problems and progresses. *Applied Microbiology and Biotechnology*, *100*(22), 9469–9482. https://doi.org/10.1007/s00253-016-7870-4

Anderson, K. E., Allen, N. O., Copeland, D. C., Kortenkamp, O. L., Erickson, R., Mott, B. M., & Oliver, R. (2024). A longitudinal field study of commercial honey bees shows that non-native probiotics do not rescue antibiotic treatment, and are generally not beneficial. *Scientific Reports*, *14*(1954). https://doi.org/10.1038/s41598-024-52118-z

Arredondo, D., Castelli, L., Porrini, M. P., Garrido, P. M., Eguaras, M. J., Zunino, P., & Antúnez, K. (2018). *Lactobacillus kunkeei* strains decreased the infection by honey bee pathogens *Paenibacillus larvae* and *Nosema ceranae*. *Beneficial Microbes*, *9*(2), 279–290. https://doi.org/10.3920/BM2017.0075

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bicker, G. (1999). Histochemistry of classical neurotransmitters in antennal lobes and mushroom bodies of the honeybee. *Microscopy Research and Technique*, *45*(3), 174–183. https://doi.org/10.1002/(SICI)1097-0029(19990501)45:3<174::AID-JEMT5>3.0.CO;2-U

Bigio, G., Schürch, R., & Ratnieks, F. L. W. (2013). Hygienic behavior in honey bees (Hymenoptera: Apidae): Effects of brood, food, and time of the year. *Journal of Economic Entomology*, *106*(6), 2280–2285. https://doi.org/10.1603/EC13076

Boomsma, J. J., & Gawne, R. (2018). Superorganismality and caste differentiation as points of no return: How the major evolutionary transitions were lost in translation. *Biological Reviews of the Cambridge Philosophical Society*, *93*(1), 28–54. https://doi.org/10.1111/brv.12330

Borges, D., Guzman-Novoa, E., & Goodwin, P. H. (2021). Effects of prebiotics and probiotics on honey bees (*Apis mellifera*) infected with the microsporidian parasite *Nosema ceranae*. *Microorganisms*, *9*(3). https://doi.org/10.3390/microorganisms9030481

Bottacini, F., Milani, C., Turroni, F., Sánchez, B., Foroni, E., Duranti, S., Serafini, F., Viappiani, A., Strati, F., Ferrarini, A., Delledonne, M., Henrissat, B., Coutinho, P., Fitzgerald, G. F., Margolles, A., Sinderen, D. van, & Ventura, M. (2012). *Bifidobacterium asteroides* PRL2011 genome analysis reveals clues for colonization of the insect gut. *PLoS ONE*, *7*(9), e44229. https://doi.org/10.1371/journal.pone.0044229

Boutin, S., Alburaki, M., Mercier, P.-L., Giovenazzo, P., & Derome, N. (2015). Differential gene expression between hygienic and non-hygienic honeybee (*Apis mellifera* L.) hives. *BMC Genomics*, *16*(500). https://doi.org/10.1186/s12864-015-1714-y

Bulson, L., Becher, M. A., McKinley, T. J., & Wilfert, L. (2021). Long-term effects of antibiotic treatments on honeybee colony fitness: A modelling approach. *The Journal of Applied Ecology*, *58*(1), 70–79. https://doi.org/10.1111/1365-2664.13786

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Chatterjee, A., Bais, D., Brockmann, A., & Ramesh, D. (2021). Search behavior of individual foragers involves neurotransmitter systems characteristic for social scouting. *Frontiers in Insect Science*, *1*. https://www.frontiersin.org/articles/10.3389/finsc.2021.664978

Chmiel, J. A., Daisley, B. A., Pitek, A. P., Thompson, G. J., & Reid, G. (2020). Understanding the effects of sublethal pesticide exposure on honey bees: A role for probiotics as mediators of environmental stress. *Frontiers in Ecology and Evolution*, *8*. https://doi.org/10.3389/fevo.2020.00022

Chmiel, J. A., Pitek, A. P., Burton, J. P., Thompson, G. J., & Reid, G. (2021). Meta-analysis on the effect of bacterial interventions on honey bee productivity and the treatment of infection. *Apidologie*, *52*(5), 960–972. https://doi.org/10.1007/s13592-021-00879-1

Crailsheim, K., Schneider, L. H. W., Hrassnigg, N., Bühlmann, G., Brosch, U., Gmeinbauer, R., & Schöffmann, B. (1992). Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): Dependence on individual age and function. *Journal of Insect Physiology*, *38*, 409–419. https://doi.org/10.1016/0022-1910(92)90117-V

Cremer, S., Armitage, S. A. O., & Schmid-Hempel, P. (2007). Social Immunity. *Current Biology*, *17*(16), 693–702. https://doi.org/10.1016/j.cub.2007.06.008

Cui, Y., Miao, K., Niyaphorn, S., & Qu, X. (2020). Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. *International Journal of Molecular Sciences*, *21*(3). https://doi.org/10.3390/ijms21030995

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Al, K. F., Chernyshova, A. M., Faragalla, K. M., Burton, J. P., Thompson, G. J., & Reid, G. (2020a). Novel probiotic approach to counter *Paenibacillus larvae* infection in honey bees. *The ISME Journal*, *14*(2), 476–491. https://doi.org/10.1038/s41396-019-0541-6

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Gibbons, S., Chernyshova, A. M., Al, K. F., Faragalla, K. M., Burton, J. P., Thompson, G. J., & Reid, G. (2020b). *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Communications Biology*, *3*(1). https://doi.org/10.1038/s42003-020-01259-8

Daisley, B. A., Pitek, A. P., Torres, C., Lowery, R., Adair, B. A., Al, K. F., Niño, B., Burton, J. P., Allen-Vercoe, E., Thompson, G. J., Reid, G., & Niño, E. (2023). Delivery mechanism can enhance probiotic activity against honey bee pathogens. *The ISME Journal*, *17*(9). https://doi.org/10.1038/s41396-023-01422-z

Daisley, B. A., & Reid, G. (2021). BEExact: A metataxonomic database tool for high-resolution inference of bee-associated microbial communities. *mSystems*, *6*(2), e00082-21. https://doi.org/10.1128/mSystems.00082-21

Damico, M. E., Beasley, B., Greenstein, D., & Raymann, K. (2023). Testing the effectiveness of a commercially sold probiotic on restoring the gut microbiota of honey bees: A field study. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-023-10203-1

DeGrandi-Hoffman, G., Chen, Y., Rivera, R., Carroll, M., Chambers, M., Hidalgo, G., & de Jong, E. W. (2016). Honey bee colonies provided with natural forage have lower pathogen loads and higher overwinter survival than those fed protein supplements. *Apidologie*, *47*(2), 186–196. https://doi.org/10.1007/s13592-015-0386-6

Eccles, L., Kempers, M., Gonzales Mijares, R., Thurston, D., & Borges, D. (2016). *Canadian Best Management Practices for Honey Bee Health – CAPA*. https://capabees.com/canadian-best-management-practices-for-honey-bee-health/

Erez, T., Bonda, E., Kahanov, P., Rueppell, O., Wagoner, K., Chejanovsky, N., & Soroker, V. (2022). Multiple benefits of breeding honey bees for hygienic behavior. *Journal of Invertebrate Pathology*, *193*, 107788. https://doi.org/10.1016/j.jip.2022.107788

Evans, J. D. (2003). Diverse origins of tetracycline resistance in the honey bee bacterial pathogen *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, *83*(1), 46–50. https://doi.org/10.1016/s0022-2011(03)00039-9

Evans, J. D., & Spivak, M. (2010). Socialized medicine: Individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, *103*, 62–72. https://doi.org/10.1016/j.jip.2009.06.019

Evivie, S. E., Huo, G.-C., Igene, J. O., & Bian, X. (2017). Some current applications, limitations and future perspectives of lactic acid bacteria as probiotics. *Food & Nutrition Research*, *61*(1), 1318034. https://doi.org/10.1080/16546628.2017.1318034

Food and Agriculture Organization of the United Nations. (2020). Good beekeeping practices: Practical manual on how to identify and control the main diseases of the honeybee (*Apis mellifera*). TECA – Technologies and practices for small agricultural producers, 1. Rome. https://doi.org/10.4060/ca9182en

Fries, I., & Camazine, S. (2001). Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. *Apidologie*, *32*(3), 199–214. https://doi.org/10.1051/apido:2001122

Gao, Y., & Wu, M. (2023). Accounting for 16S rRNA copy number prediction uncertainty and its implications in bacterial diversity analyses. *ISME Communications*, *3*(1), 1–9. https://doi.org/10.1038/s43705-023-00266-0

Genersch, E. (2010). Honey bee pathology: Current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97. https://doi.org/10.1007/s00253-010-2573-8

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome datasets are compositional: And this is not optional. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.02224

Gloor, G. B., Wu, J. R., Pawlowsky-Glahn, V., & Egozcue, J. J. (2016). It's all relative: Analyzing microbiome data as compositions. *Annals of Epidemiology*, *26*(5), 322–329. https://doi.org/10.1016/j.annepidem.2016.03.003

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME Journal*, *5*(10), 1571–1579. https://doi.org/10.1038/ismej.2011.41

Hristov, P., Shumkova, R., Palova, N., & Neov, B. (2020). Factors associated with honey bee colony losses: A mini-review. *Veterinary Sciences*, *7*(4). https://doi.org/10.3390/vetsci7040166

Hunt, G. J. (2007). Flight and fight: A comparative view of the neurophysiology and genetics of honey bee defensive behavior. *Journal of Insect Physiology*, *53*(5), 399–410. https://doi.org/10.1016/j.jinsphys.2007.01.010

Iorizzo, M., Testa, B., Ganassi, S., Lombardi, S. J., Ianiro, M., Letizia, F., Succi, M., Tremonte, P., Vergalito, F., Cozzolino, A., Sorrentino, E., Petrarca, S., De Cristofaro, A., & Coppola, R. (2021). Probiotic properties and potentiality of *Lactiplantibacillus plantarum* strains for the biological control of chalkbrood disease. *Journal of Fungi*, *7*(5). https://doi.org/10.3390/jof7050379

Jones, J. C., Fruciano, C., Marchant, J., Hildebrand, F., Forslund, S., Bork, P., Engel, P., & Hughes, W. O. H. (2018). The gut microbiome is associated with behavioural task in honey bees. *Insectes Sociaux*, *65*(3), 419–429. https://doi.org/10.1007/s00040-018-0624-9

Kapheim, K. M., Rao, V. D., Yeoman, C. J., Wilson, B. A., White, B. A., Goldenfeld, N., & Robinson, G. E. (2015). Caste-specific differences in hindgut microbial communities of honey bees (*Apis mellifera*). *PLoS ONE*, *10*(4), e0123911. https://doi.org/10.1371/journal.pone.0123911

Kassambara A. (2023). rstatix: Pipe-friendly framework for basic statistical tests. R package version 0.7.2, URL https://CRAN.R-project.org/package=rstatix.

Kern, L., Abdeen, S. K., Kolodziejczyk, A. A., & Elinav, E. (2021). Commensal inter-bacterial interactions shaping the microbiota. *Current Opinion in Microbiology*, *63*, 158–171. https://doi.org/10.1016/j.mib.2021.07.011

Kešnerová, L., Mars, R. A. T., Ellegaard, K. M., Troilo, M., Sauer, U., & Engel, P. (2017). Disentangling metabolic functions of bacteria in the honey bee gut. *PLoS Biology*, *15*(12), e2003467. https://doi.org/10.1371/journal.pbio.2003467

Koskella, B., & Bergelson, J. (2020). The study of host–microbiome (co)evolution across levels of selection. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1808), 20190604. https://doi.org/10.1098/rstb.2019.0604

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, *82*, 1–26. https://doi.org/10.18637/jss.v082.i13

Kwong, W. K., Medina, L. A., Koch, H., Sing, K.-W., Soh, E. J. Y., Ascher, J. S., Jaffé, R., & Moran, N. A. (2017). Dynamic microbiome evolution in social bees. *Science Advances*, *3*(3), e1600513. https://doi.org/10.1126/sciadv.1600513

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature Reviews Microbiology*, *14*(6). https://doi.org/10.1038/nrmicro.2016.43

Lapidge, K. L., Oldroyd, B. P., & Spivak, M. (2002). Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. *Naturwissenschaften*, *89*(12), 565–568. https://doi.org/10.1007/s00114-002-0371-6

Leclercq, G., Francis, F., Gengler, N., & Blacquière, T. (2018). Bioassays to quantify hygienic behavior in honey bee (*Apis mellifera* L.) colonies: A Review. *Journal of Apicultural Research*, *57*, 1–11. https://doi.org/10.1080/00218839.2018.1494916

Leclercq, G., Pannebakker, B., Gengler, N., Nguyen, B. K., & Francis, F. (2017). Drawbacks and benefits of hygienic behavior in honey bees (*Apis mellifera* L.): A review. *Journal of Apicultural Research*, *56*(4), 366–375. https://doi.org/10.1080/00218839.2017.1327938

Liberti, J., & Engel, P. (2020). The gut microbiota—brain axis of insects. *Current Opinion in Insect Science*, *39*, 6–13. https://doi.org/10.1016/j.cois.2020.01.004

Liberti, J., Kay, T., Quinn, A., Kesner, L., Frank, E. T., Cabirol, A., Richardson, T. O., Engel, P., & Keller, L. (2022). The gut microbiota affects the social network of honeybees. *Nature Ecology & Evolution*, *6*(10). https://doi.org/10.1038/s41559-022-01840-w

Linn, M., Glaser, S. M., Peng, T., & Grüter, C. (2020). Octopamine and dopamine mediate waggle dance following and information use in honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1936), 20201950. https://doi.org/10.1098/rspb.2020.1950

Louca, S., Doebeli, M., & Parfrey, L. W. (2018). Correcting for 16S rRNA gene copy numbers in microbiome surveys remains an unsolved problem. *Microbiome*, *6*(1). https://doi.org/10.1186/s40168-018-0420-9

Ludvigsen, J., Amdam, G. V., Rudi, K., & L'Abée-Lund, T. M. (2018). Detection and characterization of streptomycin resistance (strA-strB) in a honeybee gut symbiont (*Snodgrassella alvi*) and the associated risk of antibiotic resistance transfer. *Microbial Ecology*, *76*(3), 588–591. https://doi.org/10.1007/s00248-018-1171-7

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., Tickle, T. L., Weingart, G., Ren, B., Schwager, E. H., Chatterjee, S., Thompson, K. N., Wilkinson, J. E., Subramanian, A., Lu, Y., Waldron, L., Paulson, J. N., Franzosa, E. A., Bravo, H. C., & Huttenhower, C. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLoS Computational Biology*, *17*(11), e1009442. https://doi.org/10.1371/journal.pcbi.1009442

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1). https://doi.org/10.14806/ej.17.1.200

Martinson, V. G., Moy, J., & Moran, N. A. (2012). Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and Environmental Microbiology*, *78*(8), 2830–2840. https://doi.org/10.1128/AEM.07810-11

Masterman, R., Smith, B. H., & Spivak, M. (2000). Brood odor discrimination abilities in hygienic honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. *Journal of Insect Behavior*, *13*(1), 87–101. https://doi.org/10.1023/A:1007767626594

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Momot, J. P., & Rothenbuhler, W. C. (1971). Behaviour genetics of nest cleaning in honeybees. VI. interactions of age and genotype of bees, and nectar flow. *Journal of Apicultural Research*, *10*(1), 11–21. https://doi.org/10.1080/00218839.1971.11099665

Motta, E. V. S., Powell, J. E., Leonard, S. P., & Moran, N. A. (2022). Prospects for probiotics in social bees. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *377*(1853), 20210156. https://doi.org/10.1098/rstb.2021.0156

Naeger, N. L., & Robinson, G. E. (2016). Transcriptomic analysis of instinctive and learned reward-related behaviors in honey bees. *Journal of Experimental Biology*, *219*(22), 3554–3561. https://doi.org/10.1242/jeb.144311

Newton, D. C., & Ostasiewski, N. J. J. (1986). A simplified bioassay for behavioral resistance to American foulbrood in honey bees. *The American Bee Journal*, *126*(4), 278–281.

Nordström, E. A., Teixeira, C., Montelius, C., Jeppsson, B., & Larsson, N. (2021). *Lactiplantibacillus plantarum* 299v (LP299V®): Three decades of research. *Beneficial Microbes*, *12*(5), 441–465. https://doi.org/10.3920/BM2020.0191

Nour, I., Fattouh, F., & Eladawi, H. (2014). Chemically defined medium for optimization of proteolytic activity of *Lactobacillus bulgaricus* 761N. *International Journal of Life Science and Medical Research*, *4*, 46–56. https://doi.org/10.5963/LSMR0404002

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., … Weedon, J. (2022). vegan: Community Ecology Package. R package version 2.6-4, URL https://CRAN.R-project.org/package=vegan.

Oliver, R. (2021). *Observations on Pollen Subs*. Scientific Beekeeping. https://scientificbeekeeping.com/observations-on-pollen-subs/

Ontario Beekeeper's Association (n.d.) *A Guide to Feeding Bees.* https://www.ontariobee.com/sites/ontariobee.com/files/GuideFeedingBees.pdf

Oxley, P. R., Spivak, M., & Oldroyd, B. P. (2010). Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (*Apis mellifera*). *Molecular Ecology*, *19*(7), 1452–1461. https://doi.org/10.1111/j.1365-294X.2010.04569.x

Palarea-Albaladejo, J., & Martín-Fernández, J. A. (2015). zCompositions—R package for multivariate imputation of left-censored data under a compositional approach. *Chemometrics and Intelligent Laboratory Systems*, *143*, 85–96. https://doi.org/10.1016/j.chemolab.2015.02.019

Pérez-Sato, J. A., Châline, N., Martin, S. J., Hughes, W. O. H., & Ratnieks, F. L. W. (2009). Multi-level selection for hygienic behaviour in honeybees. *Heredity*, *102*(6). https://doi.org/10.1038/hdy.2009.20

Pino, A., Benkaddour, B., Inturri, R., Amico, P., Vaccaro, S. C., Russo, N., Vaccalluzzo, A., Agolino, G., Caggia, C., Miloud, H., & Randazzo, C. L. (2022). Characterization of *Bifidobacterium asteroides* isolates. *Microorganisms*, *10*(3). https://doi.org/10.3390/microorganisms10030655

Powell, J. E., Martinson, V. G., Urban-Mead, K., & Moran, N. A. (2014). Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Applied and Environmental Microbiology*, *80*(23), 7378–7387. https://doi.org/10.1128/AEM.01861-14

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Raymann, K., & Moran, N. A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current Opinion in Insect Science*, *26*, 97–104. https://doi.org/10.1016/j.cois.2018.02.012

Raymann, K., Shaffer, Z., & Moran, N. A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biology*, *15*(3), e2001861. https://doi.org/10.1371/journal.pbio.2001861

Rodríguez, M. A., Fernández, L. A., Daisley, B. A., Reynaldi, F. J., Allen-Vercoe, E., & Thompson, G. J. (2023). Probiotics and in-hive fermentation as a source of beneficial microbes to support the gut microbial health of honey bees. *Journal of Insect Science*, *23*(6), 19. https://doi.org/10.1093/jisesa/iead093

Rothenbuhler, W. C. (1964). Behavior genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed blood. *American Zoologist*, *4*, 111–123. https://doi.org/10.1093/icb/4.2.111

Savio, C., Mugo-Kamiri, L., & Upfold, J. K. (2022). Bugs in bugs: The role of probiotics and prebiotics in maintenance of health in mass-reared insects. *Insects*, *13*(4). https://doi.org/10.3390/insects13040376

Smutin, D., Lebedev, E., Selitskiy, M., Panyushev, N., & Adonin, L. (2022). Micro"bee"ota: Honey bee normal microbiota as a part of superorganism. *Microorganisms*, *10*(12). https://doi.org/10.3390/microorganisms10122359

Spivak, M. (1996). Honey bee hygienic behavior and defense against *Varroa jacobsoni.* *Apidologie*, *27*(4), 245–260. https://doi.org/10.1051/apido:19960407

Spivak, M., & Downey, D. L. (1998). Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *91*(1), 64–70. https://doi.org/10.1093/jee/91.1.64

Spivak, M., & Gilliam, M. (1998). Hygienic behaviour of honey bees and its application for control of brood diseases and varroa. *Bee World*. https://www.tandfonline.com/doi/abs/10.1080/0005772X.1998.11099408

Spivak, M., & Reuter, G. S. (1998). Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie*, *29*(3), 291–302. https://doi.org/10.1051/apido:19980308

Stanimirović, Z., Glavinić, U., Ristanić, M., Aleksić, N., Jovanović, N., Vejnović, B., & Stevanović, J. (2019). Looking for the causes of and solutions to the issue of honey bee colony losses. *Acta Veterinaria*, *69*(1), 1–31. https://doi.org/10.2478/acve-2019-0001

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K., & Schmidt, T. M. (2015). rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Research*, *43*(D1), D593-598. https://doi.org/10.1093/nar/gku1201

Tamarit, D., Ellegaard, K. M., Wikander, J., Olofsson, T., Vásquez, A., & Andersson, S. G. E. (2015). Functionally structured genomes in *Lactobacillus kunkeei* colonizing the honey crop and food products of honeybees and stingless bees. *Genome Biology and Evolution*, *7*(6), 1455–1473. https://doi.org/10.1093/gbe/evv079

Uzunov, A., Costa, C., Panasiuk, B., Meixner, M., Kryger, P., Hatjina, F., Bouga, M., Andonov, S., Bienkowska, M., Conte, Y. L., Wilde, J., Gerula, D., Kiprijanovska, H., Filipi, J., Petrov, P., Ruottinen, L., Pechhacker, H., Berg, S., Dyrba, W., … Büchler, R. (2014). Swarming, defensive and hygienic behaviour in honey bee colonies of different genetic origin in a pan-European experiment. *Journal of Apicultural Research*, *53*(2), 248–260. https://doi.org/10.3896/IBRA.1.53.2.06

Van den Boogaart, K. G., Tolosana-Delgado, R., & Bren, M. (2024). *compositions: Compositional Data Analysis* (2.0-7) [Computer software]. https://cran.r-project.org/web/packages/compositions/index.htmlv

Vásquez, A., Forsgren, E., Fries, I., Paxton, R. J., Flaberg, E., Szekely, L., & Olofsson, T. C. (2012). Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. *PLoS ONE*, *7*(3), e33188. https://doi.org/10.1371/journal.pone.0033188

Vernier, C. L., Chin, I. M., Adu-Oppong, B., Krupp, J. J., Levine, J., Dantas, G., & Ben-Shahar, Y. (2020). The gut microbiome defines social group membership in honey bee colonies. *Science Advances*, *6*(42), eabd3431. https://doi.org/10.1126/sciadv.abd3431

Větrovský, T., & Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS ONE*, *8*(2), e57923. https://doi.org/10.1371/journal.pone.0057923

Wang, Y., Ma, L.-T., & Xu, B.-H. (2015). Diversity in life history of queen and worker honey bees, *Apis mellifera* L. *Journal of Asia-Pacific Entomology*, *18*(2), 145–149. https://doi.org/10.1016/j.aspen.2014.11.005

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., … Yutani, H. (2019).

Welcome to the Tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Wickham H., François R., Henry L., Müller K., Vaughan D. (2023). dplyr: A grammar of data manipulation. R package version 1.1.4, URL https://CRAN.R-project.org/package=dplyr.

Williams, N. T. (2010). Probiotics. *American Journal of Health-System Pharmacy*, *67*(6), 449–458. https://doi.org/10.2146/ajhp090168

Wilson-Rich, N., Dres, S. T., & Starks, P. T. (2008). The ontogeny of immunity: Development of innate immune strength in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, *54*(10), 1392–1399. https://doi.org/10.1016/j.jinsphys.2008.07.016

Zhang, Z., Mu, X., Cao, Q., Shi, Y., Hu, X., & Zheng, H. (2020). *Honeybee gut microbiota modulates host behaviors and neurological processes* (p. 2020.12.19.423587). bioRxiv. https://doi.org/10.1101/2020.12.19.423587

Zhang, Z., Mu, X., Shi, Y., & Zheng, H. (2022). Distinct roles of honeybee gut bacteria on host metabolism and neurological processes. *Microbiology Spectrum*, *10*(2), e02438-21. https://doi.org/10.1128/spectrum.02438-21

Chapter 4

4 General Conclusion

This thesis represents an original study on an important applied aspect of beekeeping: the hygienic capabilities of a colony. Hygienic bees are a priority for beekeepers due to the multitude of diseases bees face as domesticated, social-living insects (Erez et al., 2022). In addition to the practical applications, the study presented here also advances the gut-brain axis framework from the level of the individual to the inter-individual or colony level. The gut-brain axis is vital to the health and well-being of host organisms (Wiley et al., 2017), and taking advantage of this mechanism in an agricultural context could have major implications for the environment and the food chain. My second chapter is a synthetic literature review on the potential for probiotic supplementation in managed honey bee hives, and in my third and empirical chapter, I conducted a study to explore this prospect in hives at Western University's Research Park. Overall, the work presented here is foundational and will open up future avenues of investigation into how social behaviour can be influenced by the microbiota.

4.1 The gut-brain axis and eusociality

Social insects offer a unique opportunity to study the gut-brain axis. Typically, the gut-brain axis is involved in the health and welfare of individuals, supporting the digestion, physiology, and immunity of the host organism (Jandhyala et al., 2015). There is also increasing evidence that the gut-brain axis can influence the behaviour of organisms (Desbonnet et al., 2014; Salim et al., 2021; Sarkar et al., 2020). For eusocial animals, like the Western honey bee, which lives in large (20 000-80 000 individuals) colonies of closely related kin (Andersson, 1984), this effect can be reflected up from the level of the individual to influence whole colonies, an effect I call "social amplification". In support of this concept, studies have shown that the microbiota can indeed influence behaviour in social living organisms (Liberti et al., 2022; Sarkar et al., 2020; Vernier et al., 2020; Vernier et al., 2023); what remains to be identified is how this might affect major social

behaviours in honey bees, such as foraging, defense, and hygiene in free-living colonies of bees (i.e. not in the lab).

Honey bee social behaviour is rooted in the concept of inclusive fitness. Inclusive fitness theory argues that eusocial insects, which are characterized by a reproductive division of labour (among other traits), forego direct fitness benefits (i.e. production of offspring) in order to help raise and care for their queen mother's offspring, which provides them with additional, indirect fitness (i.e. production of non-offspring kin) that may exceed the fitness benefits offered via direct reproduction (Hamilton, 1963). This is supported by the high degree of relatedness in colonies, which, for honey bees and all other Hymenoptera, is established by the sex determination system haplodiploidy (Rautiala et al., 2019), and is in line with kin selection theory, which posits that relatedness asymmetries can favour the evolution of divisions of labour and eusociality (Andersson, 1984). Honey bees divide not only reproductive labour, but tasks within the colony by a mechanism known as temporal polyethism, or age-based task allocation (Beshers et al., 2001). These theories and the complex divisions of labour within colonies show that the social behaviours exhibited in hives have evolved for specific and interconnected purposes, and are vital to the colony's survival.

4.2 Relevance to scientific beekeeping

Apiculture is crucial to the environment (Hung et al., 2018) and contributes significantly to the Canadian economy both through commercial pollination and the production of honey and other products (Agriculture and Agri-food Canada, 2022). If the potential of the gut-brain axis could be reliably harnessed, probiotics could be used for a range of desired behaviours and health-related traits in managed apiaries. There are currently probiotic supplements for bees, but these products need to be further field-validated and scientifically evaluated for their efficacy (Damico et al., 2023). A more in-depth understanding of the gut-brain axis framework, as it affects whole hives, would allow for more diversity and more precision in the intended effects of probiotic products on the market. Overall, this serves to show the importance of apiculture to our society and our

food chain (Verde, 2014) and demonstrates that probiotics may be an invaluable resource to protect the health of these managed colonies (Motta et al., 2022).

Alternatively, it is possible that the probiotic treatment in this study truly had no effect, and that the slight increases in hygiene after a single probiotic treatment were coincidental. Although probiotics have been shown to help bees recover from antibiotic-induced dysbiosis and protect against pathogens (Daisley et al 2020a, 2020b), there is thus far no existing evidence that probiotics can directly affect social behaviours in a living colony of honey bees. Alternatives to probiotic use that could aid in colony survival include RNA interference, which can be used to impair viral replication or silence essential genes of ectoparasites such as *Varroa* (Garbian et al. 2012; Maori et al. 2009; Smeele et al. 2023). Queens can also be vaccinated with inactive forms of pathogens, which are then passed down to their offspring by transgenerational immune priming (Dickel et al., 2022), conferring protection against the inoculated pathogen. Although the two solutions mentioned above are promising, they require further work to be practical solutions for beekeepers. A simpler alternative, evidence shows that natural therapies such as essential oils can be used against certain pathogens (Hýbl et al. 2021; Tutun et al. 2018), and beekeepers do indeed use natural remedies such as cinnamon in the treatment of chalkbrood.

There are many ministries, councils, and associations that regulate apiculture in Canada and provide resources for beekeepers, including the Canadian Association of Professional Apiculturists, Canadian Honey Council, Ontario Beekeeper's Association, Ontario Ministry of Agriculture, Food, and Rural Affairs, and Agriculture and Agri-Food Canada. Part of the mandate of these organizations is to protect and improve Canada's apiculture industry through innovation, research, and applied science. For example, the Ontario Beekeepers Association places an emphasis on bee breeding and has multiple programs to ensure high-quality bees, such as the Ontario Bee Breeders Association and the Tech Transfer Program, which, among other research initiatives, provides hygienic testing services for beekeepers to inform them on which hives to breed.

4.3 Objectives, goals, and insights

In my thesis, the two primary objectives I addressed were whether probiotic treatment (in the form of *Lactiplantibacillus plantarum* and *Bifidobacterium asteroides* in a BioPatty) could alter the gut microbiota of worker bees in a managed apiary, and to determine whether probiotic treatment could impact the prevalence of hygienic behaviour within that same apiary. The first objective was addressed via my 16S rRNA gene sequencing, while the second objective was addressed through my freeze-kill brood assay hygienic testing. I found that probiotic treatment altered the gut microbiota of bees from living hives, but this effect was not consistent with treatment and was fine-scale, and may have been induced by repeated applications of pollen substitutes and changes in foraging over the duration of the experiment. Additionally, I saw small changes in hygiene within treatment groups, but overall this effect was not significant, thus necessitating a larger field study to confirm the findings of this project.

The goal of this thesis was to provide evidence of a functional gut-brain axis as it might affect the social behaviours of honey bees, with the hope of providing functional information on best practices to beekeepers. Slight differences in honey bee behaviour and changes in the gut suggested an acute impact of the BioPatty, however, this effect had no statistical significance, perhaps in part because of a low sample size. Despite this, the work presented here shows promise and has drawn upon many different areas of biology to lay a foundation that can be continued not only by myself but also by future students in my lab. This thesis has provided some insights on how the microbiota might be harnessed to affect social behaviours in apiculture and provides information and protocols that could be used going forward to further explore the gut-brain axis pathway.

4.4 Conclusions

In conclusion, this thesis has contributed valuable information to the gut-brain axis framework as a mechanism to influence social behaviours, but steadfast conclusions cannot be drawn from this work due to a lack of statistical significance in the linear mixed model and a low sample size of nine colonies. My hypothesis that the application

of probiotic strains could affect the concentration of olfactory-associated neurotransmitters and subsequently impact the threshold response of nurse bees to olfactory cues emitted from the brood was not supported in my full statistical model. My initial prediction was that hygiene would be increased through probiotic supplementation, an effect which appeared to be supported after a single round of treatment, but a second round of treatment did not support this and instead increased the variability in hygienic scores.

Due to the lack of conclusive support for my hypothesis, I plan to repeat my experiment this summer (August 2024) to bring up my sample size using an additional 15 colonies, to a total of 24 colonies across two summers of experimental work. This increase in sample size will hopefully allow me to draw a more confident conclusion from the foundational work that I have discussed in this thesis. Regardless of the outcome of the second experiment, both studies will contribute new knowledge to the growing field of probiotics for honey bees and will create new directions for future avenues of investigation. This project could also promote discussion of alternatives to antibiotics and colony loss in domestic honey bees.

4.5 References

Agriculture and Agri-Food Canada. (2021). *Statistical Overview of the Canadian Honey and Bee Industry and the Economic Contribution of Honey Bee Pollination*. https://agriculture.canada.ca/en/sector/horticulture/reports/statistical-overview-canadian-honey-and-bee-industry-2021

Andersson, M. (1984). The evolution of eusociality. *Annual Review of Ecology and Systematics*, *15*, 165–189.

Beshers, S. N., Huang, Z. Y., Oono, Y., & Robinson, G. E. (2001). Social inhibition and the regulation of temporal polyethism in honey bees. *Journal of Theoretical Biology*, *213*(3), 461–479. https://doi.org/10.1006/jtbi.2001.2427

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Al, K. F., Chernyshova, A. M., Faragalla, K. M., Burton, J. P., Thompson, G. J., & Reid, G. (2020a). Novel probiotic approach to counter *Paenibacillus larvae* infection in honey bees. *The ISME Journal*, *14*(2), 476–491. https://doi.org/10.1038/s41396-019-0541-6

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Gibbons, S., Chernyshova, A. M., Al, K. F., Faragalla, K. M., Burton, J. P., Thompson, G. J., & Reid, G. (2020). *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Communications Biology*, *3*(1), Article 1. https://doi.org/10.1038/s42003-020-01259-8Damico, M.E., Beasley, B., Greenstein, D., Raymann, K. (2023). Testing the effectiveness of a commercially sold probiotic on restoring the gut microbiota of honey bees: A field study. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-023-10203-1

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G., & Cryan, J. F. (2014). Microbiota is essential for social development in the mouse. *Molecular Psychiatry*, *19*(2). https://doi.org/10.1038/mp.2013.65

Dickel, F., Bos, N. M. P., Hughes, H., Martín-Hernández, R., Higes, M., Kleiser, A., & Freitak, D. (2022). The oral vaccination with *Paenibacillus larvae* bacterin can decrease susceptibility to American Foulbrood infection in honey bees—A safety and efficacy study. *Frontiers in Veterinary Science*, *9*, 946237. https://doi.org/10.3389/fvets.2022.946237

Erez, T., Bonda, E., Kahanov, P., Rueppell, O., Wagoner, K., Chejanovsky, N., Soroker, V. (2022). Multiple benefits of breeding honey bees for hygienic behavior. *Journal of Invertebrate Pathology*, *193*, 107788. https://doi.org/10.1016/j.jip.2022.107788

Garbian, Y., Maori, E., Kalev, H., Shafir, S., & Sela, I. (2012). Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. *PLoS Pathogens*, *8*(12), e1003035. https://doi.org/10.1371/journal.ppat.1003035

Hamilton, W. D. (1963). The evolution of altruistic behavior. *The American Naturalist*, *97*(896), 354–356. https://doi.org/10.1086/497114

Hung, K.-L. J., Kingston, J. M., Albrecht, M., Holway, D. A., & Kohn, J. R. (2018). The worldwide importance of honey bees as pollinators in natural habitats. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1870), 20172140. https://doi.org/10.1098/rspb.2017.2140

Hýbl, M., Bohatá, A., Rádsetoulalová, I., Kopecký, M., Hoštičková, I., Vaníčková, A., & Mráz, P. (2021). Evaluating the efficacy of 30 different essential oils against *Varroa*

destructor and honey bee workers (*Apis mellifera*). *Insects*, *12*(11). https://doi.org/10.3390/insects12111045

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N. (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology*, *21*(29), 8787–8803. https://doi.org/10.3748/wjg.v21.i29.8787

Liberti, J., Kay, T., Quinn, A., Kesner, L., Frank, E.T., Cabirol, A., Richardson, T.O., Engel, P., Keller, L. (2022). The gut microbiota affects the social network of honeybees. *Nature Ecology and Evolution*, *6*(10). https://doi.org/10.1038/s41559-022-01840-w

Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., & Sela, I. (2009). IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Molecular Biology*, *18*(1), 55–60. https://doi.org/10.1111/j.1365-2583.2009.00847.x

Motta, E.V.S., Powell, J.E., Leonard, S.P., Moran, N.A. (2022). Prospects for probiotics in social bees. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *377*(1853), 20210156. https://doi.org/10.1098/rstb.2021.0156

Rautiala, P., Helanterä, H., & Puurtinen, M. (2019). Extended haplodiploidy hypothesis. *Evolution Letters*, *3*(3), 263–270. https://doi.org/10.1002/evl3.119

Salim, S., Banu, A., Alwa, A., Gowda, S.B.M., Mohammad, F. (2021). The gut-microbiota-brain axis in autism: What *Drosophila* models can offer? *Journal of Neurodevelopmental Disorders*, *13*, 37. https://doi.org/10.1186/s11689-021-09378-x

Sarkar, A., Harty, S., Johnson, K.V.A., Moeller, A.H., Carmody, R.N., Lehto, S.M., Erdman, S.E., Dunbar, R.I.M., Burnet, P.W.J. (2020). The role of the microbiome in the neurobiology of social behaviour. *Biological Reviews*, *95*, 1131–1166. https://doi.org/10.1111/brv.12603

Smeele, Z. E., Baty, J. W., & Lester, P. J. (2023). Effects of deformed wing virus-targeting dsRNA on viral loads in bees parasitised and non-parasitised by *Varroa destructor*. *Viruses*, *15*(11). https://doi.org/10.3390/v15112259

Tutun, H., Koç, N., & Kart, A. (2018). Plant essential oils used against some bee diseases. *Turkish Journal of Agriculture - Food Science and Technology*, *6*(1), Article 1. https://doi.org/10.24925/turjaf.v6i1.34-45.1502

Verde, M. M. (2014). Apiculture and food safety. *Cuban Journal of Agricultural Science*, *48*(1). https://www.cjascience.com/index.php/CJAS/article/view/423

Vernier, C.L., Chin, I.M., Adu-Oppong, B., Krupp, J.J., Levine, J., Dantas, G., Ben-Shahar, Y. (2020). The gut microbiome defines social group membership in honey bee colonies. *Science Advances*, *6*, eabd3431. https://doi.org/10.1126/sciadv.abd3431

Vernier, C. L., Nguyen, L. A., Gernat, T., Ahmed, A. C., Chen, Z., & Robinson, G. E. (2024). Gut microbiota contribute to variations in honey bee foraging intensity. *The ISME Journal*, *18*(1), wrae030. https://doi.org/10.1093/ismejo/wrae030

Wiley, N. C., Dinan, T. G., Ross, R. P., Stanton, C., Clarke, G., & Cryan, J. F. (2017). The microbiota-gut-brain axis as a key regulator of neural function and the stress response: Implications for human and animal health. *Journal of Animal Science*, *95*(7), 3225–3246. https://doi.org/10.2527/jas.2016.1256

Appendices

Appendix A. Table of raw scores from individual collars and hives.

The overall hygiene score was calculated as an average in Excel. Collar hygiene scores were calculated from counts of freeze-kill brood assay pictures in ImageJ using the formula (number of cells frozen–number of non-empty cells after the test/(number of cells frozen)). Bif stands for *Bifidobacterium asteroides* treatment, Lac for *Lactiplantibacillus plantarum*, and Veh for Vehicle control. The two collars were not statistically different from each other, therefore the average was taken for downstream analysis.

Hive	Collar 1	Collar 2	Overall Hygiene Score
H1T1 (Bif)	0.6145	0.4066	0.51055
H1T2 (Bif)	0.6867	0.381	0.53385
H1T3 (Bif)	0.3721	0.3659	0.369
H2T1 (Veh)	0.9302	0.7826	0.8564
H2T2 (Veh)	0.8333	0.7995	0.8164
H2T3 (Veh)	0.7011	0.7791	0.7401
H3T1 (Lac)	0.9254	0.7059	0.81565
H3T2 (Lac)	0.8919	0.9846	0.93825
H3T3 (Lac)	0.8519	0.7582	0.80505
H4T1 (Bif)	0.7123	0.4545	0.5834
H4T2 (Bif)	0.5584	0.625	0.5917
H4T3 (Bif)	1	0.7925	0.89625
H5T1 (Veh)	0.3174	0.4923	0.40485
H5T2 (Veh)	0.7229	0.5814	0.65215
H5T3 (Veh)	0.7442	0.8293	0.78675
H6T1 (Lac)	0.3208	0.382	0.3514
H6T2 (Lac)	0.4588	0.3371	0.39795
H6T3 (Lac)	0.6901	0.1948	0.44245
H7T1 (Bif)	0.6216	0.6548	0.6382
H7T2 (Bif)	0.8987	0.4839	0.6913
H7T3 (Bif)	0.8028	0.7821	0.79245
H8T1 (Veh)	0.4253	0.6588	0.54205
H8T2 (Veh)	0.1935	0.1579	0.1757
H8T3 (Veh)	0.5192	0.7966	0.6579
H9T1 (Lac)	0.6282	0.5301	0.57915
H9T2 (Lac)	0.6471	0.6966	0.67185
H9T3 (Lac)	0.4217	0.5542	0.48795

Appendix B. Taxonomy of the 100 most abundant sequence variants (SVs) in the 16S analysis.

These represent the 100 most abundant SVs in my honey bee gut samples, out of a total of 469 SVs present after pruning (retained samples with >1000 reads, SVs present at >1% relative abundance in any sample, SVs with >0.01% abundance in every sample, SVs with >100 total reads across samples and SVs with 0% abundance in at least half the samples were discarded). For space, the designation "Kingdom" has been removed from this table (as it is simply Bacteria for all), as well as the base pair sequence of each species. The entire dataset of 469 SVs is available upon request but was not included due to the length of the document.

	Phylum	Class	Order	Family	Genus	Species
SV_1	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	asteroides
SV_2	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	apis
SV_3	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_4	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	apicola
SV_5	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	melliventris
SV_6	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_7	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Frischella	perrara
SV_8	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	asteroides
SV_9	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Rhizobiaceae	Bartonella	apis
SV_10	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_11	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus	NA
SV_12	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_13	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_14	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_15	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	alvi
SV_16	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_17	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_18	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_19	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	kimbladii
SV_20	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacillus	NA
SV_21	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_22	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA

SV_23	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_24	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_25	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	apicola
SV_26	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_27	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	apicola
SV_28	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_29	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_30	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	NA
SV_31	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_32	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_33	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	panisapium
SV_34	Proteobacteria	Alphap-roteobacteria	Acetobacterales	Acetobacteraceae	Commensalibacter	NA
SV_35	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_36	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_37	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_38	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacillus	NA
SV_39	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	NA
SV_40	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	apicola
SV_41	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	alvi
SV_42	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_43	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_44	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	NA
SV_45	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_46	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_47	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_48	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_49	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_50	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_51	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_52	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Apilactobacillus	NA
SV_53	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_54	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	apicola
SV_55	Proteobacteria	Alpha-proteobacteria	Acetobacterales	Acetobacteraceae	Commensalibacter	NA
SV_56	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Rhizobiaceae	NA	NA
SV_57	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	alvi

SV_58	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Lelliottia	NA
SV_59	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Erwiniaceae	Pantoea	NA
SV_60	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	NA
SV_61	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_62	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_63	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_64	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	NA
SV_65	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_66	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_67	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	Lelliottia	NA
SV_68	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Erwiniaceae	Pantoea	NA
SV_69	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_70	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Frischella	NA
SV_71	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_72	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	apicola
SV_73	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_74	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_75	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_76	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Frischella	NA
SV_77	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_78	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Enterobacteriaceae	NA	NA
SV_79	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_80	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	alvi
SV_81	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	NA
SV_82	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Rhizobiaceae	NA	NA
SV_83	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_84	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_85	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_86	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_87	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_88	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
SV_89	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA

SV_90	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_91	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Bombilactobacillus	NA
SV_92	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Frischella	NA
SV_93	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_94	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus	NA
SV_95	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Frischella	NA
SV_96	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA
SV_97	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_98	Proteobacteria	Gamma-proteobacteria	Burkholderiales	Neisseriaceae	Snodgrassella	NA
SV_99	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Tyzzerella	NA
SV_100	Proteobacteria	Gamma-proteobacteria	Enterobacterales	Orbaceae	Gilliamella	NA

Curriculum Vitae

Name:	Sophie Killam
Post-secondary Education and Degrees:	University of Windsor Windsor, Ontario, Canada 2018-2022 B.Sc.H. Biological Sciences The University of Western Ontario London, Ontario, Canada 2022-2024 M.Sc. Ecology and Evolutionary Biology (in progress)
Honours and Awards:	Queen Elizabeth II Graduate Scholarship in Science and Technology 2023-2024
Related Work Experience:	Biology for Science, Teaching Assistant The University of Western Ontario Fall 2022 Scientific Methods in Biology, Teaching Assistant The University of Western Ontario Winter 2023 Animal Behaviour, Teaching Assistant The University of Western Ontario Fall 2023
Volunteer Experience:	Biology Graduate Research Forum Chair and Head of Finance March 2023-November 2023 Biology Barbecue Planning Committee Student Representative June 2023-September 2023 London Bug Day Termite and Honey Bee Volunteer September 2023 Canadian Bee Gut Project Royal Winter Fair Volunteer October 2023

Publications/Conferences:

Killam SM, Daisley BA, Kleiber ML, Lacika JF, Thompson GJ (Submitted ms: 1422265) A case for microbial therapeutics to bolster hive health and performance of honey bees. Frontiers in Bee Science.

Killam, SM.* & Thompson, GJ. (June 28th 2024) The potential to manipulate the brain-gut axis of Western honey bees to promote hygienic behaviour. Animal Behaviour Society. London, ON, Canada. [Accepted Talk]

Killam SM, Chernyshova AM, Daisley BA, Kleiber ML, Allen-Vercoe E, Thompson GJ*. (March 28, 2024). Behavioural studies into the brain-gut axis of honey bees: How gut microbes can affect complex social behaviour of hives. Biology & Genomics of Social Insects, Cold Spring Harbor, USA. [Talk]

Killam, SM.* & Thompson, GJ. (October 17th 2023). Effects of lactic acid-producing bacteria supplementation on the hygienic behaviour of Western honey bees. Entomological Society of Canada Joint Annual Meeting. Saskatoon, SK, Canada. [Talk]

Killam, SM.* & Thompson, GJ. (October 13th 2023). Effects of lactic acid-producing bacteria supplementation on the hygienic behaviour of Western honey bees. BeeCon. Toronto, ON, Canada. [Talk]

Daisley BA, Mallory E, Chernyshova AM, Pitek AP, Rodríguez MA, Killam SM, Lacika JF, Allen-Vercoe E, Thompson GJ*. (September, 2023). The Canadian Bee Gut Project. Apimondia International Apicultural Congress, Santiago, Chile. [Poster]

Killam SM[1], Chernyshova AM[1], Daisley BA, Pitek AP, Lacika JF, George SA, Burton JP, Allen-Vercoe E, Thompson GJ*. (September 7th, 2023). Behavioural studies into the brain-gut axis of honey bees: How gut microbes can affect complex social behaviour of hives. Apimondia International Apicultural Congress, Santiago, Chile. [Talk, 1 - joint first author]

Killam, SM.* & Thompson, GJ. (May 27th 2023). Effects of lactic acid-producing bacteria supplementation on the hygienic behaviour of Western honey bees. Ontario Ecology, Ethology and Evolution Colloquium. London, ON, Canada. [Poster]