# ‘OMICS’ APPROACHES TO UNDERSTAND ECOLOGY AND PHYSIOLOGY OF BEES

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**Title**

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University’s regulations and meets the accepted standards for the degree of

**DOCTOR OF PHILOSOPHY**

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## ABSTRACT

Bees face several ecological and physiological challenges due to poor nutrition and abiotic stress. This dissertation explores various aspects related to these challenges, including the microbial ecology of honey bees and the alfalfa leafcutting bee, as well as the cold physiology of the alfalfa leafcutting bee. The first study delves into the dynamics of gut microbiota in overwintering adult worker honey bees, *Apis mellifera*. Through 16S rRNA gene amplicon sequencing, variations in the microbial composition among the bee strains, due to types of storage conditions, and the month of storage were examined. Overall, in this study a stable gut microbiota was observed in the bees both in the indoor and outdoor storage conditions during the winter months with slight differences in the abundance of bacterial species between bee strains. This study highlights the resilience of honey bee gut microbiota under cold stress conditions. The second study was focused on assessing the gut microbial ecology in the solitary bee, *Megachile rotundata*. The study investigated the significance of gut microbiota in these bees and in particular the effect of the *Apilactobacillus* clade on the larval and prepupal development, their weight, and survival. Results indicate that reliance on non-host specific environmental bacteria may not significantly impact the fitness of *M. rotundata*. The study highlights the role of environmentally collected bacteria in shaping bee nutrition and health. The third study explored the regulation of diapause initiation in the *M. rotundata*. By analyzing the gene expression using RNA-seq during diapause initiation, the research identified differentially expressed genes associated with oxidative stress, cell signaling, and other diapause-related pathways. The study provides insights into the molecular differences between diapausing and non diapausing individuals, contributing to a broader understanding of the cold physiology of *M. rotundata*. Together, these multi ‘omics’ studies contribute invaluable knowledge to the fields of bee

biology, ecology and physiology, offering insights into the intricate relationships between bees, their microbiota, and the environmental factors influencing their life cycle.

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## DEDICATION

My Ph.D. is dedicated to the resilient and courageous sons of Punjab, whose unwavering sacrifice and commitment, during my years of study, embody the spirit of Punjab and the agrarian struggle. These brave souls, who laid down their lives advocating for the rights of farmers, stand as a testament to the strength deeply rooted in the soil of Punjab. In profound dedication to those sons of Punjab who were either killed or lost, this tribute extends its heartfelt condolences to the families that bore the weight of immeasurable loss. Moreover, it stands as a heartfelt tribute to the brave hearts who stood at the forefront of the farmers' protests, challenging injustices with determination and ensuring that Punjab remains a symbol of strength and unity. Their sacrifices form a forever mark in the history and echo that will inspire and vibrate through generations to come. This dedication further extends to the vibrancy, rich culture, and the collective spirit of Punjab.

May the land of five rivers continue to thrive and prosper!

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# CHAPTER 1: INTRODUCTION

Bees are important for the pollination of agricultural crops and trees (Hill and Webster, 1995). In 2005, the total economic value of pollination was nearly $172 billion throughout the world which accounted for about 10% of the world's crop production consumed by humans (Gallai et al., 2009). Between $235 and $577 billion (U.S.) worth of annual global food production relies on the direct contribution of pollinators (inflated to 2015 US$) (Lautenbach et al., 2012). And, the percentage of the cultivated area requiring pollination is increasing yearly (Aizen et al., 2008). Although the economic value of honey bees is significant, wild solitary bees are also recognized for their contribution to sustaining agricultural production (i.e market cap of $4 billion). The value of insect-pollinated crops in the United States was estimated to be $15.1 billion in 2009, $3.4 billion of which was attributed to non-Apis bees (Calderone, 2012). Unfortunately, honey bees are declining and facing a lot of challenges for meeting agriculture demands (Aizen and Harder, 2009). So, it is important to study not only social bees but solitary bees too, which can be used as an alternative to honey bee pollination. Solitary bees are excellent and efficient pollinators that pollinate not only legume crops like alfalfa, birdsfoot trefoil, and sweet clover and but several other crops like berries, some citrus, carrot seed (Tepedino and Parker, 1986), canola (Soroka et al., 2001), and blueberry (Stubbs et al., 1994).

Global agriculture has become increasingly pollinator-dependent. Despite the rise in demand for pollinator-dependent crops, the availability of commercially managed bees has grown far slower due to several ecological and physiological constraints. To gather more insights into the reasons for this deficit, my research has concentrated on: (1) understanding gut microbial ecology of overwintering honey bees, (2) studying the interplay between ecology, nutrition, and the microbiome of the alfalfa leafcutter bee, *Megachile rotunadata*, and (3) the cold physiology

of *M. rotundata*. This multifaceted approach has allowed me to explore the physiological and ecological challenges of solitary and social bee species.

## Importance of social bee microbiome

The decline in populations of both honey bees and wild bees has been linked to several ecological factors such as inadequate nutrition, pesticide exposure, habitat deterioration, increased parasite levels, and a decrease in genetic variation (Cornman et al., 2016). While genome wide surveys of social bee populations have challenged the significance of these causes, poor nutrition has been consistently associated with poor bee health. One of the most important ecological aspect that has shown distinct role on host nutrition is the gut microbiome. In honey bees, these microbes affect host nutrition, contribute to the digestion of macromolecules, endocrine signaling, neutralization of dietary toxins, weight gain, and pathogen resistance (Zheng et al., 2018). Moreover, the gut associated bacteria can have profound effects on physiology, development, and behavior. Environmental and developmental factors like diet, age, climate, chemicals, antibiotics, and temperature stress alter the gut microbiome, eventually effecting immune function, metabolism, behavior, growth, and development. The honey bee gut microbiome contains five core, highly conserved, and host-specific bacterial species that comprise 95% of the total microbes inside the gut. The hindgut of every adult worker contains *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus Firm-5, Lactobacillus Firm-4*, and *Bifidobacterium* species (Raymann and Moran, 2018). This tightly linked host-specific core gut microbiota is actively transmitted to the next generation via social contact.

Genomic and metagenomic studies indicate that bacteria residing in the social bee gut play a role in breaking down macromolecules, providing nutrients, and neutralizing dietary toxins (Moran, 2015). In temperate climates, honey bees rely on stored carbohydrates to sustain

them throughout the winter (Quinlan et al., 2023), and serve as an energy source for thermoregulation (Southwick and Heldmaier, 1987). Glycogen serves as a primary energy source, and its levels significantly decreased during the mid-phase of overwintering. However, glycerol, along with certain low-molecular-weight sugars and polyols such as mannitol, sorbitol, and trehalose, were identified as the primary cryoprotectants in the overwintering bees. Their levels increase during the winter period (Qin et al., 2019).

Thus, the core bacteria play crucial roles in aspects such as, fermenting complex carbohydrates, aiding in digestion, and synthesizing essential nutrients (Engel et al., 2012; Lee et al., 2015). Furthermore, some of the symbiotic microorganisms residing within the social bees have been observed fermenting carbohydrates (Martinson et al., 2014), while some others may engage in syntrophic interactions to partition metabolic resources (Kwong et al., 2014). Both in honey bees and bumble bees, maternally inherited and bee-specific *Lactobacilli* carry genes related to carbohydrate utilization and phosphotransferase systems that are essential for sugar uptake (Kwong et al., 2014; Ellegaard et al., 2015). Core bacterial communities may be key to cold stress resilience and overwintering success.

Alterations in the bee's physiological state throughout different seasons may impact the diversity and composition of the gut microbiome (Carey and Duddleston, 2014). Insects that overwinter experience substantial seasonal variations in feeding habits (Hahn and Denlinger, 2007), the composition of intestinal contents (Olsen and Duman, 1997), the immune system (Ferguson and Sinclair, 2017), and physiological functioning (Denlinger and Lee, 2010). The overwintering health and survival of colonies remain a significant concern among the beekeeping industry (Steinhauer et al., 2021). However, beekeepers have achieved favorable outcomes by overwintering honey bee colonies indoors under controlled cold climate conditions at 5 °C - 7 °C

and 25% relative humidity (Owens et al., 1971; Degrandi-Hoffman et al., 2019). Yet, it is unclear whether temperature during cold storage alters the composition of the honey bee gut microbiome during the winter months. Considering the significance of gut microbiota in winter bees, it is crucial to understand the changes and differences in microbes between bees stored at 6°C and overwintering bees in natural conditions during the subsequent months. Therefore, in Chapter 2, I test the hypothesis that the composition of the honey bee gut microbiome could be impacted by the overwintering temperature. Two types of treatment bees (viz. Bolton bees claimed to be cold hardy and Mann lake bees) were either housed indoors at a consistent temperature or subjected to natural temperature fluctuations. We used 16s rRNA gene sequencing to identify the bacterial communities present in the gut of the adult honey bee workers from each treatment. This study will enhance our understanding of the importance of several factors, such as bee strain, months, and storage status, and how resilient or sensitive to change the gut microbiota is to these factors.

**Importance of solitary bee gut microbiome**

Unlike social bees, solitary bees do not share food and their gut microbiome with one another. The transfer of plant-associated bacteria to the digestive systems of larval and adult solitary bees occurs via pollen ingestion. Given the functional contrast observed between how bacteria are transmitted socially versus environmentally in bees, there's a need to explore the connection between pollen-related microbes and the fitness of solitary bees. The bacterial diversity found in the brood cell provisions of solitary bees has been investigated in the past for above the ground - stem and cavity nesting species (Voulgari-Kokota et al., 2019). The solitary bees in the *Megachilidae* family host highly diverse and fluctuating bacterial communities from different families, such as *Acetobacteraceae, Bacillaceae, Clostridiaceae, Enterobacteriaceae,*

*Lachnospiraceae*, *Lactobacillaceae*, *Methylobacteriaceae*, *Moraxellaceae*, and *Oxalobacteraceae* (McFrederick and Rehan, 2016; McFrederick et al., 2017; Voulgari-Kokota et al., 2018) . It has been suggested that the composition of the microbiota in solitary bees is unique to each species, but individual bacteria might not exclusively associate with specific bee species. These bacterial taxa are acquired through foraging on pollen and nectar of host plants (Vannette, 2020; Keller et al., 2021). Multiple studies have observed alterations in the bacterial community within both the pollen provisions and larvae over time and by location (Mohr and Tebbe, 2006; Keller et al., 2013; Voulgari-Kokota et al., 2018). Previous research utilizing trophic biomarkers has indicated that microbes could serve as direct prey for bees, making bees an omnivorous organism which feeds on both plant and microbial-derived food rather than strict herbivory (Steffan et al., 2019). This observation may hold true for solitary bees where microbes play a crucial role as a source of larval nutrition. In addition, microbial activity in pollen provision aids in unlocking nutrients that are otherwise confined within the indigestible exine of the pollen (Steffan et al., 2019). Studies conducted by feeding sterilized pollen provisions to Osmia sp., have demonstrated that the presence of the naturally occurring microbial community is crucial for larval growth and development (Dharampal et al., 2019; Dharampal et al., 2020) which is the only study that shows that a decreasing density of bacteria in pollen provisions can lead to increased larval mortality and slower growth in solitary bees.

Researchers have noted the presence of a distinct cohort of bacterial species in wild bees. One prevalent bacterial species in wild bees is *Apilactobacillus micheneri* which is a taxon closely related to *Apilactobacillus kunkeei*. *A. kunkeei* is commonly found in honey bee's crop and hive materials. In contrast to *A. kunkeei, A. micheneri* is more abundant in wild bee guts and pollen provisions. The genus *Apilactobacillus* comprises three distinct species: *A. micheneri, A.*

*timberlakei, and A. quenuiae*. These are collectively referred to as the *A. micheneri* clade in solitary bees. Moreover, the investigation comparing larvae and pollen provisions across three genera of *Megachilidae* identified a monophyletic Lactobacillus clade common to this group (McFrederick et al., 2017). Thus, there exists a combination of shared and diverse bacterial communities within the pollen provisions of related Megachilid species.

The *Apiactobacillus* clade is an abundant pollen and gut bacteria which possesses resistance to a broad range of antibiotics (McFrederick et al., 2014). A. micheneri is predicted to have a beneficial function for their host bees (McFrederick et al., 2012). Previous study suggests that *A. micheneri* can thrive in pollen and nectar-rich environments such as wild bee pollen provisions and their guts. Secondly, the *A. micheneri* genome contains pectate lyase which aids in pollen digestion, and has been shown to cause increase in the weight of bees (Zheng et al., 2017; Vuong et al., 2019). Thirdly, the divergence in genes of *A. micheneri* involved in osmotolerance, pH, and temperature tolerance suggests that these bacteria have adapted to wild bee guts (Vuong et al., 2019). In Chapter 3, the goal was to study the function *A. micheneri* and the microbiome as a whole by conducting fitness bioassays. Larvae were reared on natural pollen (pollen provisions containing environmentally collected microbiota) and sterile pollen (pollen provision lacking microbial communities). To study the role of the *Apilactobacillus* clade, *A. micheneri* was added to the natural pollen as well as the sterile pollen. To replicate a control group used in other studies, pollen provisions were treated with a mixture of antibiotics. For the individuals reared on different treatment groups, larval and prepupal weights, days taken to develop from egg to fifth instar larvae, and the weight of cocoon were recorded. To understand the community composition, the bacterial species present inside *M. rotundata* larvae from each

treatment were identified using 16s rRNA gene sequencing. These results will improve our knowledge about the role of the solitary bee microbiome in development and survival.

## Diapause in alfalfa leaf cutting bees

Diapause is an adaptation characteristic that enables insects to avoid unfavorable conditions by suspension of development. Concurrently, insects also dimmish their metabolic and physiological processes by arresting their cell cycle, and down regulating various pathways like Wnt/β-catenin pathway, FOXO signaling pathway, and the insulin signaling pathway (Nakagaki et al., 1991; Denlinger, 2002; Koštál et al., 2009; Hahn and Denlinger, 2011; Koštál et al., 2017; Shimizu, Tamai, et al., 2018; Shimizu, Mukai, et al., 2018). These adaptations confer distinct advantages to insects, particularly during brief periods of the year when they are essential for their development and reproduction. Guided by appropriate environmental cues, the diapausing insects will reinitiate active development when favorable conditions return (Koštál, 2006a). Numerous comprehensive reviews provide valuable insights into different facets of insect diapause like photoperiodic regulation, regulation due to various environmental cues, the hormonal changes, and various molecular mechanisms (Denlinger, 2002; Denlinger, 2008).

Insects store the metabolic reserves in three forms of macronutrients i.e., lipids, carbohydrates, and amino acids, and two micronutrient forms which are vitamins and minerals. During diapause initiation, individuals alter their metabolic demands and pursue an alternative developmental pathway compared to those undergoing regular metamorphosis. Diapausing individuals increase the production of glycerol, several classes of heat shock proteins (Koštál, 2006b), amino acids, and carbohydrates to synthesize protective molecules (Block, 2011). In addition, triglycerides are also the most important energy reserve in most diapausing insects and account for 80–95% of total lipid content. Some diapausing species accumulate larger

triacylglyceride stores than non-diapausing individuals to mitigate the metabolic demands of diapause. Triacylglyceride stores are mobilized from intracellular lipid droplets and play a role in modifying cell membrane fluidity and the deposition of cuticle waxes during the diapause preparatory phase (Tauber et al., 1986; Danks, 1987; Hahn and Denlinger, 2007).

Additionally, fat body glycogen is another key player which provides a major role in diapausing insects. Fat body glycogen reserves serve two primary functions in diapausing insects: firstly, they are converted to glucose or trehalose to be transported out of the fat body and used by tissues to fuel catabolism (Denlinger and Lee, 2010), secondly, they are metabolized to produce various sugar-alcohol and sugar-based cryoprotectant molecules (Jurenka et al., 1998; Michaud and Denlinger, 2006; Tomčala et al., 2006). In addition to accumulating reserves during the diapause preparatory phase, diapausing individuals suppress several physiological pathways and respiratory metabolism to reduce metabolic cost (Denlinger, 2003).

In diapausing organisms, the insulin signaling pathway plays a crucial role in reducing metabolism (Hahn and Denlinger, 2011) and initiating the accumulation of energy reserves (Satake et al., 1997). This process is facilitated by a critical downstream member of the pathway, the fork-head transcription factor (FOXO) (Baker and Thummel, 2007). When insulin is present, FOXO is suppressed. However, in the absence of insulin, FOXO becomes activated and moves from the cytoplasm into the nucleus. In the nucleus, FOXO initiates various responses, one of which is the accumulation of fat (Baker & Thummel, 2007). Furthermore, insulin signaling is a crucial pathway in metabolic reorganization, nutrient regulation, growth, and development in insects. It interacts with other pathways, such as Target of Rapamycin (TOR) signaling and AMP-activated protein kinase (AMPK), which are potential regulators of reserves for insect diapause. These pathways collaborate with insulin signaling to control body size and nutritional

status in insects (Grewal, 2009; Hahn and Denlinger, 2011). Beside such cell signaling modifications, diapause encompasses a wide array of mechanisms, cell cycle alterations, regulations of macronutrients and micronutrients, and stress tolerance, which has adapted and evolved to face various environmental challenges in insects. This conserved set of genes governing diapause development across insect species has been proposed by multiple research teams, referred to as the diapause "genetic toolkit" (Tammariello and Denlinger, 1998; Yocum et al., 1998; Rinehart et al., 2000; Rinehart and Denlinger, 2000; Rinehart et al., 2001; Uno et al., 2004; Zhang et al., 2004; Hayward et al., 2005).

*Megachile rotundata* is a bivoltine solitary bee that undergoes facultative diapause. Progeny within the population either enter diapause in the prepupal stage or skip diapause and become adults and produce a second generation. The environmental regulators of diapause have been reasonably well defined in *M. rotundata,* but of the molecular regulation of diapause is still in need of in-depth studies. Various transcriptomic differences have been found in *M. rotundata* while experiencing fluctuating thermal regimes (Torson et al., 2015; Torson et al., 2017), during early and late season diapause (Cambron-Kopco et al., 2022), and during various points of the insect's developmental history in diverse environmental settings (Yocum et al., 2018) . Previous studies have shown that developing a diapause toolkit with shared diapause-regulating genes may be technically challenging while conducting field and lab based studies for *M. rotundata*. This challenge arises from observed differences in gene expression profiles between diapausing prepupae of *M. rotundata* subjected to laboratory conditions versus those existing in natural field environments. Despite these transcriptomic differences attributed to varying environmental conditions, there persists a core set of transcripts collectively constituting the diapause "toolkit" essential for governing diapause regulation (Yocum et al., 2018). In another study conducted on

*M. rotundata*, genes associated with the diapause 'toolkit' were revealed to exhibit anticipated behavior across individuals undergoing diapause and those in the preparatory phase for diapause termination. This comparative transcriptome analysis suggests that various mechanisms within the diapause toolkit, such as enrichment for signaling pathways, chromatin remodeling, and activation of cell cycle processes, are also found to be involved in the diapause to post-diapause transition of *M. rotundata* (Yocum et al., 2015). Despite the intensive studies on transcriptional changes in individuals during diapause in different environments, from diapause transition to post-diapause and quiescent states, the molecular mechanisms underlining diapause initiation has remained elusive, until now. The research goal in Chapter 4 was to identify the key mechanisms and pathways during diapause initiation by comparing diapausing and non-diapausing individuals. Samples were collected during early and late field seasons. After confirming the diapause status, samples were sent for RNA sequencing. Transcriptomics analyses were performed to explore differential expression of transcripts, GO terms, and KEGG pathways involved in diapause initiation. Therefore, the fourth chapter, will enhances our understanding of *M. rotundata* physiology at the diapause initiation stage.

**Objective 1: To determine whether overwintering storage conditions impact the gut microbiota of honey bees, *Apis mellifera***

The study aims to investigate the impact of overwintering conditions on the honey bee gut microbiome. Recognizing the ecological factors influencing bee populations, the research focuses on the potential effects of temperature variations during the overwintering period. The key objectives include assessing how indoor overwintering at a stable temperature, compared to exposure to natural temperature fluctuations, influences the composition and diversity of the honey bee gut microbiome. The study predicts that indoor stored bees will exhibit differences in

bacterial communities compared to bees kept outside in natural fluctuating temperatures. Additionally, a significant difference in bacterial communities between two honey bee strains, Bolton bees, and Mann Lake bees when sampled over three months (October, November, and December) from different storage environments is also predicted. By exploring these hypotheses, the study aims to enhance our understanding of factors contributing to cold stress resilience and overwintering success, providing valuable insights for beekeeping practices and broader implications for honey bee health.

**Objective 2: To determine whether environmentally acquired gut-associated bacteria are critical for growth and survival in a solitary bee, *Megachile rotundata***

This study focuses on understanding the role of environmentally acquired gut associated microbes, particularly *A. micheneri*, in *M. rotundata*. While past studies have identified this bacterium and its clade in wild bee environments, the goal in the present study was to move beyond genomic predictions and delve into its actual functional role. Through microbial bioassays, the impact of the entire gut microbiome, as well as the specific role of *A. micheneri*, on bee development and survival will be tested. Predictions from this research include, (a) that the absence of intact gut microbiota will negatively affect overall health and survival, and (b) that introducing *A. micheneri* into larval provisions will have synergistic effects on the growth and development of *M. rotundata*. These anticipated results promise to significantly contribute to our understanding of the dynamic interactions between gut microbiota and solitary bee health and development.

## Objective 3: To identify gene expression differences associated with the transition to diapause in prepupal *Megachile rotundata*

The study focuses on unraveling the intricate molecular mechanisms and pathways that govern the initiation of diapause in *M. rotundata*, a bivoltine solitary bee exhibiting facultative diapause. The bees face a crucial decision, with individuals either entering diapause during the prepupal stage or bypassing it to become adults and contribute to the second generation. While previous studies have shed light on environmental regulators of diapause in *M. rotundata*, the molecular regulation in initiating diapause remains less explored. This study aims to identify the molecular differences between diapausing and non-diapausing individuals during the critical phase of diapause initiation. We anticipate identifying key transcripts and pathways that play a pivotal role in determining the developmental destiny of these individuals. Our prediction is that the major developmental decision involving diapause and direct development involve activity of differently expressed transcripts across various pathways, shedding light on the intricate genetic toolbox governing diapause in *M. rotundata*.

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# CHAPTER 2: HONEY BEES MAINTAIN THEIR MICROBIOME DURING OVERWINTERING STORAGE [1]


## Abstract

Honey bee gut microbiota play a number of specific roles in promoting host growth and physiology. While the gut microbiota of honey bee queens, workers, and larvae has been extensively studied, less is known about the gut microbiota of winter worker bees. This study investigates the dynamics of the gut microbiota in overwintering adult worker bees, focusing on two commercial bee strains: Bolton™ bees and Mann Lake™ bees. They were investigated under different storage conditions (indoor storage at 6°C and outdoor storage in natural conditions) during the winter months (October, November, and December). Utilizing 16S rRNA gene amplicon sequencing, we identified the microbial composition of the gut. We observed a [1]stable microbiota with slight variations in host-associated bacterial communities with *Lactobacillus* genus dominating in all the overwintering honey bee guts. Bolton bees exhibited higher abundance levels of *Bartonella*, *Bifidobacterium*, and *Wolbachia*, while Mann Lake bees showed increased abundance of *Commensalibacter*. Our results suggest that alpha diversity is influenced by the month rather than by the bee strain or storage conditions. This study also found significant difference in beta diversity by month. Overall, in this study a stable gut microbiota was observed in both indoor and outdoor environmental conditions across different months with slight abundance difference between bee strains. Considering all potential benefits of the honey


[1] Chapter 2 is in preparation for submission. Gagandeep Brar (GB) is the first author, with Lyna Ngor (LN), Quinn McFrederick (QSM), Preetpal Singh (PS), Arun Rajamohan (AR), Alex Torson (AT), Joseph Rinehart (JR), and Julia Bowsher (JB) as co-authors. GB, JR, and JB conceived and designed the study. GB and LN processed samples, and GB and PS analyzed the data. GB wrote the first version of the manuscript. All authors contributed to reviewing the final manuscript.

bee gut microbiome to health and nutrition, this study suggests that beekeepers need not be concerned about whether overwintering storage conditions affect the gut microbiome.

## Introduction

Honey bees pollinate crops worldwide (Parker et al., 2010). Winter presents a significant challenge for honey bee colonies in temperate regions. Over the last two decades, beekeepers have reported high colony losses over winter. These losses have been associated with low temperature stress, geographic relocation due to climate change, genotype, diseases, pesticides, and poor nutrition (Abou-Shaara et al., 2017; Genersch, 2010; Giannini et al., 2015; Hristov et al., 2020). In preparation for overwintering, a honey bee colony undergoes notable alterations in both the behavior and physiology of the worker bees. These changes include shifts in endocrine profiles, decreased individual activity, increased nutrient reserves, extended longevity, and thermoregulating cluster at the colony level (Genersch, 2010). Low temperatures affect honey bee survival (Q. Wang et al., 2016) through higher larval mortality and reduced life expectancy in adulthood (Steinhauer et al., 2021). To mitigate these deleterious effects, honey bees have evolved life history strategies like cessation of brood rearing in winters, increased immunity, and increased antioxidant expression (Aurori et al., 2014). Moreover, to reduce the cold temperature stress on the bee colonies, and to improve overwintering survival, bee keepers often store the hives inside. Storing hives under controlled cold climate conditions of 5°C - 7°C and 25% relative humidity has been shown to improve survival (Degrandi-Hoffman et al., 2019) (Owens et al., 1971) (Meikle et al., 2023).

Honey bees have shown vulnerability to temperature change through negative effects on the symbiotic gut microbiota (Coulibaly et al., 2022). The honey bee gut microbiome contains five core host-specific bacterial species that are highly conserved and comprise 95% of the total

microbes (Engel et al., 2016; Zheng et al., 2018). The hindgut of every adult worker contains *Snodgrassella alvi, Gilliamella apicola, Lactobacillus Firm-5, Lactobacillus Firm-4,* and *Bifidobacterium* species. Along with these core bacteria, there are non-core species including *Bartonella apis*, *Commensalibacter spp*., and some other identified and several unidentified species that have been collected from the surrounding environment like hives and plants (Kwong & Moran, 2016; Martinson et al., 2012). These bacterial species promote honey bee growth, physiology, facilitate breakdown of toxic dietary compounds, and modulate immune functions (Engel & Moran, 2013b).

Dynamic changes to the microbiota composition have been observed throughout the seasons (Almeida et al., 2023; Castelli et al., 2022a; Ludvigsen et al., 2015). The gut microbiota has been reported to differ between winter and summer honey bees, with reduced α-diversity and higher levels of *Bartonella* and *Commensalibacter* during winter (Kešnerová et al., 2019). Moreover, temperature during different months affects the composition of the gut microbiota by gradually changing the bacterial diversity between the seasons. In subtropical conditions, precipitation affects the composition of the honey bee gut microbiota (Castelli et al., 2022b). Numerous studies have been conducted to understand the seasonal dynamics of honey bee gut microbiota. However, very few studies have explored the effects of prolonged exposure to harsh climatic conditions on honey bee gut microbiome. For instance, direct exposure to high temperatures has been found to influence the gut microbiota in both honey bees and bumble bees, rendering them more resistant to heat stress (Hammer et al., 2021). Additionally, elevated temperature has shown to affect nectar microbes where the abundance of bacteria increased in the warmer temperature, leading in bumble bee forager preference (Russell & McFrederick, 2022a).

Beyond seasonality and temperature, vast differences in gut microbiota at the strain level has also been observed in two closely related honey bee species (Ellegaard et al., 2020). The gut composition exhibits greater divergence among genetically distinct bees at both phylotype- and sequence-discrete population levels (J. Wu et al., 2021). Additionally, significant variation in both composition and function among diverse Asian honeybee populations has been reported, showing patterns of their gut microbiota (Su et al., 2022). A previous study showed genetic divergence and functional convergence of gut bacteria in the eastern honey bee, *Apis cerana* and the western honey bee, *Apis mellifera* (Y. Wu et al., 2022). *Apis cerana* indica has a higher diversity in gut microbes compared to *Apis florea* (Khan et al., 2023). Differences in gut microbes between strains of *Apis mellifera* may influences the performance of those honey bee strains under different climate conditions.

The goal of this study was to assess whether overwintering storage conditions disrupt the stability and diversity of the gut microbes. To understand long term storage effects on gut microbiota, we compared the whole gut microbiota of two commercial strains of *Apis mellifera* i.e., claimed cold hardy bees https://boltonbees.com/pages/mn-hardy-hives (Bolton Bees, MN) that are bred and reared in Minnesota and Italian bees (Mann lake Bees, MN) of unknown rearing. Hives were stored either at a constant 6°C indoor or kept outside in natural conditions during winter. We predicted that hives stored outside in natural fluctuating temperatures will have different gut bacterial communities due to cold stress compared to hives stored in constant temperatures. We also predicted that bacterial communities would change when sampled over a three month period of falling temperatures, viz. October, November, and December.

## Material and Methods

### Insect samples

Honey bee hives were managed outside in the field from the first week of May to the last week of October and then some of them were shifted to refrigerated cargo containers for overwintering storage (6°C) in November while a few others were left outdoors in the field. First sampling was done on 10/17/2022 (base sampling of outdoor bees), followed by shifting of some hives inside storage on 10/21/2022. The second sampling was done on 11/15/2022 from hives both inside and outside. The third sampling was done 12/14/2022, again for both inside and outside hives (Table. 1)

Table 1. Representation of honey bee sample collected from various storage environments across different months.

| | Bee strain | | | |
|---|---|---|---|---|
| Months | Mann lake Bees: Outside (3 hives) | | Bolton Bees: Outside (3 hives) | |
| October | 3 bees/hive | 3 bees/hive | 3 bees/hive | 3 bees/hive |
| | Mann lake Bees | | Bolton Bees | |
| | Storage - Constant 6°C (3 hives) | Outside (3 hives) | Storage - Constant 6°C (3 hives) | Outside (3 hives) |
| November | 3 bees/hive | 3 bees/hive | 3 bees/hive | 3 bees/hive |
| December | 3 bees/hive | 3 bees/hive | 3 bees/hive | 3 bees/hive |

### DNA extraction

Three bees from each treatment were used for whole gut dissection after surface sterilizing bees using 1% sodium hypochlorite followed by three washing using sterilized water in sterile conditions. Whole gut samples were prepared by bead-beating the samples on a Qiagen Tissuelyser for 6 min at 30 Hz to disrupt recalcitrant cells. Samples for cell lysis were prepared by combining two 3-mm chromium steel beads and approximately 50 µl of 0.1 & 0.5 mm ZR

BashingBeads™ inside lysis tube (Biospec, Bartlesville, OK) with 750 µl of ZymoBIOMICS™ lysis solution and 20 µl of proteinase K. A subsequent round of bead beating involved rotating the samples for 6 minutes at 30 Hz, followed by an incubation period at 56°C for one hour. DNA was extracted using ZymoBIOMICS™ DNA microprep kit collection (Zymo research, Irvine, California) including 2 blank extractions as a no template control for further downstream analysis.

**PCR Amplification and Illumina Sequencing**

16S rRNA gene libraries for paired-end reads were generated following a previously published protocol (McFrederick & Rehan, 2016; Russell & McFrederick, 2022). The V5-V6 region of the 16S rRNA gene was amplified using 16S rRNA gene primers (799F mod3: CMGGATTAGATACCCKGG and 1115R: AGGGTTGCGCTCGTTG), each incorporating a unique barcode sequence. PCR1 reactions were conducted with 2 µl of DNA, 10 µl of 2× Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 10 µl of ultrapure water, and 0.5 µl of 10 µM 799F-mod3 and 0.5 µl of 10 µM 1115R primers. The reaction condition for PCR1 was 94°C for 3 minutes, 94°C for 45 seconds, 52°C for 1 minute, 70°C for 1:30 minutes, repeated step2 29X, and 72°C for 10 minutes to amplify this region. We ran gel electrophoresis to confirmed if 16s rRNA primers were successfully attached to our samples. To complete the Illumina adapter sequence, we initiated the process by cleaning the PCR product with exonuclease and shrimp alkaline phosphatase. Exonuclease was applied to remove excess primers, while shrimp alkaline phosphatase was used to eliminate residual deoxynucleoside triphosphates (dNTPs). Four µL of the 1x ExoSAP was mixed with each 7µL PCR1 sample. This reaction was incubated at 37˚C for 30 minutes and then 95˚C for 5 minutes. Following this initial cleanup, the purified PCR products were employed as templates for a second PCR. This

subsequent PCR (PCR2) utilized 1 µl of the cleaned PCR product as a template, using the same primers (PCR2F and PCR2R), and was conducted under conditions identical to the initial PCR. For PCR2, a 1 µl aliquot of Exo-Sap PCR1 product was utilized to conduct the second step of Illumina library preparation. In PCR2, the Exo-Sap PCR1 products were further amplified, incorporating linker poly-A primers for recognition on the sequencing platform. The PCR2 reaction conditions included an initial denaturation at 94°C for 3 minutes, followed by cycles of 94°C for 45 seconds, 58°C for 1 minute, 72°C for 1 minute and 30 seconds, with step 2 repeated 14 times, and a final extension at 72°C for 10 minutes, with final volume of 25 µl. To ensure uniform DNA input quantities, Invitrogen DynaMag TM-96 Side Skirted kit was employed for the normalization step across all samples prior to submitting a final volume of 10 µl for sequencing. The 16 rRNA gene sequencing was performed on NextSeq2000 P2 600 cycle kit (2x300xN/A) that produced total of 382.53 M reads at Q30 of 88.96%.

**Bioinformatics**

QIIME 2-2019 was used for the visualization and trimming of low-quality ends in reads from raw 16S rRNA sequence libraries. Subsequently, DADA2 was utilized for the assignment of sequences to amplicon sequence variants (ASVs), which represent 16S rRNA gene sequences with 100% matches. This process involved the removal of chimeras and reads with more than two expected errors. Taxonomy was assigned to the ASVs through the sklearn classifier, trained specifically for the 799-1,115 region of the 16S rRNA gene, utilizing the SILVA database. Additionally, local BLASTn searches against the NCBI 16S microbial database (accessed October 2023) were conducted. Features were filtered from the resultant ASV table, corresponding to contaminants identified in the blanks using the R package ‘decontam’ (version 1.10.0) at a conservative threshold of 0.5. This process aimed to identify contaminants while also

eliminating ASVs identified as chloroplast and mitochondria. To standardize the number of sequences per library, alpha rarefaction in QIIME2 was employed, and 4000 reads per sample were selected. This approach ensured the retention of 107 out of 108 samples while capturing most of the diversity.

Generalized Linear Mixed-effects Models (GLMMs) was used to model the Shannon diversity index. All GLMMs were fitted using the lme4 package (Bates et al., 2015) and lmer Test package (Kuznetsova et al., 2017) in R (R core team, 2022), using R studio version 4.2.2. Bee strain, storage treatment (exposure to natural vs. storage at 6 °C temperature), and sampling month were incorporated as fixed effects. Hive ID was incorporated as a random effect to account for the repeated measuring of each hive (Harrison et al., 2018). A stepwise backward selection process was employed for model selection. Initially, a full model was run with all fixed effects and random effects. Then all possible interactions among fixed effects were examined, followed by the systematic removal of one fixed effect at a time. The process continued until no further model improvement (e.g., lower AIC) could be observed. A difference of $\leq 2$ in the AIC indicated that models were similar, in which case the simpler model (i.e., with fewer parameters) was selected as the best-fit model (Meurisse et al., 2021). The best fit model was diagnosed for model assumptions through residual diagnostic plots using the 'DHARMa' package (Hartig, 2018). Finally, package 'ggeffects' version 0.16.0 (Lüdecke, 2018) was used to extract means, standard deviations, and 95% confidence intervals for the predicted values of Shannon diversity index for different sampling times. Tukey HSD tests for multiple comparisons between treatments was employed with estimated marginal means comparisons (EMMs) using the 'emmeans' function (Russell V. Lenth, 2024).

For beta diversity analysis, Adonis Bray–Curtis distance dissimilarities and nonmetric multidimensional scaling (NMDS) ordination were conducted in R v4.3.0 with ‘vegan’ package. The 'phyloseq’ (ver 1.34.0) R package was used for beta diversity analysis. Permutation analysis of variance (PERMANOVA) was applied using the ‘adonis’ function on distance matrices with 999 permutations. The differences between bee gut microbiomes including distance as a dependent variable and months (October, November, and December), bee strain (Bolton bees and Mann lake bees) and Storage Status (Inside and Outside) as independent variable were assessed using separate Adonis models. In all models, bee hives were accounted as a block-strata, where permutations were allowed among levels of all the variable, but within each level of bee hives (random variable), no permutations occurred.

To understand the effect of different variables on the abundance of candidate bacterial species, Generalized Linear Mixed-effects Models (GLMMs) was used to model the abundance of particular bacterial species. Bee strain, storage treatment (exposure to natural vs. storage at 6 °C), and sampling month were incorporated as fixed effects. Hive ID was incorporated as a random effect to account for the repeated measures of each hive (Harrison et al., 2018). The fitted GLMM and lm was diagnosed for issues with heterogeneity, overdispersion or missing co-variates using the residual plots. To evaluate the significance of fixed effect, analysis of variance tables for the GLMM was calculated using Wald chi-square tests. Tukey HSD tests was employed for multiple comparisons between treatments with estimated marginal means comparisons (EMMs) using the ‘emmeans’ function (Lenth, 2024).

# Results

## Alpha diversity

To investigate alpha diversity patterns, linear mixed models (LMM) that included Shannon diversity matrix as a response variable was applied. (Table 2). The most parsimonious, best-fit model included month as a fixed effect, and random effects for hive ID (different hives in each treatment) (Table 3). In contrast to the predictions, alternative models incorporating bee strain and storage treatment did not significantly improve the best-fit model. The random effects structure in the best fit model showed a relatively small variation among hives (For hives and replications, SD = 0.1604 & 0.0406, respectively), when compared to the residual error of the model (SD = 0.3560). This indicates variation between hives and the replications do not account for the majority of the variation in Shannon diversity. Furthermore, Shannon diversity index in October did not differ significantly from November ($p = 0.1275$), and December ($p = 0.398$), whereas it is significantly lower in November compared to December ($p = 0.0045$). Month explained only 8% of the variation in Shannon diversity, whereas the full model explained 23% of the variation in Shannon diversity. Overall, results indicate a temporal variation in Shannon diversity, but the strain of bee and whether hives were stored inside or outside had no effect (Figure 1).

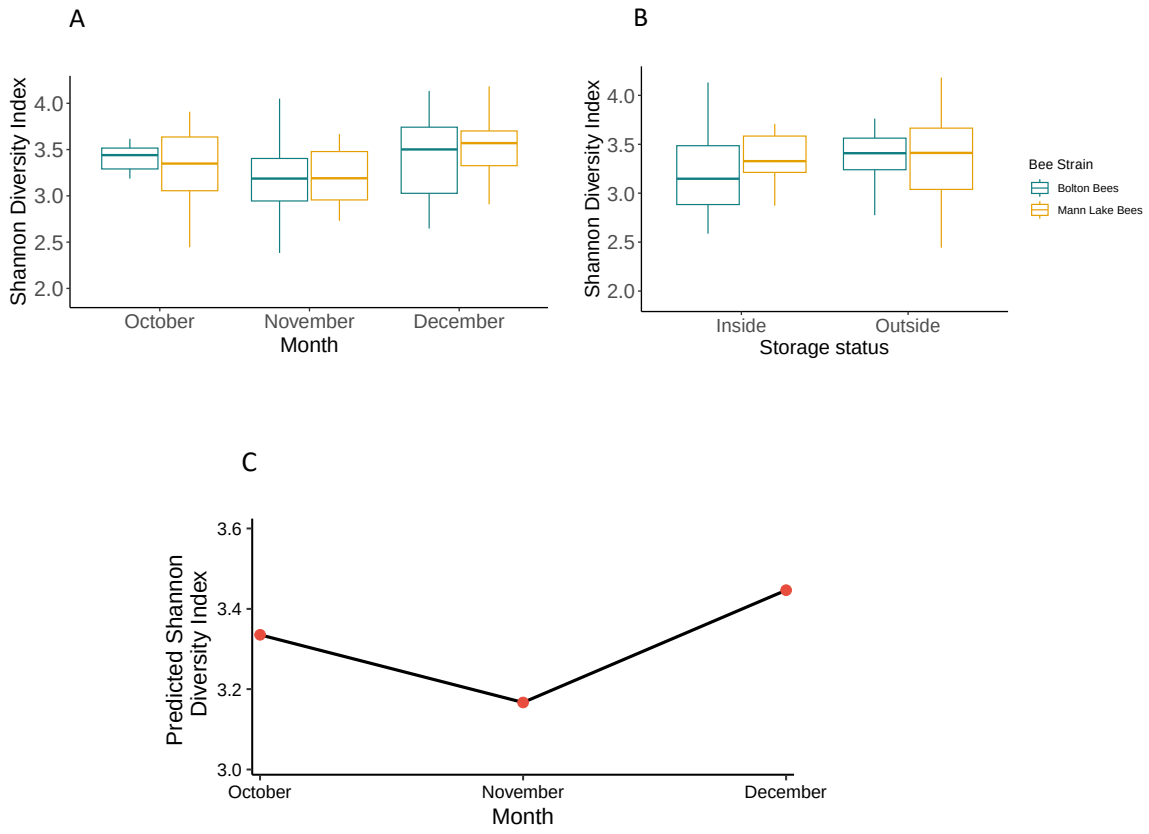


Figure 1. Plot shows alpha-diversity: Shannon diversity Index for Bolton bees and Mann Lake bees during different months (A) and different storage conditions (B)The line inside the box represents the median, while the whiskers represent the lowest and highest values within the 1.5 interquartile range. The outliers and individual sample values are shown as dots. (C) Model predictions for effect of sampling time on Shannon diversity index. Grey shading represents the 95% confidence interval.

Table 2. Comparisons of Akaike Information Criterion (AIC) value for different Generalized Linear Mixed Models (GLMMs) with relevant predictor and random variables. Best fit GLMM with the lowest AIC value is highlighted in bold. Δ AIC ≤2 indicated similar models, which leads to the selection of simpler model (i.e. with less parameters).

| Model | AIC |
|---|---|
| *Model: Shannon diversity index~* | |
| Storage status + month + bee strain + 1\|hive ID | 110.0 |
| Storage status * month * bee strain + 1\|hive ID | 112.8 |
| Storage status * month + bee strain + 1\|hive ID | 106.8 |
| Storage status + month * bee strain + 1\|hive ID | 114.6 |
| Storage status * bee type + strain + 1\|hive ID | 113.7 |
| Storage status + month + 1\|hive ID | 108.0 |
| Month + bee strain + 1\|hive ID | 108.1 |
| Storage status + bee strain + 1\|hive ID | 116.2 |
| Month * bee strain + 1\|hive ID | 110.9 |
| Storage status * bee strain + 1\|hive | 118.2 |
| Storage status + 1\|hive ID | 114.3 |
| Bee strain + 1\|hive ID | 116.4 |
| **Month + 1\|hive** ID | **106.1** |
| 1\|hive ID | 112.5 |

Table 3. Results of best fit Generalized Linear Mixed Model from Shannon diversity index.

| Model | | β Estimate ± SE | df | *t* value | *p* value | Variance | SD |
|---|---|---|---|---|---|---|---|
| *Shannon diversity index ~ Month + 1\|hive ID* | | | | | | | |
| Fixed Effects | (Intercept) | 3.33555 ± 0.07521 | 33.48419 | 44.352 | $< 2\times10^{-16}$ [a] | | |
| | Month (Reference: October | | | | | | |
| | November | -0.16872 ± 0.08512 | 95.20089 | -1.982 | 0.0503 | | |
| | December) | 0.11101 ± 0.08447 | 95.08469 | 1.314 | 0.1920 | | |
| Random Effects | Hive (Intercept) | | | | | 0.02506 | 0.1583 |
| | Residual | | | | | 0.1284 | 0.3584 |
| Number of observations = 107; groups: Hive, 12; | | | | | | | |

[a] Statistically significant at $p \leq 0.05$. SE = standard error; SD = standard deviation

**Beta diversity**

To investigate beta diversity patterns, we conducted PERMANOVA using adonis2 package in R, testing various models with distance matrices (phyloseq) as a response variable. This study aimed to assess the factors influencing the distance matrices, and a series of models were assessed to identify the factors influencing these matrices. The most parsimonious and best-fit model included storage status and month in interaction with bee strain as a fixed effect, and block (strata) as bee hive ID; *Distance ~ Storage status + Month * Bee strain, strata = hive* ID (Table 4). The overall composition of bee gut microbiomes differed by bee type (adonis F = 3.19, df = 1, $R^2$ = 0.0242, P = 0.002), month (adonis F = 5.09, df = 2, $R^2$ = 0.0872, P = 0.001),

but not storage status (adonis F = 0.57, df = 1, $R^2$ = 0.0047, P = 0.887). The interaction between month and bee strain was significant (adonis F = 1.57, df = 2, $R^2$ = 0.0269, P = 0.001). Month explained only 8.7% of the variation, whereas the bee type, and interaction between month and bee strain explained 2.4%, and 2.6% of the variation, respectively. Overall, these findings suggest that the microbial dynamics in overwintering honey bee workers are influenced by bee strain over the months, with stability observed in storage conditions (Figure 2). Pairwise comparison showed significant difference in gut microbiota when compared October – November (*p* value = 0.002), November – December (*p* value = 0.001), and October - December (*p* value = 0.001).

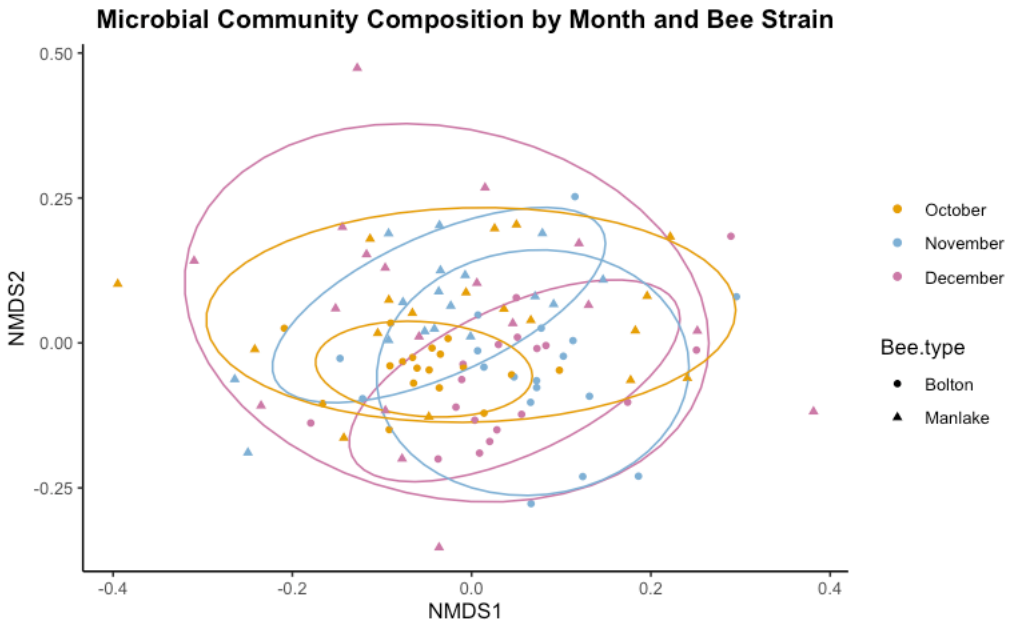


Figure 2. Non-metric multidimensional scaling (NMDS) performed with Bray-Curtis dissimilarity. Each point on the plot represents a honey bee gut sample and samples that share greater similarity are ordinated closer together. Ellipses on the plot indicate 95% confidence intervals.

Table 4. Best fit permutation analysis of variance (PERMANOVA) model applied using the adonis function with distance matrices with 999 permutations and bee hives accounted as a blocks – strata.

| Model | | df | Sum of squares | $R^2$ | F | Pr (>F) |
|---|---|---|---|---|---|---|
| *Distance ~ Storage status + Month * Bee strain, strata = hive ID)* | | | | | | |
| Fixed Effects | Storage Status | 1 | 0.0894 | 0.00487 | 0.5701 | 0.887 |
| | Month | 2 | 1.5975 | 0.08702 | 5.0950 | 0.001 |
| | Bee stain | 1 | 0.5001 | 0.02724 | 3.1903 | 0.001 |
| | Month * bee strain | 2 | 0.4945 | 0.02694 | 1.5771 | 0.021 |
| | Residual | 100 | 15.6767 | 0.85394 | | |
| | Total | 106 | 18.3581 | 1.00000 | | |

a Statistically significant at $p \leq 0.05$.

**Taxonomical abundance**

Following the removal of barcodes and primer sequences through QIIME2 (Demultiplexing), we obtained a collective sum of 34,879,494 paired-end 16S rRNA gene amplicon reads. The average read count per sample was 317,086, with an overall average quality score of 34. Across the 108 samples analyzed, the range of reads per sample varied from a minimum of 56,533 to a maximum of 1,314,819. Analyzing the taxonomic profile revealed a predominance of *Firmicutes* (70.96%), *Proteobacteria* (20.18%), and *Actinobacteriota* (5.83%) at the phylum level. We investigated the presence of the core gut microbiota and identified only a limited number of reads for *Snodgrassella*. *Lactobacillus* Firm-5 and *Lactobacillus* Firm-4 were not detected in our samples. We focused on examining the relative abundance of two non-core (*Bartonella* and *Commensalibacter)* and two core (*Gilliamella* and *Bifidobacterium*) microbes, along with the endosymbiont *Wolbachia*. The bacterial species-specific analysis for

relative abundance was performed using mixed model with formula: *Abundance ~ Storage status + Month + Bee strain + (1 | hive ID*).

Considering the importance of genus *Bartonella* in a previous study (Kešnerová et al., 2019), we analyzed the abundance of two *Bartonella* species: *Bartonella apis* and another denoted as *uncultured Bartonella*. *Bartonella apis* abundance did not differ by month (df = 99.11, F value = 2.27, *p*-value = 0.1085), bee strain (df = 38.26, F value = 0.1824, *p*-value = 0.6717), or storage status (df = 38.26, F value = .1824, *p*-value = 0.6717). The *Bartonella uncultured* significantly differed by month ( df = 98.72, F value = 4.74, *p*-value = 0.0107) while storage status (df = 34.58, F value = 0.0528, *p*-value = 0.81954) and bee strain (df = 11.559, F value = 0.1322, *p*-value = 0.72274) were not significant. We found a significant difference in abundance between December and November (SE = 1.03, df =97, *p*-value: 0.0085), but not between October and December (SE = 1.21, df =112, Tuckey HSD, *p*-value = 0.358) or October and November (SE = 1.21, df =112, Tuckey HSD, *p*-value = 0.456). For *Commensalibacter (Commensalibacter Acetobacteraceae_bacterium)*, bee strain (SE = 1.48, df =102, Tuckey HSD, *p*-value < 0.0001) was the only factor significantly influencing abundance with Mann lake bees having a higher abundance than Bolton bees. The effect of storage status (df = 107, F value = 0.261, *p*-value = 0.6097) and month (df = 107, F value = 1.6663, *p*-value = 0.1938) was not statistically significant.

Bee strain is a significant predictor of *Bifidobacterium* (*Bifidobacterium uncultured_Bifidobacterium*) abundance where *Bifidobacterium* showed higher abundance in Bolton bees compared to Mann Lake bees (SE = 0.077, df =102, Tuckey HSD, *p*-value = 0.0014). The storage status (df = 107, F value = 0.0682, *p*-value = 0.7944) and month (df = 107, F value = 0.8069, *p*-value = 0.4489) variables did not contribute significantly to the variation in

this model. The storage status (df = 37.34, F value = 6.67, *p*-value = 0.013) and month (df = 99.15, F value = 14.18, *p*-value = $3.808\times10^{-6}$) significantly affected the abundance of *Gilliamella* (*Unclassified  Gilliamella*), while Bee strain did not show a significant effect (df = 12.10, F value = 0.675, *p*-value = 0.426). Pairwise comparison showed significant differences in abundance during October and December (SE = 0.197, df =112, Tuckey HSD, *p*-value < 0.0001), November and December (SE = 0.166, df =97.5, Tuckey HSD, *p*-value = 0.002), but no significant difference during November and October (SE = 0.197, df =112, Tuckey HSD, *p*-value = 0.5361). A significant effect of the storage status on the abundance of *Gilliamella* was seen between the Inside and Outside bees (SE = 0.215, df =45, Tuckey HSD, *p*-value = 0.0272). storage status (df = 48.66, F value = 0.0377, *p*-value = 0.846) and Month (df = 99.42, F value = 0.97, *p*-value = 0.38) is not a significant predictor of abundance in *Wolbachia* (*Unclassified Wolbachia*). *Wolbachia* abundance was significantly different between the bee strains (df = 11.78, F value = 5.352 *p*-value = 0.03959). The results suggest a potential difference in gut microbial abundance between Bolton bees and Mann lake bees, but the significance was not very strong (SE = 0.243, df =14, Tuckey HSD, *p*-value = 0.0570). Overall, this taxonomic data emphasizes the marginal differences in gut microbiome composition between bee types, revealing a temporally stable microbial composition that is not influenced by storage conditions in overwintering worker honey bee (Figure 3 (for October), and supplementary figure 1 (for November and December)).

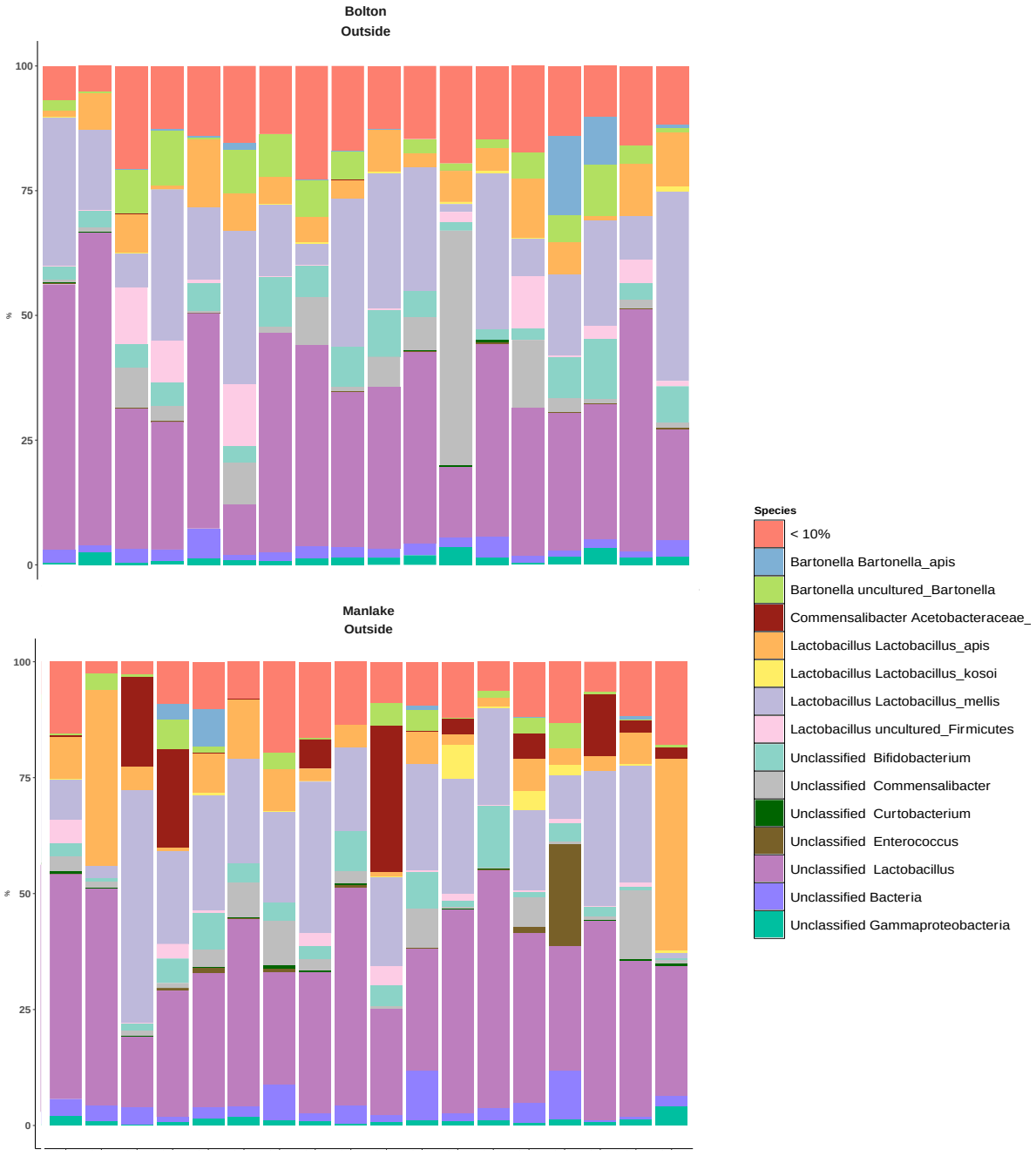


Figure 3. Relative abundances of the top fifteen bacterial species present in honey bee gut in different storage conditions in October. Less than 10% is a category of low abundance species that made up less than 10% of the median number of reads. Each column represents an individual bee. The relative abundance, represented in percentages, is shown on the y-axis.

## Discussion

Transcriptomic studies have shown that the loss of honey bee workers during the winters is due to weather conditions which is the primary stress factor, with starvation and diseases being secondary factors (Zhang et al., 2023). The honey bee gut microbiome has emerged as one of the most important physiological aspect to investigate due to its purported effects on honey bee health. The gut bacterial species within honey bee undergo alterations due to various environmental and developmental stresses, with temperature being a crucial one (Raymann & Moran, 2018). The honey bee gut microbiome has been shown to play functional roles in nutrition acquisition, including fermenting complex carbohydrates, aiding in digestion, and synthesizing essential nutrients (Kwong & Moran, 2016). Due to the two-way interaction, health benefits of the gut microbiota to bees and bees providing an optimum environment for these gut bacterial species to reside in, studying the gut microbiota in overwintering bees has become essential for ensuring the health and resilience of overall bee colony, particularly during extended periods of forage dearth in winter.

We used a 16srRNA gene amplicon sequencing approach to study the gut microbiota of overwintering adult worker bees between (a) two commercial bees (Bolton bees and Mann Lake bees), (b) different storage status/conditions and, (c) different months during storage/ a season. In this study, it was hypothesized that temperature would be one of the most important environmental stressors affecting the gut microbiota in overwintering honey bees. Surprisingly, we found no changes in the diversity, composition, or abundance of the microbiota when the bees were stored at constant 6°C or outside during the winter. Despite the physiological stress induced by cold temperatures on honey bee physiology, this study demonstrates that honey bees have the potential to acclimatize their gut microbiota despite the cold stress conditions. One

possible explanation for these findings is that honey bees rely on specific bacterial species to maintain their health and utilize the functions performed by the gut microbiota, and therefore the bees retain and maintain these bacterial species to extract sufficient resources from them. Interestingly, the overwintering bees were dominated by the *Lactobacillus* genus, which contributed to an increased bacterial load across all samples, regardless of storage conditions. As the most important genus in the guts of honey bees (Forsgren et al., 2010a; Vásquez et al., 2012), *Lactobacillus* plays an important role in the health of bees by showing probiotic characteristics. *Lactobacilli* have shown an ability to digest flavonoids and other compounds present in the pollen wall (Kešnerová et al., 2017), to inhibit pathogens (Forsgren et al., 2010b; Sabaté et al., 2009; Yoshiyama & Kimura, 2009) and to release shorth chain fatty acids and vitamins used by midgut cells as an energy resource (Den Besten et al., 2013). A higher abundance of *Lactobacillus* during overwintering phase may support heat production by worker bees, stabilizing colonies temperature. The only difference that was observed was the significantly high levels of *Commensalibacter* in Mann Lake bees compared to Bolton bees where the later one showed significantly higher levels of *Bartonella* (in some instances) Bifidobacterium, and Wolbachia as compared to Bolton bees.

Bolton bees represent genetic lines known for their resilience in Minnesota's harsh climate. This genetic line, named after the location where the parent Queen overwintered (Minnesota), is derived from the base stock called MN Hygienic. The queens from the MN-Hardy line, used as grafts for queen production, have successfully endured the challenges of a prolonged and frigid Minnesota winter. Typically, such severe winters could affect honey stores, cleansing flights, and brood-laying, but these MN-Hardy Queens are claimed to have demonstrated ability to thrive under these cold winter conditions

(https://boltonbees.com/pages/mn-hardy-hives). On the other hand, “OHB Italian” Mann Lake bees, sourced from Olivarez Honey Bees (OHB) in Northern California, are accompanied by claims for their hive performance, disease resistance, and overall robust health. The Italian Queens from OHB have reportedly undergone extensive breeding and careful genetic selection. The incorporation of traits such as "Minnesota Hygienic" and "VSH" (Varroa Sensitive Hygiene) further strengthens their innate ability to resist diseases and combat mite infestations. These two distinct types of bees have been specifically employed for their resilience to withstand the challenging winters typical of the Midwest, making them interesting subjects to study the gut microbiome during the overwintering period. In this study, differences in bacterial composition at the species level were observed between both bee types. Bolton bees exhibited significantly higher abundance of *Bartonella* (in some instances), *Bifidobacterium*, and *Wolbachia*, whereas Mann lake showed significantly higher abundance of *Commensalibacter* when compared to Bolton bees. In social bees, the transmission of symbiotic bacteria in the gut occurs among successive generations of siblings through social interactions. In a previous study on honey bees, the colonization of specific sets of bacteria, such as *Lactobacillus* Firm5 and *Bifidobacterium* Bifido-1.2, in genetically varied hosts strongly suggests that the genotype significantly influences the microbiota structure (J. Wu et al., 2021). Another study in honey bees has identified marked differences in the core gut microbial community when comparing different lineages, which include Maltese honey bees (lineage A) to the Italian honey bees (lineage C). Notably, Maltese honey bees exhibited an inverse proportion of *Lactobacillaceae* and *Bartonellaceae* when compared to Italian honey bees (Gaggìa et al., 2023). Functionality of these differences between bee strains have not been studied. This finding underscores the extent of strain-level diversity within the bacterial communities. Our study showed extensive overlapping

of the gut microbial strains among both bee types and difference in specific bacterial species. The results of the current study raises several fundamental questions regarding the evolution and maintenance of stable microbiota with slight change in specific host-associated bacterial communities.

The findings in this study reveal varying degrees of presence of bacterial species in overwintering honeybees across different months. There were significant differences in the richness and evenness (Shannon Diversity Index) between October and November but no differences were noted in the bee sampled in November and compared to December samples. Additionally, beta diversity shows difference in species diversity in different months. Before overwintering, foragers collect pollen, water, nectar, and propolis. Remarkably, the overwintering honeybee samples exhibited consistent dominance in species composition with no significant difference in their abundance across different months. However, a significant difference in beta diversity was observed and this is consistent with findings from prior studies (Bleau et al., 2020; Liu et al., 2021). These findings could be interpreted as that during the timeframe when the honey bee workers are confined to their hives without access to foraging, there is a lack of exposure to new environmental microorganisms to colonize and populate the bee gut. Previous studies have noted that the gut microbiota differs between winter and summer honey bees, with the long-lived winter bees exhibiting a stable microbiota with reduced α-diversity and higher levels of *Bartonella* and *Commensalibacter* (Kešnerová et al., 2019). This lower community alpha diversity with *Bartonella* and *Commensalibacter* as dominant bacterial species may confer certain physiological benefits. However, these studies also collectively reveal minor variations in gut microbial communities in temperate honey bee colonies during winter, indicating a shift in dominance, with the non-core bacterium *Bartonella* surpassing the core

bacterial species (Kešnerová et al., 2019; Liu et al., 2021; Papp et al., 2022). Another recent study also highlighted the significance of *Bartonella*, highlighting its expanded capability to convert metabolic wastes such as lactate and ethanol into pyruvate, which potentially provides energy for the host as well as other symbionts (Li et al., 2022). Considering the importance of *Bartonella* in previous studies, the current study analyzed two different species of *Bartonella* for their taxonomic abundance. However, the study shows *Lactobacillus* genus as dominant, present in higher abundance, contrasting with the findings of a previous study where the *Bartonella* genus showed dominance in winter bees. Lower diversity in the gut microbiota was observed in both summer and winter, with predominance of *Gilliamella apicola* and *Snodgrasella alvi* (Castelli et al., 2022b). Overall, we observed that the honey bee gut microbiota remains stable across different months whether under storage of natural conditions in mid-western part of the United States.

In this study, it is possible that months- or storage conditions-dependent variations in the hind gut core bacteria are either not detectable, or are being over-influenced by the non-core – non stable midgut bacteria. It is possible that slight differences in the relative abundance of bee-associated bacterial species are influenced by changes in other non-core hive-associated bacteria. A previous study (Engel & Moran, 2013a) identified co-evolutionary relationship of core gut bacteria and honey bee that is generally very similar and share the same core bacterial species. Measuring slight differences between bee strains in terms of absolute abundance would be challenging due to costs associated with increasing the depth of 16S sequencing. Additionally, 16S rRNA data cannot measure absolute abundances, only the relative abundance and general compositional nature of the microbiome. To enhance bacterial species detection resolution and functional profiling at the gene level, future studies could employ shotgun sequencing instead of

16S amplicon sequencing. Our study was also limited in timeframe, only sampling in the fall and early winter. We observed beta diversity drastically decline in the month of November, and it is likely that this pattern could change if bees were sampled during subsequent months, such as January, February, and March.

## Conclusion

In conclusion, our study shows that the gut microbiota of overwintering honey bees is dominated by one specific bacterial genus, *Lactobacillus*, which contribute to the bees' ability to withstand environmental stressors due to known functionality (Den Besten et al., 2013; Forsgren et al., 2010b, 2010a; Kešnerová et al., 2017; Sabaté et al., 2009; Vásquez et al., 2012; Yoshiyama & Kimura, 2009). Our study of the gut microbiota in overwintering honey bees reveals significant stability across different months and storage conditions. However, there was a significant difference in alpha and beta diversity, which was influences by month irrespective of storage conditions and different bee strain. Furthermore, our analysis reveals minor differences in the gut microbiota between different bee types, viz. Bolton and Mann Lake bees. Despite the microbial differences among the bee types, the overall stability of the gut microbiota across different months and storage conditions suggests a remarkable resilience of honey bee gut bacteria to environmental changes. This resilience may be attributed to the co-evolutionary relationship between honey bees and their gut microbiota, which has developed over millions of years (Engel & Moran, 2013a). Finally, our findings suggest that beekeepers need not be overly concerned about changes in the gut microbiota during the winter months, as the bacteria appear to remain stable regardless of storage conditions. Overall, our study contributes to the growing body of research on the gut microbiota of honey bees and its role in bee health during winter. By demonstrating the stability of the gut microbiota across different months of storage and storage

conditions, this study provides important insights for beekeepers and researchers alike. Further research is needed to explore the mechanisms underlying the resilience of honey bee gut bacteria and its implications for bee health.

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# CHAPTER 3: ENVIRONMENTALLY ACQUIRED GUT-ASSOCIATED BACTERIA ARE NOT CRITICAL FOR GROWTH AND SURVIVAL IN A SOLITARY BEE, *MEGACHILE ROTUNDATA* [2]


## Abstract

Social bees have been extensively studied for their gut microbial functions, but the significance of the gut microbiota in solitary bees remain less explored. Solitary bee females provision their offspring with pollen from various plant species, harboring a diverse microbial community that colonizes larvae guts. The *Apilactobacillus* is the most abundant microbe, but evidence concerning the effects of *Apilactobacillus* and other provision microbes on growth and survival are lacking. We hypothesized that the presence of *Apilactobacillus* in abundance would enhance larval and prepupal development, weight, and survival, while the absence of intact microbial communities was expected to have a negative impact on bee fitness. We reared larvae of the solitary bee *Megachile rotundata* on pollen provisions with naturally collected microbial communities (Natural pollen) or devoid of microbial communities (Sterile pollen). We also assessed the impact of introducing *Apilactobacillus micheneri* by adding it to both types of pollen provisions. Feeding larvae with sterile pollen + *A. micheneri* led to the highest mortality, followed by natural pollen + *A. micheneri*, and sterile pollen. Larval development was significantly delayed in groups fed with sterile pollen. Interestingly, larval and prepupal weights did not significantly differ across treatments compared to natural pollen-fed larvae. 16S rRNA


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gene sequencing found a dominance of the endosymbiont *Sodalis* when *A. micheneri* was introduced to natural pollen. The presence of *Sodalis* with abundant *A. micheneri* suggests potential crosstalk between microbial species, which may shape bee nutrition and health. However, the overall results of this study suggest that environmentally acquired gut bacteria do not impact the fitness of *M. rotundata*.

## Introduction

Insect-bacteria relationships extend from tight knit symbiotic mutualisms to commensal or parasitic interactions. Bacteria can be endosymbionts, living in specialized structures or guts of insects and enhancing insect fitness by providing essential nutrients (Lee et al., 1993; Douglas, 2003; Scully et al., 2014). Insects that depend on plants for food, such as bees, can benefit from microbes that digest plant tissues, facilitating the insect's access to carbohydrates, proteins, lipids, water-soluble vitamins, inorganic elements, and minerals (Brodschneider and Crailsheim, 2010) (Warnecke et al., 2007). Social bees serve as a model for symbiotically associated microbiome studies (Kwong, Medina, et al., 2017), but the microbiome of social bees is very different than that of solitary bees. In social bees, the gut harbors a core group of microbial species that is common across environments, is transmitted via social interactions, and are beneficial to the host's health. Unlike social bees, solitary species lack the transmission routes to acquire gut symbionts from nest mates. Bacteria present on plants are transferred to the guts of larval and adult solitary bees through pollen consumption, but these bacteria are not necessarily mutualistic and can be neutral or detrimental to fitness. Considering this functional disparity between social transmission and environmental transmission of bacteria in bees, the ecological relationship between pollen associated microbes and the fitness of solitary bees should be investigated.

The gut microbiota of social bees is species-specific and symbiotic. The gut microbiome plays a crucial role in promoting weight gain, hormone signaling (Zheng et al., 2017) and immune system function (Emery et al., 2017; Kwong, Mancenido, et al., 2017). Honey bee workers are dominated by five core, highly conserved host-specific bacterial species that comprise 95% of the total microbes inside the gut. The hindgut of every adult worker across the globe contains *Snodgrassella alvi*, *Gilliamella apicola*, two species of *Lactobacillus (Lactobacillus Firm-5 and Lactobacillus Firm-4.),* and *Bifidobacterium* species (Raymann and Moran, 2018). A few environmental bacteria that are present in pollen and hive surfaces also colonize the foregut and midgut but are not stably associated with the host (Martinson et al., 2012). Honey bee gut bacteria produce metabolites that promote host growth and physiology, facilitate the breakdown of toxic dietary compounds, and modulate immune functions in the gut (Engel and Moran, 2013). *Gilliamella apicola* which form a continuous lining layer over the ileum with *Snodgrassella alvi*, potentially contribute to an increase in the weight of honey bee workers by enabling bees to break down pollen using genes related to pectate lyase and hydrolases (Zheng et al., 2017; Zheng et al., 2019). Several bacteria, including *Lactobacillus* and *Bifidobacterium*, hamper the pathogens responsible for American and European foulbrood diseases (Vásquez et al., 2012; Killer et al., 2014). The detailed functions and roles of the gut microbiota in social bees inevitably raises the question of whether gut associated microbiota in solitary bees is of comparable significance and performs similar functions for the host.

Solitary bee gut microbiota comprises highly diverse, fluctuating, and non-host specific bacterial communities that are acquired from the pollen of multiple plant species (Voulgari-Kokota, Grimmer, et al., 2019). The hypothesis that the environment is the main source of bacterial transmission is supported by the presence of the same bacteria on flowers and in

association with multiple wild bee species (McFrederick et al., 2016; Kapheim et al., 2021), correlations between pollen sources and specific bee-associated bacteria (McFrederick and Rehan, 2016; Russell and McFrederick, 2022), and correlations between the bacterial communities present in pollen provisions and the guts of solitary bee larvae and adults (Keller et al., 2021;Voulgari-Kokota, Ankenbrand, et al., 2019) . Based on maternal foraging preference and geographical location, pollen provisions have a diverse and environmentally acquired microbial community from multiple bacterial families, including *Acetobacteraceae*, *Bacillaceae*, *Clostridiaceae*, *Enterobacteriaceae*, *Lactobacillaceae*, *Methylobacteriaceae*, *Moraxellaceae*, and *Sphingomonadaceae* (Voulgari-Kokota, McFrederick, et al., 2019). These bacteria colonize the larval gut but are lost during metamorphosis. The gut microbiome is regained when the emerged adults start foraging pollen (Mcfrederick et al., 2014). Based on genomic data, acidophilic bacteria present within solitary bee pollen have been suggested to safeguard against the proliferation of mold growing inside the nests (Mcfrederick et al., 2012). The pollen-borne microbiome can ferment the pollen mass, indirectly offering insects various nutritional advantages (Rosa et al., 1999; Rosa et al., 2003; Pimentel et al., 2005). Other environmentally acquired bacteria isolated from nests of solitary bees have bioactivity against disease causing fungi and bacteria (Potts et al., 2005). Thus, the pollen-borne microbiome may be important for growth, development, and survival in solitary bees.

*Apilactobacillus* species dominate the brood provisions of most solitary bee species (Mcfrederick et al., 2014). Comparative genomics studies have shown that *Apilactobacillus micheneri* has a pectate lyase gene that may help the larva digest pollen (Vuong et al., 2019), similar to the role of *Gilliamella* sp. in honey bee workers (Zheng et al., 2017). Additionally, *A. micheneri* can thrive in acidic environments, potentially inhibiting opportunistic pathogens

(Vuong et al., 2019). However, these findings are limited to genomic data and have not been tested by experimental manipulations and bioassays. *A. micheneri* may have detrimental effects on its host because *Lactobacilli* have a tendency to produce harmful metabolites like histamines and tyramines (Daliri et al., 2018). The direct effect of the microbiome on the biology of solitary bees must be investigated through microbial bioassays, empirically testing these genomic findings.

In this study, we conducted fitness bioassays and metabarcoding of gut microbial communities in *Megachile rotundata* larvae. We reared larvae on pollen provisions containing environmentally collected microbial communities (Natural pollen) and pollen provision lacking microbial communities (Sterile pollen). To study the role of the *Lactobacillus* clade in bee nutrition, *A. micheneri* was added to the natural pollen and sterile pollen. As reported in a prior study, wherein *A. micheneri* was determined to be the most abundant bacterial species in larval gut of *M. rotundata*, the same treatment group was established through the use of pollen provisions treated with a mixture of antibiotics (McFrederick et al., 2014). We used 16s rRNA gene sequencing to identify the bacterial communities present inside *M. rotundata* larvae from each pollen treatment to correlate the phenotype outcomes with the absence, presence, and changes in bacterial genera. We hypothesized that, (a) the absence of gut microbiota will deteriorate the overall health and survival and (b) adding *A. micheneri* to the larval provision will have positive effects on the physiology of *M.rotundata.* While we found evidence that the gut microbiome was beneficial in some respects, *A. micheneri* appeared to be not beneficial in all context and was pathogenic when present in abundance.

## Material and methods

### Animal collection

Alfalfa leafcutter bee nesting boxes were set up in a single location in the university alfalfa field plots near North Dakota State University (Cass County, Fargo, ND) where adult females foraged, collected pollen and nectar from surrounding vegetation. Mothers laid eggs on the top of pollen provision inside a cavity (paper straws) provided in nesting boxes. Straws having freshly laid eggs and pollen inside brood cells were collected on a daily basis. Straws were cut open using sterilized razor blades and eggs were sexed based on the position of the brood cell inside the straws, as *M rotundata* assign the front cell positions to male offspring (Yocum et al., 2014). To avoid sex-specific difference in growth rate and development, we used males by only collecting eggs from the first two cells. Eggs were collected from pollen provision using size zero paint brush (#1 Camel Hair Bristle, Wooster) and were distributed randomly across the treatments.

### Pollen treatments

*M. rotundata* provision are a mixture of pollen and nectar made of 33% to 36% of pollen and 64% to 67% nectar by weight, but the majority of the mass is pollen containing 1.3 million pollen grains and is 47% of sugar by weight (36). Fresh larval provisions were collected in the first 14 days of July and pooled to avoid floral and microbial variation. Field collected provisions were divided in half: one half was set aside (natural pollen: control) keeping the microbial community intact, and the other half was sterilized using gamma-irradiation (28 kGy for 12 h) at an off-site facility (VPT Rad – Radiation Lab & Test Services, Chelmsford, MA). Sterility of pollen was confirmed by plating on different agar plates (Lactobacillus MRS (de Man, Rogosa, and Sharpe agar) agar, trypticase soy agar + 5% defibrinated sheep blood, and LB agar) and

observing for growth after 3 days. Treatments were made using natural pollen (N) and sterilized pollen (S) placed in 96 well plates (150 mg of pollen/cell) (Figure 4, Table 5). Pure culture of *A. micheneri* was grown overnight in MRS broth with 2 % fructose at 25 °C. Our hypothesis was that *A. micheneri* was a beneficial microbe, so we increased the load to determine whether there would be improved growth and survival. The addition of *A. micheneri* was done on pollen provisions having an intact microbiome (NAm) and was also added to sterile provision to test whether *A. micherenri* would be beneficial when acting alone (SAm). Approximately 50,000 cells of *A. micheneri* were added to 150 mg of pollen in each cell of a 96 well plate in two doses. The number of bacterial cells was counted using a hemocytometer under a microscope (Agilent BioTek). The first dose was given before eggs were placed on the provision, and the second dose was 10 days afterwards. The same number of bacterial cells were embedded in 150 mg of sterile pollen (SAm) to know the effect of single bacterial species on growth and development of *M. rotundata*. An antibiotic cocktail (AC) treatment was made according to McFrederick (2014) consisting of 3ug/µl each of rifampicin, tetracycline, ampicillin, chloramphenicol, and erythromycin that was fed 3µl every other day for 8 days (McFrederick et al., 2014). McFrederick (2014) showed that *A. micherenri* was the most abundant bacterial species in *M. rotundata*, and that it was resistant to antibiotics. This antibiotic cocktail from the previous study served as another treatment group to determine if we could obtain reproducible results. All treatments had 3 replicates of 96 well plates for a sample size of 288 individuals per treatment.

Freshly laid eggs were transferred from field-collected straws on top of the pollen placed in 96 well plates using a fine point round brush and were allowed to develop till fifth instar larvae. A different approach was used to place eggs on a sterile diet to maintain their sterility. Surface sterilization of eggs was done using two washes of contact lens wash solution (BioTrue)

and three washes of sterilized water (Xu and James, 2009). To avoid sinking into pollen, eggs were placed on black autoclaved filter paper placed in sterilized petri-plate. When eggs started showing 1st instar emergence, they were transferred to sterile pollen inside in 96 well plates. No surface sterilization was done to eggs transferred to natural pollen. Data were collected for various parameters, including the number of days taken for development from egg to 5th instar larvae, the weights of 5th instar larvae and prepupa (recorded 4 days after cocoon spinning was completed), the weight of cocoons spun by 5th instar larvae, and the percent mortality of larvae. Larval weight was measured at the end of the feeding period and therefore is a measure of the maximum weight prior to pupation. We also measured prepupal weight, which is the weight of the bee after initiating metamorphosis and spinning the cocoon, but prior to molting into the pupal stage. All individuals in this study were reared at 25°C and 70% RH. For comparing the effect of different treatments on larval weight, prepupal weight, cocoon weight, and days taken to develop from $1^{st}$ instar to $5^{th}$ instar, the non-parametric Kruskal-Wallis test and Dunn's test with bonferroni-type adjustment were used at alpha = 0.05.

Table 5. Table describing pollen treatments fed to *Megachile rotundata* larvae.

| Treatments | Description | Dose |
|---|---|---|
| Natural pollen (N) | Pollen having naturally occurring intact microbiome | 3µl of PBS |
| Natural pollen + *A. micheneri* (NAm) | *Apilactobacillus micheneri* added to natural pollen | ~50,000 cells in PBS fed twice (1st day and 10th day) |
| Sterile pollen (S) | Sterilized pollen using gamma-irradiation (28 kGy for 12 h) | 3µl of PBS |
| Sterile pollen + A. micheneri (SAm) | *Apilactobacillus micheneri* added to sterile pollen | ~50,000 cells in PBS fed twice (1st day and 10th day) |
| Antibacterial cocktail (AC) | 3µg/ul each of rifampicin, tetracycline, ampicillin, chloramphenicol, and erythromycin (in PBS) added to natural pollen (McFrederick, 2014) | 3µl of of antibacterial cocktail was fed every other day for 8 days |

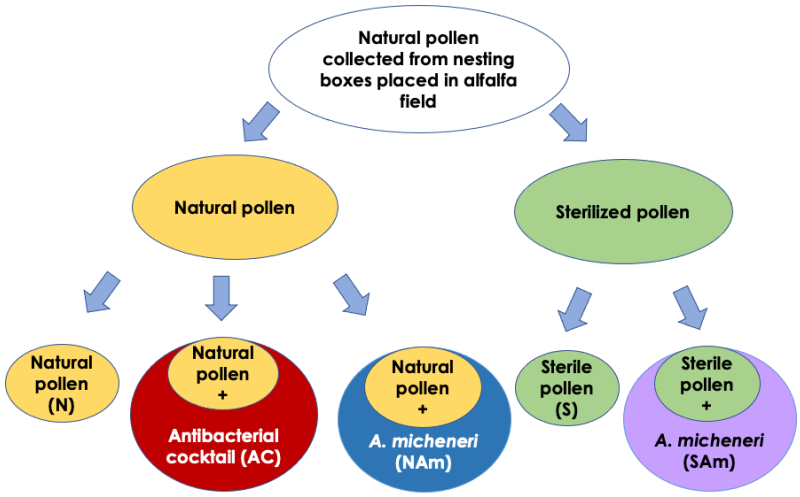


Figure 4. Illustration of different treatments used to feed *Megachile rotundata* larvae for growth and survival study.

**16s rRNA gene sequencing**

Twenty 5$^{th}$ instar larvae of *M. rotundata* from each treatment were snap-frozen for microbial analysis. The larvae were surface sterilized using 1% sodium hypochlorite followed by three washes using sterilized water in sterile conditions. Whole larvae were used for extraction of

DNA using tissue collection plates (Qiagen, Germantown, MD) followed by bead beating the samples on a Qiagen Tissue Lyser for 6 min at 30 Hz for recalcitrant bacterial cell lysis. Samples for cell lysis were prepared by adding two 3-mm chromium steel beads and ~50 µl of 0.1-mm zirconia beads (Biospec, Bartlesville, OK) in 180 µl of Qiagen buffer ATL and 20 µl of proteinase K. A second round of bead beating was done by rotating plates for 6 min at 30 Hz followed by incubation at 56°C for an hour. Qiagen DNeasy Blood and Tissue protocol was used for the rest of the DNA extraction process and 3 blank extractions were included as a no template control for further downstream analysis.

**PCR Amplification and Illumina Miseq Analysis**

16s rRNA gene libraries for paired end reads were prepared using previously described protocol by (McFrederick and Rehan, 2016; Russell and McFrederick, 2022).We used the 16s rRNA gene primers (799F mod3, CMGGATTAGATACCCKGG and 1115R, AGGGTTGCGCTCGTTG) having unique barcode sequence (38)to amplify V5 -V6 region of the 16s rRNA gene. To amplify this region, we performed PCRs using 4 µl of DNA ,10 µl of 2 × Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 10 µl of ultrapure water, and 0.5 µl of 10 µM 799F-mod3, 0.5 µl of 10 µM 1115R primers with an annealing temperature of 52°C for 25 cycles. To complete Illumina adapter sequence, we first cleaned the PCR product with exonuclease and shrimp alkaline phosphatase to remove excess primers and deoxynucleoside triphosphates (dNTPs), respectively, then we used the cleaned PCR products as the template for a second PCR. We performed the second PCR with 1 µl of cleaned PCR product as a template with primers PCR2F (CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC) and PCR2R (CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC) (Kembel et al.) under

identical conditions to the initial PCR. 18 µl of PCR product was normalized using SequalPrep Normalization plates (Thermo Fisher Scientific, Waltham, MA) and 5 µl of normalized product from each sample was pooled into a single sample. In order to perform Ultraclean sequencing, the pooled library was cleaned with AMPure XP beads (Beckman Coulter, Brea, CA) to remove primer-dimers and excess master mix components. Finally, library quality was assessed using 2100 Bioanalyzer (Agilent, Santa Clara, CA) and multiplexed libraries were sequenced using Miseq Reagent Kit with MiSeq sequencer (Illumina) with 2 X 300 cycles, at the IIGB Genomics Core, UC Riverside.

**Bioinformatics**

QIIME 2-2019 was used to visualize and trim the low-quality ends of reads from raw 16S rRNA sequence libraries. DADA2 (Callahan et al., 2016) was used to assign sequences to amplicon sequence matches (ASVs; 16S rRNA gene sequences that are 100% matches) followed by removing chimeras and reads with more than two expected errors. Taxonomy was assigned to the ASVs using the sklearn classifier trained to the 799-1,115 region of the 16S rRNA gene with the SILVA database (Bokulich et al., 2018; Quast et al.). We also conducted local BLASTn searches against the NCBI 16S microbial database (accessed June 2022). Features were filtered out from the resulting ASV table that corresponded to contaminants as identified in our blanks and R package decontam (version 1.10.0) (Davis et al., 2018) at conservative threshold at 0.5 to identify contaminants along with removal of ASVs identified as chloroplast and mitochondria. Alpha rarefaction in QIIMME2 was used to normalize the number of sequences per library and 12,000 reads per sample were selected to retain samples and still capture the majority of the diversity. Alpha diversity was analysed using the Pielou eveness index using the Kruskal-Wallis test in QIIME2 and beta diversity was tested using Adonis Bray─Curtis distance dissimilarities

and nonmetric multidimensional scaling (NMDS) ordination in R v4.3.0 (Bunn and Korpela, 2013) with the package vegan (J, 2009). Betadisper function in the vegan package was used to check for differences in dispersion between treatment groups. Permutational multivariate analysis of variance (PERMANOVA) analysis based on rarefied Bray–Curtis matrices (pairwise BH-FDR correction) was performed in QIIME2 to determine the statistical significance of differences in bacterial communities between treatments.

## Results

### Growth and survival

We predicted that sterile pollen would have a negative effect on larval and prepupal weight, and that addition of *A. micheneri* would increase weight. Neither of these predictions were supported. We found an overall effect of treatments on larval weight (Kruskal-Wallis chi-squared = 110.22, df = 4, $p < 2.2e\text{-}16$) where only antibiotic treatment had a significant ($p < 0.05$), negative effect on larval and prepupal weight in pairwise comparisons to natural pollen. There was no significant effect of treatment on larval weight when individuals were fed on natural pollen + *L. micheneri* ($p = 0.22$), sterile pollen ($p = 0.60$), and sterile pollen + *A. micheneri* ($p = 0.62$) as compared to natural pollen (control) fed individuals (Figure. 5A). Pairwise comparisons showed no significant effect of the addition of *A. micheneri* on prepupal weight as compared to control (Figure 5B; natural pollen + *A. micheneri* ($p = 0.60$), sterile pollen ($p = 1.0$), sterile pollen + *A. micheneri* $p = 0.104$). Prepupae developed on the antibiotic cocktail also weighed significantly less as compared to all other treatments and control ($p < 0.05$). There was an overall effect of treatment on prepupal weight (Kruskal-Wallis chi-squared = 81.765, df = 4, $p < 2.2e\text{-}16$), but that was due to the antibiotic treatment weighing less than control. None of

the other treatments were significantly different from control, indicating that *A. micheneri* and gut microbes in general do not influence the final weight gained at the end of the larval stage.

The microbiome influenced the duration of the larval stage (Kruskal-Wallis chi-squared = 245.5, df = 4, $p < 2.2e-16$). Larval development (number of days until cocoon spinning started) was significantly delayed when individuals were fed on sterile pollen ($p < 0.05$), sterile pollen + *A. micheneri* ($p < 0.05$), and the antibiotic cocktail ($p < 0.05$) as compared to control. There was no significant difference in developmental days when larvae grew on natural pollen + *A. micheneri* compared to control ($p = 1.0$) (Figure 5C). After cocoon spinning was over, weight of cocoon spun by larvae in different treatments was measured. Larvae spun significantly heavier cocoons when fed with sterile pollen ($p = 0.001$), sterile pollen + *A. micheneri* ($p= 0.02$), and natural pollen + *A. micheneri* ($p < 0.05$) as compared to control. Antibiotic cocktail-fed individuals spun significantly lighter cocoon as compared to all other treatments and control ($p = 0.007$) (Figure. 5D).

The difference in microbiome significantly affected survival of the larvae (Kruskal-Wallis chi-squared = 11.9009, df = 4, $p = 0.02$). Pairwise comparisons showed those individuals fed on sterile pollen + *A. micheneri* has significantly higher mortality than individuals fed on natural pollen ($p = 0.0077$, alpha = 0.05) or sterile pollen ($p = 0.0060$, alpha = 0.05). Antibacterial cocktail fed ($p = 0.0313$, alpha = 0.05) and natural pollen + *A. micheneri* ($p = 0.0384$, alpha = 0.05) fed individuals have significantly higher mortality than individuals fed on natural pollen. No other pairwise comparison for mortality showed a significant difference. The Schneider-Orelli formula (corrected % mortality ± SE) was used to calculate corrected percentage mortality (46). Sterile pollen + *A. micheneri* pollen feeding caused the highest mortality (36.98 ± 7.37 %) followed by the antibiotic cocktail (14.45 ± 4.3 %), natural pollen +

*A. micheneri* fed (6.30 ± 1.6 %), sterile pollen fed (3.75 ± 1.17 %), and natural pollen fed (0.82 ± 1.04 %) (Table 5). These results suggest that *A. micheneri* have a negative effect on larval survival.

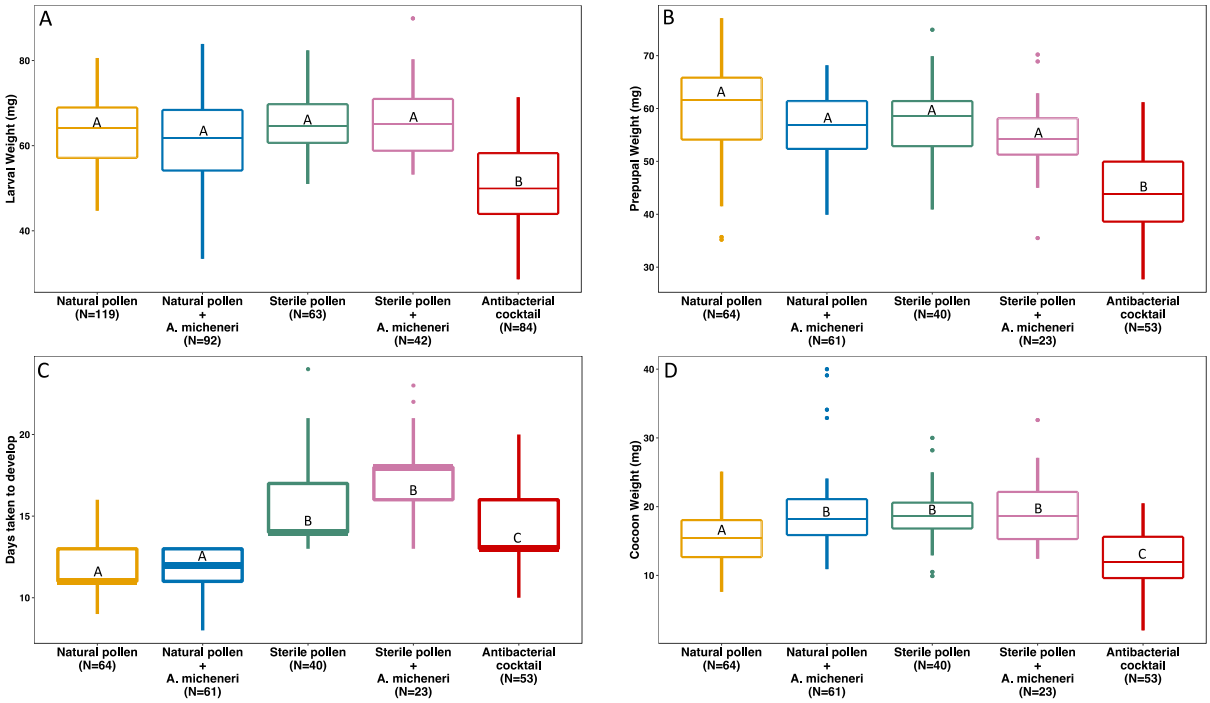


Figure 5. Larval weight (A), prepupal weight (B), days taken to develop from 1st instar to 5th instar (C), and cocoon weight (D) after feeding on pollen treatments. The central line of the boxplot represents the medians, boxes comprise the 25-75 percentile and whiskers denote the range. Data was analyzed using Dunn test (p-values adjusted with the Bonferroni method). Different letters indicate significant difference between treatments.

Table 6. Data shows the corrected mortality percentage using Schneider-Orelli's formula after feeding larvae on different pollen treatments. * represents the significant difference of a treatment from natural pollen and ** represents the significant difference of a treatment from sterile pollen (Dunn test, Kruskal-Wallis chi-squared = 11.9009, df = 4, p = 0.02).

| Treatments | Corrected % mortality ± SE |
|---|---|
| Natural pollen (N) | 0.82 ± 1.04 |
| Natural pollen + *A. micheneri* (NAm) | 6.30 ± 1.6 * |
| Sterile pollen (S) | 3.75 ± 1.17 |
| Sterile pollen + *A. micheneri* (SAm) | 36.98 ± 7.37 *, ** |
| Antibacterial cocktail (AC) | 14.45 ± 4.3 * |

**Bacterial taxonomic profile and relative abundance**

Following demultiplexing using QIIME we obtained a total of 9,669,732 paired end 16s rRNA gene amplicon reads with an average of 94,801 reads per sample and an average quality of 38. Across the 102 samples, the lowest number of reads was 32,441 and the greatest was 186,025. The taxonomic profile shows that samples were dominate by the members of *Firmicutes, Proteobacteria*, and *Actinobacteriota* at the phylum level.

Bees fed on natural pollen (N) had microbiota dominated by *Lachnospiraceae* family (Clostridia), *Sodalis*, and *Apilactobacillus micheneri*, with highly variable abundances between individuals. We predicted that bees fed on Natural pollen + *A. micheneri* (NAm) would be dominated by the *A. micheneri* because this bacterial species was fed to them in abundance. Surprisingly, these bees were dominated by the endosymbiont *Sodalis*, although *Apilactobacillus micheneri* was found in all samples. In the treatment where *A. micheneri* was added into sterile pollen (SAm) we detected *Apilactobacillus micheneri,* but the samples were dominated by a diversity of bacterial species that represent <2.5% of the relative abundance of the total microbiome (Figure 6). Overall, an average reads per sample of *A. micheneri* across treatments were N- 14409 (SE ± 4253. 75), NAm- 13587(SE ± 3398.26), S- 276 (SE ± 86.84), SAm- 3595 (SE ± 1173.67), AC- 38853 (SE ± 6002. 89) . We observed that larva fed on sterile pollen still had 89,932 reads on average, which indicates that individuals were not axenic. Although the most prevalent bacteria, found in other treatments, decreased in sterile pollen, species with less than 2.5% relative abundance were still detected. Prior to transferring eggs to the pollen, we tested that sterilized pollen was still sterile by plating on multiple agars. We observed no growth of any microbe after 3 days. Therefore, bacterial present in these samples likely came from other environmental sources besides the pollen. A similar trend was seen in mason bees, where sterile

pollen fed to larvae of *O. bicornis* showed low count bacterial reads in the bees (Voulgari-Kokota et al., 2020). *A. micheneri* was dominant in larvae fed on pollen treated with antibiotics (AC), reproducing results found by McFrederick *et al*., 2014, which showed selection for a higher relative abundance of this bacterial species.

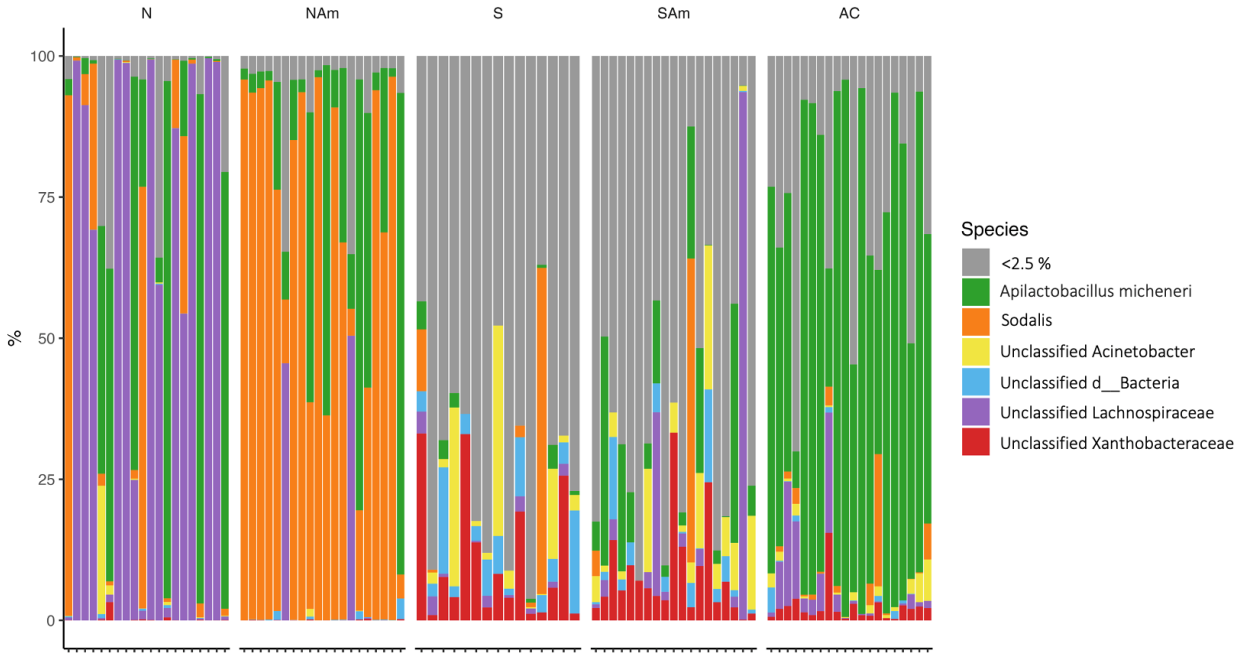


Figure 6. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. Treatments were: natural pollen (N), natural pollen with added *A. micheneri* (NAm), Sterile pollen (S), sterile pollen with *A. micheneri* (Sam), and natural pollen with an antibacterial cocktail (AC). Less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. Each column represents an individual bee. The relative abundance, represented in percentages, is shown on the y-axis.

**Diversity analysis**

Alpha diversity of bacteria, as calculated by Pielou evenness, was significantly different between Natural pollen + *A. micheneri* (NAm) compared to control and all other treatments. The Pielou evenness was higher in the sterile pollen + *A. micheneri* (SAm) and sterile pollen (S) treatment followed by subsequent decline in evenness in antibiotic cocktail (AC) treated pollen and natural pollen (N). There were no significant differences obtained in the values of diversity

between Natural pollen (N) and antibiotic cocktail treatment (AC) and sterile pollen (S) and sterile + *A. micheneri* (SAm) treatment (Kruskal–Wallis test at $p > 0.05$) (Figure 7). Bray Curtis dissimilarities showed that beta diversity of bacteria differed by the treatments (Bray Curtis bacteria, adonis $F = 3.66$, $R2 = 0.141$, $df = 4$, $p = 0.001$). Beta dispersion analysis revealed significant clustering of bacterial communities in antibacterial cocktail treatment (AC) and natural pollen + *A. micheneri* (NAm) (Figure 8, adonis $F = 9.58$, $df = 4$, $p = 0.001$ and betadisper $F = 14.69$, $df = 4$, $p < 0.001$). Pairwise comparisons using PERMANOVA show the significant difference between pollen with antibiotics (AC), sterile pollen (S), and sterile pollen with added *A. micheneri* (SAm) as compared to Natural pollen (N) (Table B1). The introduction of an antibiotic cocktail to pollen (AC) resulted in a significant alteration of the bacterial community, with *A. micheneri* dominating. Furthermore, irrespective of the pollen sterility in NAm, the addition of *A. micheneri* did not induce a change in the composition of the larval bacterial community when compared to sterile pollen.

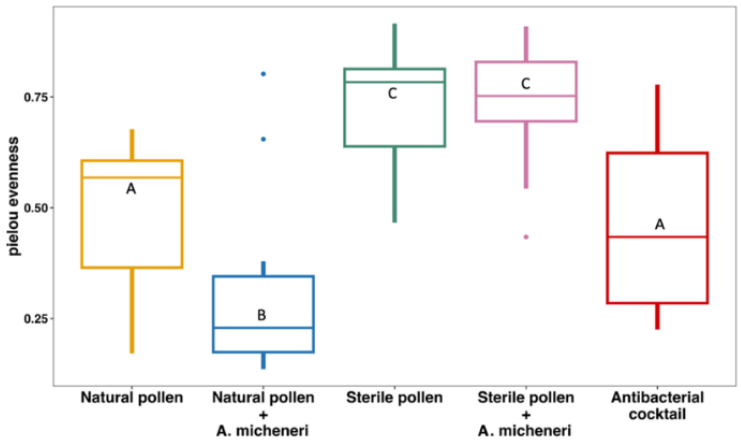


Figure 7. Pielou evenness indices using bacterial ASVs. Bacterial diversity is reduced in Natural pollen with added *A. micheneri* (NAm), as compared to Sterile pollen (S), Sterile pollen with added *A. micheneri* (SAm), and Natural pollen treatment (N; control). The central line of the boxplot represents the median, boxes comprise the 25-75 percentile and whiskers denote the range. Different letters indicate significant differences detected between treatments using a pairwise Kruskal-Wallis test.

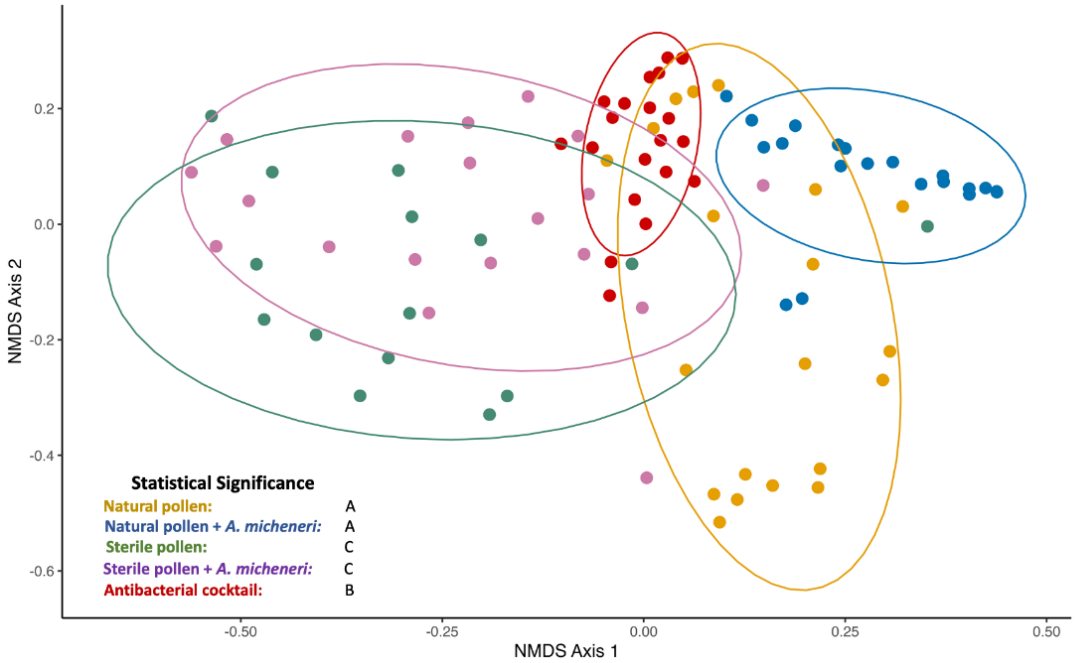


Figure 8. Nonmetric multidimensional scaling (NMDS) ordination of Bray─Curtis distance matrices representing bacteria from different treatments. Letters indicate significant differences detected between treatments. Overall antibacterial cocktail treatment samples were the most significantly different from control and other treatments. Ellipses denote 95% confidence intervals.

## Discussion

The goals of this study were to determine whether absence of environmentally acquired, non-symbiotically associated microbiome negatively affects the larval growth and development of *M. rotundata*. Additionally, the study aimed to investigate whether providing excess amount of *A. micheneri* enhances the fitness of *M. rotundata*. We found that developmental time was delayed when larvae ingested sterilized pollen provisions compared to control group. However, there was no significant difference in body weight at the end of larval period across all the treatment except antibiotic fed larvae. This suggests that *M. rotundata* larvae are able to attain full body weight in the absence of an intact pollen microbiome, possibly compensating by increasing developmental time. Similar trends have been observed in other invertebrates where axenic insects exhibit phenotypic differences and delayed development when compared to

gnotobiotic groups. A delay in development has been observed in axenic individuals of *Aedes aegypti* (Correa et al., 2018 Feb 13), *Drosophila* (Ridley et al., 2012), and *Caenorhabditis elegans* (Szewczyk et al., 2006). This implies that eliminating the pollen microbiome primarily impacts developmental time, suggesting a role for the microbiome in facilitating weight gain, but not being required for weight gain.

Our results show that the pollen microbial community is not essential for weight gain in *M. rotundata* larvae. Larvae reared on sterile pollen didn't show any reduced effect on larval and prepupal weight compared to pollen with intact bacterial communities. Similar to our study, previous studies have shown that there was no significant difference in larval development or prepupal weight when *M. rotundata* individuals were reared on a sterile pollen using gamma irradiation as compared to the natural pollen having all the environmental bacteria (Inglis et al., 2015). Moreover, another study showed bees weighed more when fed a sterile diet compared to pollen that had naturally occurring bacteria (Inglis et al., 1992). Pollen sterilized using propylene oxide to get rid of all microbes had no differences in mortality of *M. rotundata* larvae when compared to pollen having natural microbiota (Inglis et al., 1992). A previous study in *M. rotundata* demonstrated that larvae fed on pollen mixed with antibiotics had reduced weight and increased mortality (McFrederick et al., 2014). However, based on the antibiotic treatment in this study, that observed pattern may be due to direct toxicity of antibiotics and may not be due to a dysfunctional microbiota. Our results both support (survival and development) and contrast (weight) a previous study conducted on the solitary bee *Osmia ribifloris* where natural pollen with intact microbial communities was mixed with sterile pollen and fed to larvae to determine the effect of the microbiome on host weight (Dharampal et al., 2019). *O. ribifloris* larvae fed an increasingly sterile diet exhibited a significant decrease in wet weight, growth rate, and survival.

The study in *O. ribifloris* is the only report of a negative impact of sterile pollen on the weight of the host in solitary bees (Dharampal et al., 2019). While sterile pollen may not consistently influence bee weight, it remains essential to delve deeper into the microbial interactions for a holistic understanding of their effects on solitary bee biology and health.

A second goal of our study was to determine whether *A. micheneri* is beneficial to the development of *M. rotundata* larvae. Previous genomic studies have suggested that the presence of *A. micheneri* might be beneficial to its host. It could potentially optimize the absorption of nutrients in the gut, leading to improved growth and survival (Vuong et al., 2019). To test the role of *A. micheneri*, we added it to both control pollen and sterile pollen, with the expectation that it would colonize the gut. However, neither treatment had an overabundance of *A. micheneri* in the gut, although *A. micheneri* was present in the gut of larvae fed on natural pollen. Our sequencing results reveal that when *A. micheneri* was added to sterilized pollen (SAm), instead of *A. micheneri* being dominant, we observed an increase in the presence of rare bacteria. This suggests that the larval fed on SAm wasn't solely *A. micheneri* but a mix of rare bacteria and *A. micheneri*. Ingesting pollen having mixture of rare taxonomical bacteria and *A. micheneri* (SAm) had more severe consequences on larval survival than ingesting sterilized pollen that was dominated by rare bacteria (S). Adding an excess of *A micheneri* to natural pollen did not increase the amount of *A. micheneri* in the gut, nor did it have an effect on larval weight, development time or survival. Whether *A. micheneri* is beneficial or detrimental to the bee host may therefore depend on context. For example, *A. micheneri* might not be directly detrimental to bees but may lead to a pollen microbiome that increase larval mortality in the right context. In a different context *A. micheneri* may even be beneficial. This idea is supported by the mortality data from pollen provisions that received no treatment (N). *A. micheneri* was present in all

samples and dominated 8 of those samples in the natural pollen treatment, and the survival rate of those bees was 99 percent. *Apilactobacillus* clade bacteria was the most abundant bacteria in larval gut when fed on antibacterial cocktail, which is similar to what has been demonstrated previously. Additionally, the earlier study illustrated the resistance of the *Apilactobacillus* clade to a combination of rifampicin, tetracycline, ampicillin, chloramphenicol, and erythromycin (McFrederick et al., 2014). However, the poor performance of larvae on the antibacterial cocktail can be explained by either a negative effect of *A. micheneri*, or the toxic effects of the antibiotics themselves. Thus, the effects of an excessive load of *Apilactobacillus* bacteria on survival in solitary bees still remains unclear.

Interestingly, when *A. micheneri* was added to pollen with the natural microbiota (NAm), the endosymbiont *Sodalis* dominated the bacterial communities in the larvae. Our taxonomical data shows that when *Sodalis* dominated the overall microbiome, *A. micheneri* was present in minimal quantities (Table B2). Similar patterns were observed in Osmia aglaia, Lactobacillus was absent when Sodalis dominated at 96% prevalence (Saeed and White, 2015). In the halictid bees *Halictus ligatus* and *Lasioglossum pilosum*, *Sodalis* was absent when Lactobacillus dominated the microbiome at 94% and 9%, respectively (Saeed and White, 2015). Similar trend was also seen in Osmia excavate, where dominance of *Sodalis* decreased the abundance of *A. micheneri* and vice-versa (Liu et al., 2023). *Sodalis* is best studied in the tsetse fly Glossina morsitans and rice weevil Sitophilus oryzae, and it functions differently in these two insect species. In weevils, *Sodalis pierantonius* play an important role in exoskeleton development and nutrition (Vigneron et al., 2014). whereas no clear function has been documented for *S. glossinidius* in tsetse flies (Balmand et al., 2013). In several Hymenopterans, *Sodalis* is maternally inherited and can potentially compromise reproductive compatibility (Figueroa et al.,

2021). Symbiosis between halictid bees and *Sodalis* appears to be in its early life stages of evolution; *Sodalis* strains are vertically transmitted and found at higher prevalence in solitary versus social halictids. This suggests that the prevalence of *Sodalis* differs between bee species but still remains unclear whether *Sodalis* resides within the lining of the host gut, present in bacteriocytes, or transmitted vertically from mother to their offspring.

DNA barcoding using 16S provides relative abundance data, but not absolute abundances. This limits our ability to make specific conclusions regarding the role of *Sodalis* in determining the total microbial composition and diversity in *M. rotundata*. For example, *Sodalis* might be repressing *A. micheneri* in one treatment vs another, but this is hard to definitively demonstrate without measuring absolute abundance of both species across all treatments. Analyzing absolute abundance using qPCR or detecting bacterial species using shotgun sequencing metagenomics can identify potential crosstalk between the *Apilactobacillus* clade and *Sodalis*. Moreover, when *A. micheneri* was introduced to sterilized pollen (SAm), neither *Sodalis* nor *A. micheneri* emerged as dominant members of the microbiome. This raises the question whether *A. micheneri* requires the presence of other bacteria to sustain itself and engage in interactions with *Sodalis*. Our work paves the way for future studies aimed at locating *Sodalis*, understanding its symbiotic functions, and the crosstalk of gut microbiome-endosymbiont-host physiology interactions.

## Conclusion

This study highlights that the dependency for the growth and survival on gut associated bacteria is not a universal phenomenon across bee species. Environmentally acquired non-host specific bacteria might not shape solitary bee fitness in all host species. More phenotypic traits like adult reproduction, flight performance, and overwintering survival should be included to

better understand the functions performed by of gut microbiomes in solitary bees. Furthermore, this research opens new avenues for understanding interactions between gut microbiomes and endosymbionts. A future goal arising from our study would be to study the functional role of *Sodalis* bacteria in solitary bees using histological, immunological, and network analyses. This understanding will help us to better understand the importance of environmentally gathered microbiomes for solitary bee survival, growth, and development.

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# CHAPTER 4: TRANSCRIPTOMICS REVEALS COMPLEX MOLECULAR MECHANISMS OF PREPUPAL DIAPAUSE IN *MEGACHILE ROTUNDATA* [3]


## Abstract

Environmental cues regulate diapause initiation in facultatively diapausing insects. In the alfalfa leafcutting bee, *Megachile rotundata*, the major cues for diapause are maternally regulated. Many candidate pathways associated with diapause progression have been identified in *M. rotundata* diapause and post-diapause quiescent individuals. However, the molecular mechanisms and functions of the same pathways during diapause initiation are largely unknown. The goal of this study was to understand gene expression during diapause initiation and to identify genes that were differentially expressed at that decision point. Samples were collected during early and late in the field season, and after confirming diapause status, RNA sequencing was performed on the samples. Diapause-destined and non-diapause-destined prepupae were compared and differentially expressed genes involved in oxidative stress, cell signaling, and other diapause-related pathways were identified. Key findings include cell cycle arrest, downregulation of insulin signaling, suppression of Wnt signaling and immune responses, upregulation of lipid biosynthesis, and potential involvement of autophagy-related mechanisms. These results shed light on the molecular networks governing diapause initiation in *M. rotundata*.


[3] Chapter 4 is in preparation for submission. Gagandeep Brar (GB) is the first author, with Preetpal Singh (PS), Alex Torson (AT), George Yocum (GY), Joseph Rinehart (JR), and Julia Bowsher (JH) as co-authors. GB and PS collected all the field data. PS and JH analyzed field data. GB and AT developed the pipeline for transcriptome data and contributed to data visualization. GB, AT, and JH contributed to experimental design and writing of the manuscript.

## Introduction

Insects overcome seasonally reoccurring stressors using a variety of physiological adaptations. The most important physiological adaptation against harsh winters is the diminished metabolic activity and developmental arrest associated with diapause (Denlinger, 2002; Gill, et al., 2017). Diapause is associated with metabolic suppression, stress tolerance, and nutrient storage (Deng et al., 2018). These mechanisms are regulated by suppression of signaling pathways like insulin (Wadsworth et al., 2015), mTOR (target of rapamycin) (Miki et al., 2020), Wnt (Chen and Xu, 2014), lipid metabolism (Hahn and Denlinger, 2007), and cell cycling arrest (Koštál et al., 2009) which are common in all diapausing insects irrespective of life stage.

The regulatory mechanisms of diapause are complex. The "toolkit" of conserved pathways has been studied intensively in recent years, but many underlying gene networks are still poorly understood. Most of these signaling systems are interconnected and work together to facilitate the diapause response. Insulin signaling appears to be a promising candidate for growth regulation and regulating energetic reserves during diapause. In the cotton bollworm, *Helicoverpa armigera*, insulin-like peptides are present in higher levels in the hemolymph of non-diapausing than in the diapausing pupae (Li et al., 2017; Zhang et al., 2017). Low levels of insulin-like peptides may downregulate Akt, leading to activation of transcription *factor forkhead box O transcription factor* (FOXO), and promoting the diapause phenotype. Fat accumulation and enhancement of stress tolerance are linked to the cross talk between increased expression of FOXO and downregulation of insulin signaling pathway that might be critical for diapause (Zhang et al., 2017). FOXO acts as a master switch that mediates multiple downstream effects related to diapause such as regulation of cell cycle and fat hypertrophy. While FOXO is a

crucial participant in diapause, not all aspects of diapause are triggered by this pathway. Cyclins and Cyclin-dependent kinases play an important role in cell cycle arrest (Poupardin et al., 2015).

Lipid storage during diapause preparation is associated with a decrease in digestive proteases and an increase in the expression of fatty acid synthase (Robich and Denlinger, 2005). The composition of the fat body is regulated through subcellular mechanisms that interact with the brain-endocrine system. In this intricate system, the brain plays a central role, receiving input from various organs via signaling responses. TOR signaling is a sister pathway to insulin signaling, sometimes collectively referred as insulin/TOR pathway, sharing many key elements that may sense nutritional status and cue the accumulation of additional reserves prior to diapause (Colombani et al., 2003). Several transcriptomic studies have revealed a role of TOR signaling in diapause regulation of Diptera, Hymenoptera, and Lepidoptera (Ragland and Keep, 2017). Yet, it is unknown how the fat body interprets and transduces amino acids using TOR prior to and after diapause. Chromatin remodeling and cell cycle arrest related genes crosstalk with insulin signaling and upstream gene related to TOR signaling (Yocum et al., 2015). The Wnt pathways are linked to insulin, TOR, ecdysone, and JH, suggesting multiple connections to the regulation of diapause. Most of the genes associated with Wnt pathways are downregulated during diapause (Ragland and Keep, 2017). Therefore, the regulation of insect diapause seems to be governed by the crosstalk between the various pathways at the transcriptomic level.

*Megachile rotundata*, the alfalfa leafcutter bee, exhibits facultative diapause where individuals either pause their development during pre-pupal stage or develop into adults in summer. Day length may mediate diapause because eggs laid during longer days tend to develop into non-diapausing individuals than if the mother bee experiences a shorter day length. The mother seems to play a significant role in the diapause status of offspring, either by directly

sensing photoperiod and transmitting that information to the egg (Pitts-Singer, 2020) or by providing some other maternal effect (Wilson et al., 2021). The ovaries of *M. rotundata* have different expression profiles under long and short days, suggesting that environmental cues are transmitted to the egg (Hagadorn et al., 2023). While many studies have noted the importance of daylength, temperature may also impact diapause initiation in *M. rotundata* prepupa. But studies related to regulation of diapause at transcript level is still unknown. To identify differentially expressed transcripts associated with diapause, expression profiles between diapausing and non-diapausing individuals during early and late season were compared. The prediction in this study was that the major developmental decisions between diapause and direct development of offspring involve numerous genes across different pathways.

## Material and Methods

### Insect collection

Nest cavities lined with paper straws that contained offspring were collected and placed inside an incubator at 25°C for five days until they developed into the 5th instar larvae (Pitts-Singer and Cane, 2010). *M. rotundata* builds linear nests within each straw. The larva from the first brood cell in each nest was removed and snap frozen four days after the cocoon spinning event (pre-pupal stage). Individuals in the first brood cells were chosen from straws that had at least six brood cells so that the remaining individuals in the nest could continue to develop and be monitored for diapause status. These five individuals from the straw were allowed to develop for 7 more days, and then were X-rayed (Faxitron 43804N, Faxitron Bioptics, Tucson, AZ, USA) to confirm diapausing status. The frozen samples that had all their nestmates going into diapause were denoted as a diapausing prepupa and the samples that had all the nestmates skipping diapause and growing into adults were denoted as non-diapausing prepupa. Five diapausing and

five non-diapausing samples were collected in the last week of July (early season) and second week of August (late season) and used for RNA extraction. To determine the probability that designating the diapause status of the first position individual based on the diapause status of their nestmates could be incorrect, a larger population of nests were examined. The diapause status of individuals from 845 nests (4,465 individuals) from the same field season was determined. Of those nests, only seven had a diapausing larvae in the first cells position while the rest of the nest were non-diapausers, which is a 0.8% failure rate for correctly predict non-diapausing individual. Only four nests had non-diapausers in the first position with the rest of the nest being diapausers, which is a 0.5% failure rate for correctly predict diapausing individuals. Only 20 individuals were selected for sequencing from this population and the likelihood that their status was misassigned was 1.3%.

**RNA sample processing, library preparation, and sequencing**

Total RNA was extracted from the same prepupae using QIAGEN All prep- DNA/RNA mini prep Extraction Kit (Qiagen, 80204, Hilden, Germany). After analyzing the quantity and purity of total RNA using a spectrophotometer, integrity was measured using Qubit (concentration no less than 20 ng/µl). Total RNA was dissolved in RNAase free water and further assessed at the University of Georgia Sequencing Facility. RNA integrity was confirmed using Agilent Bioanalyzer for high quality RNA extraction. For the RNA-seq study, 20 NGS stranded mRNA libraries from the total RNA of diapausing individuals (Early and late season) and non-diapausing individuals were prepared for sequencing on Illumina Nextseq 500 PE75 HO platform using a standard protocol from the University of Georgia Genomics Facility (Athens, GA, USA).

**Transcriptome assembly and annotation**

For RNA-seq analysis, raw paired end reads sequence were generated from Illumina Nextseq 500 having 30X sequencing depth. Quality trimming on the raw RNA-seq reads (BioProject accession: NCBI) was performed using fastp (Chen et al., 2018a) with the default quality threshold (Phred ≥ 15). Assessment of library quality was carried out pre- and post-trimming using FastQC (Version 0.11.9) (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data, no date). The quality-trimmed reads were then mapped to the *M. rotundata* genome (accession number: PRJNA66515) utilizing HISAT2 (Kim et al., 2019) Subsequent to mapping, clean transcriptomes were assembled with StringTie (Pertea et al., 2015) after generating summaries of reading mapping using MultiQC (Version 1.7) (Ewels et al., 2016). All the assemblies generated from each sample were merged using TACO (Niknafs et al., 2016) to create consensus transcriptome for downstream analysis. All quality control, mapping and transcript assembly was conducted using NDSU CCAST computing system (https://ondemand.ccast.ndsu.edu/).

**Pairwise differential expression analysis**

We used the prepDE.py script to obtain transcript count matrices to determine level of significance (p.adj < 0.05) using DESeq2 (Love et al, 2014) for differential gene expression analysis between diapausing and non-diapausing individuals. A fold change cut-off (reported in $\log_2$ scale) was not used for differential expression analysis because removal of differently expressed genes having smaller effect sizes could have larger biological effects that might change the topology of downstream functional analysis, like KEGG pathways and GO enrichments. Blast2go (Götz et al., 2008) and topGO package (Alexa, 2010) was used for annotation and enriched gene ontology (GO terms), respectively for testing pairwise differential

expression comparisons. The analysis of enrichments involved the use of combinations of 'parentChild' (Grossmann et al., 2007) algorithm with 'Fisher exact' statistics. The enrichment of KEGG metabolic pathways within pairwise differential expression comparisons was done using clusterProfiler package, version 4.2.2 (Wu et al., 2021). KEGG orthologs from differential expression comparisons were plotted using the pathview package (Luo and Brouwer, 2013).

## Results

### Differential expression and enrichment analysis

We sequenced mRNA libraries from 21 individuals across diapausing and non-diapausing individuals. The libraries ranged from 60.13 to 23.10 million read pairs (average 39.4 ± 12.7 (mean ± SD) with 92.8 ± 0.62 % of the reads greater than Q30. Summary shows that on an average, 91 ± 3.7 % of the reads mapped to the reference genome after trimming. The complete mapping is shown in the Supplementary table 1.

### Differential expression of transcripts, Gene Ontology, and KEGG pathway

Principal component analysis (PCA) of the RNA-seq profiles showed that the first component explains 24% and the second component explains 12% of the variance separating the transcripts of diapausing individuals from non-diapausing individuals. We observed complete overlap of ellipses when comparing the early season and late season individuals (Figure A). The effects of seasonality were accounted for in DESeq2. The transcripts from diapausing or non-diapausing individuals were analyzed in the model (Figure 9). 1,592 differentially expressed transcripts were analyzed when pairwise comparison was made between diapausing and non-diapausing individuals. A fold-change cut-off was not used thus preserving the downstream functional analyses such as GO enrichment and KEGG pathway. Exclusive use of the fold-change cut-off was solely intended to determine the expression directionality of transcripts.

Specifically, among the total differentially expressed transcripts during diapause, 175 transcripts were observed that exhibited increased abundance with a log2FoldChange>0; $p < 0.05$. Conversely, 1417 transcripts displaying decreased abundance with a log2FoldChange < 0 ($p < 0.05$) were identified. Next, transcripts were categorized into functional groups using GO and KEGG categories. The GO term enrichment analysis revealed that the regulation of diapause was linked to increased abundance of transcripts involved in metabolic and catabolic processes, lipid biosynthesis processes, negative regulation of gene expression, and macromolecule methylation in diapausing individuals (Supplementary table 2). In addition, the increased abundance in GO terms related to metabolic processes, cellular metabolic processes, and phosphorus metabolic process were noted in diapausing individuals (Figure 10A). In contrast, transcripts related to regulation of cellular process and biological process, cellular response to stimulus, cell communication, cell cycle, mRNA processing showed decreased abundance in diapausing individuals (Figure 10B). KEGG pathways analysis used to identify potential pathways related to diapause showed two pathways that were enriched in differentially expressed transcripts. These enriched pathways were related to Cysteine and methionine metabolism (gene ratio=13/378) and drug metabolism (gene ratio=9/378). KEGG pathway analysis showed transcripts associated to cell cycle arrest, decreased signaling pathways, decrease metabolism, arrest of DNA replication, and arrest of growth hormone synthesis in diapausing individuals. On the other hand, non diapausing individuals' transcripts associated to cell division activation, apoptosis, hormonal changes. ABC transporter activation, and biosynthesis of secondary metabolites (Supplementary table 3).

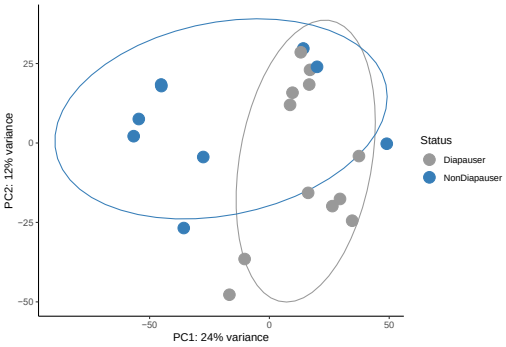


Figure 9. Principal component analysis (PCA) shows clustering of RNA-seq samples by diapausing status. While PC1, explaining 24% and PC2, explaining 12 % of the total variance, separates diapausing individuals from non-diapausing individuals. Ellipses on the plot indicate 95% confidence intervals.

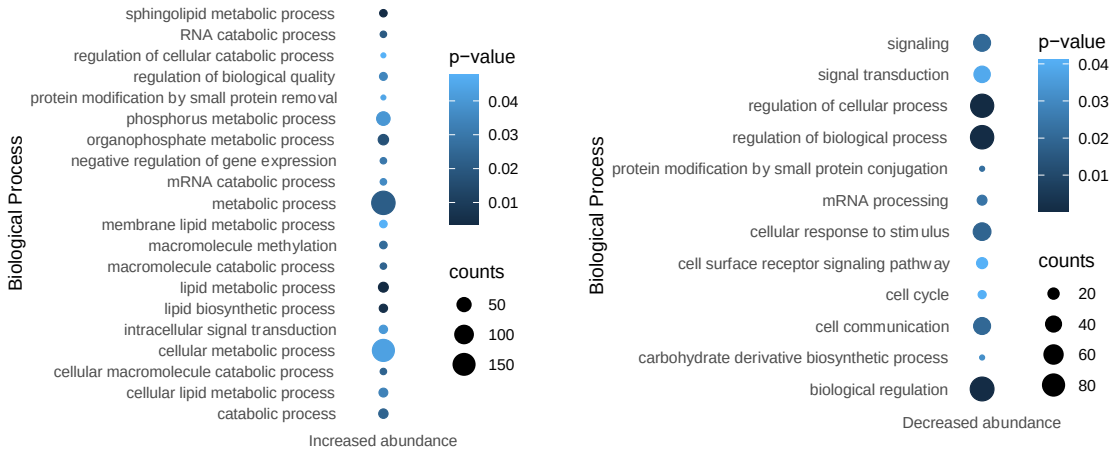


Figure 10. Bubble charts showing the differentially expressed transcript present in various GO biological processes. The y-axis represents different pathways; the x-axis indicates (A) increased abundance of transcripts in a given pathway in diapausing individuals, and, (B) decreased abundance of transcripts in a given pathway in diapausing individuals. The size of the bubble is proportional to the number of differentially expressed transcripts in the pathway; the color corresponds to the p-value, indicating statistical significance of the enrichment.

## Discussion

A core set of transcripts collectively constituting the diapause "toolkit" is essential for governing diapause regulation (Denlinger, 2023). But developing a diapause toolkit with shared diapause-regulating genes may be technically challenging for different types of studies in *M. rotundata*. For instance, investigations into the gene expression profiles of diapausing prepupae of *M. rotundata* have revealed transcriptional differences between individuals subjected to laboratory conditions compared to those existing in their natural field habitats. This underscores the influence of environmental history on gene expression patterns, suggesting that the behavior of the pathways in diapause 'toolkit' may not consistently follow a uniform pattern across all instances(George D Yocum et al., 2018). Conversely, in another study conducted on *M. rotundata*, genes associated with the diapause 'toolkit' had similar expression profiles during diapause and in post-diapause quiescence. This analysis suggests that cell signaling pathways, chromatin remodeling, and activation of cell cycle processes, are involved in the diapause to post-diapause transition of *M. rotundata* (Yocum et al., 2015). Numerous other studies have focused on the overall gene expression patterns influenced by various environmental factors, such as fluctuating thermal regimes (Torson et al., 2015; Torson et al., 2017), the timing of diapause initiation (Cambron-Kopcoet al., 2022), and developmental history in diverse environmental settings (George D. Yocum et al., 2018)Click or tap here to enter text.. Despite the transcriptional changes in individuals during diapause in different environments, diapause transition to post-diapause, and quiescent states, the clear elucidation of the diapause initiation mechanism (where the decision of diapause is made) between diapausing and non-diapausing individuals remains elusive, until now. In this study, we first report differences in transcript

expression levels and their functions associated with diapausing and non-diapausing individuals in prepupal stage of *M. rotundata.*

**Cell cycle arrest**

Numerous cell cycle checkpoints regulate cell division and we predicted that these would be arrested in diapausing *M. rotundata*. We observed decreased abundance of transcripts related to cell cycle activation for example, *PLK1*, *CDK1,2,6*, *Cyclin A* and *Cyclin B* and *Aurora B* (Ubersax et al., 2003). These crucial kinases drive cell cycle progression through the G1-S transition and G2-M transition. The interaction between Cyclin A and Cdks 1 and 2 is crucial for transitioning from G1 to S phase and progressing through the S phase. The binding of Cyclin B and Cdk1 is necessary for transitioning from G2 to M phase. During the G2 phase, PLK1 is activated and indirectly enhances the activation of cyclin B-Cdk1, which promotes entry into mitosis. Aurora B ensures proper chromosome segregation and normal progression through mitosis (Salaun et al., 2008). Similar to the observations in the current study, *Cyclin E, Cyclin B, cdks 1,2,* and *7* were observed to be downregulated in preparation for diapause in the fly, *Chymomyza costata* (Poupardin et al., 2015).

The gene expression patterns responsible for regulating the cell cycle and the insulin signaling pathway show significant variations across the phases of diapause in the alfalfa leafcutting bee, *Megachile rotundata* when exposed to natural conditions (Cambron et al., 2021). These results are also relatable to another study in *M. rotundata* where the abundance of transcripts encoding various components of the cell cycle, such *as cyclin-e, cyclin-k, cyclin-dependent kinase-2,* and *S phase kinase-associated proteins* increased during the termination of diapause, when the larval cells were preparing to resume the cell cycle (Yocum et al., 2015). The analysis of gene expression patterns in this study also revealed the suppression of hormonal

signaling pathways involved in development in larvae that are destined for diapause. Consequently, there was a decrease in the activity of transcription factors, which ultimately resulted in the arrest of the cell cycle (Koštál et al., 2017). Overall, the results from the current research suggest that cell cycle arrest is associated with the initiation of diapause in *M. rotundata*.

**Insulin signaling pathways**

FOXO (forkhead transcription factor), a downstream molecule in the insulin signaling pathway, mediates the diapause response like cell cycle arrest, suppressed metabolism, and enhanced stress response (Sim and Denlinger, 2013). The current study predicted that insulin signaling would be downregulated in diapausing individuals. While insulin signaling appears to remain lower during diapause in this study, common transcripts are observed to be shared across different signaling pathways, including the *5'-AMP-activated protein kinase* (AMPK), *hormone-sensitive lipase* (HSL), *serrate,* and *fringe.*

It is known that the energy-sensing AMPK directly phosphorylates FOXO at different regulatory sites, enhancing its transcriptional activity, and leading to the expression of specific target genes involved in energy metabolism and stress resistance (Greer et al., 2009). Once FOXO is activated, it promotes certain features of the diapause phenotype. This activation occurs in the presence of low levels of insulin-like peptides along with the downregulation of Protein kinase B (PKB), also known as Akt (Zhang et al., 2022). Therefore, AMPK acts as an energy sensor, regulating cellular metabolism by interacting with various interconnected pathways. In the present study, we observed an increase in abundance of *5'-AMP-activated protein kinase*, AMP-activated protein kinase (AMPK) signaling, FOXO signaling, longevity regulation, and adipocytokine signaling pathways. AMPK is ubiquitously distributed across eukaryotic

organisms and serves as a pivotal sensor responsible for monitoring cellular energy and nutrient levels (Hardie, 2007; Hardie, 2011; Hardie, 2014). AMPK is activated under conditions of energy deficit and phosphorylates downstream targets. This activation prompts the induction of catabolic pathways aimed at ATP production that simultaneously suppresses energy-expensive biosynthetic processes. Considering its involvement in modulating energy utilization, AMPK would be a promising candidate for mediating metabolic rate depression. In *C. elegans*, AMPK isoform *aak-2* plays a crucial role for the extension of lifespan triggered by environmental stress (Apfeld et al., 2004). In *C. elegans*' dauer stage, the *aak-2* isoform of AMPK is essential for survival as it actively preserves lipid reserves, ensuring vital energy sources are maintained during this dormant stage (Narbonne and Roy, 2008). Activation of AMPK was observed in overwintering larvae of the goldenrod gall moth (*Epiblema scudderiana*) and the goldenrod gall fly (*Eurosta solidaginis*) (Rider et al., 2011; Rider, 2015)

Winter survival in many species relies on a combination of cold-hardiness adaptations and the initiation of a hypometabolic state by minimizing energy expenditure during this period. In mammals, triacylglycerol reserves stored in lipid droplets of adipocytes are hydrolysed during lipolysis, releasing free fatty acids and glycerol (Narbonne and Roy, 2008). In this study, the low abundance of *hormone-sensitive lipase* (HSL) was reported along with *insulin* and *protein phosphatase 1* which is involved in glycogen and lipid metabolism. HSL plays a key role in the breakdown of triglycerides, leading to the production of free fatty acids. Considering these functions, the activation of AMPK might indeed inhibit TAG breakdown by potentially inactivating HSL, a mechanism observed in *C. elegans* (Rider, 2015). In insects, the upregulation of AMPK (AMP-activated protein kinase) during diapause maintenance has been implicated in production of Adipokinetic hormones AKH, where the accumulation of AMPK results in

production of DAGs from TAGs via cAMP and the $Ca^{2+}$ signaling cascade resulting in fatty acid biosynthesis and lipid storage (Sinclair and Marshall, 2018). In this study, other components of PI3K-Akt signaling pathway, *insulin-like growth factor 1*, *cyclin-dependent kinase 6*, *cyclic AMP-responsive element-binding protein 3*, show decreased abundance in diapausing individuals suggesting activation of FOXO and networks of signals and genes that encourage diapause. Other signaling pathway like insulin and FOXO have also been suggested to be involved in this event of lipid storage (Toprak, 2020). In the current study, it was found that polo-like kinase 1, p38 MAP kinase, tyrosine-protein kinase, along with cyclin B2 and cyclin B3, and guanine nucleotide-binding protein G(o) subunit alpha are present in low in abundance showing FOXO, a key player in growth and development, and metabolism (Schmidt et al., 2002). Additionally, the late phases of cell cycle progression are negatively regulated by the serine/threonine kinase Polo-like kinase 1, a crucial cell cycle regulator, which serves as a major controller of FOXO (Yuan et al., 2014). During the late phase of the cell cycle, Polo-like kinase 1 binds and phosphorylates FOXO1 and induces the nuclear exclusion of FOXO1. This event results in the inhibition of its transcriptional activity during the late phases of the cell cycle (Liu, Kao and Huang, 2008). This marks the connection between reported genes and FOXO in cell development. Overall, our findings support a consistent trend observed in the expression profile and KEGG analysis, indicating an increased activity and abundance of 5'-AMP-activated protein kinase.

Furthermore, we found reduced abundance of *serrate* and *fringe* which are responsible for wing development (Klein and Martinez Arias, 1998), limb development (De Celis et al., 1998) and segment morphogenesis (Dearden and Akam, 2000). FOXO has been shown to directly bind to the promoter of *fringe* to regulate its transcription whereas Insulin signaling negatively regulates *fringe* expression in the germline stem cell niche. Therefore, Notch activity

is also being regulated by Insulin signaling via FOXO/fringe interaction (Yang et al., 2013). These observations suggest interconnections among various genes contributing to the generation of components that maintain diapause in *M. rotundata* through interconnected signaling pathways.

**Wnt signaling**

Wnt signaling is a highly conserved regulatory pathway that plays a pleiotropic role in tissue proliferation during insect metamorphosis (Siegfried and Perrimon, 1994)**.** The downregulation of most members of Wnt pathway is associated with the developmental arrest that regulates diapause (Ragland and Keep, 2017). Therefore, we hypothesized that Wnt signaling would be repressed during diapause in *M. rotundata*. The investigation revealed a reduction in the abundance of genes involved in the canonical Wnt pathway, including *wif-1* and *frizzled,* which play a significant role in embryogenesis and organ development. Additionally, genes associated with the Wnt/Ca2+ pathway, such as *Wnt5*, *RYK*, and *frizzled,* also exhibited decreased abundance during diapause. These genes govern cell growth and cycle proliferation and comprise two different Wnt signaling pathways: the canonical pathway, involving β-catenin, and non-canonical pathways, involving calcium signaling (Oberhofer et al., 2014). In a previous study, a significant decrease in both the Wnt/β-catenin and target of rapamycin (TOR) pathways were observed after RNAi silencing of Frizzled 2 (*Fz2*) in female *Cyrtorhinus lividipennis*. Moreover, silencing Fz2 showed additional effects like reduced expression of the vitellogenin gene (*Vg*), decreased soluble protein levels in ovaries and fat bodies, lowered juvenile hormone titers, and reduced body weight (Ge et al., 2020). Transcripts coding for cell cycle regulation, especially *polo* and *frizzled,* showed a downregulation response in diapausing individuals of *C. costata* (Poupardin et al., 2015). Interestingly, an abundance of the Wnt receptor *frizzled-4* that is

predicted to be a positive regulator and is required for activation of the signaling pathway increased during diapausing stage of *Anoplophora glabripennis* (Torson et al., 2023). Hence, the directionality of *frizzled* cannot be solely predicted based on its typical function as a positive regulator of Wnt signaling pathway because Wnt pathways genes are interconnected with other crucial signaling pathways such as ecdysone, JH, and insulin, mTOR and Notch pathway.

The Notch signaling pathway plays a significant role in regulating tissue proliferation. The connection between the Notch signaling pathway and the Wnt signaling pathway has been established through the abnormal expression of a gene, *Spen*, which is consistently upregulated during diapause in 11 different species that were studied (Ragland and Keep, 2017) and exerts pleiotropic effects by repressing Notch signaling. Hence, the observed downregulation of most members of the Wnt pathway in the present study holds true, indicating a common pattern of this pathway during diapause.

**Lipid biosynthesis**

During diapause preparation metabolic processes shift from supporting growth and development to facilitating the accumulation of reserves (Koštál, 2006). These stored resources are later utilized slowly to sustain essential cellular functions and enhance tolerance to environmental stress, ensuring long-term survival (Hahn and Denlinger, 2007). Triacylglycerol is a primary energy sources to fuel metabolism in both mammalian hibernators and many insects during diapause (Joanisse and Storey, 1996; Frank, 2011; Rozsypal et al., 2014). In this study, KEGG pathway analysis confirmed increase in expression of *phospholipase A1-like transcript* present in metabolic pathway, vitamin digestion and absorption pathway, fat digestion and absorption pathway, and glycerolipid metabolism pathway which is responsible for increased abundance of triacylglycerol lipase during diapause. Key relation of lipid metabolism and insulin

has been studied previously where it has been suggested that different insect insulin like peptides may be involved in regulating fat cell proliferation, lipid storage and, energy metabolism and other key physiology related traits for survival. For example, in *Drosophila* insulin act as a positive regulator of fat cell mass that changes in total cell number and lipid storage (DiAngelo and Birnbaum, 2009). In contrast, mutations in the gene insulin receptor and loss of insulin-like peptide-producing cells results in a significant increase in triacylglycerides in *Drosophila* (Böhni et al., 1999; Tatar et al., 2001). In honey bees, the expression levels of lipid storage protein 1 (*Lsd-1*) and long-chain-fatty acid-CoA ligase *ACSBG2-like* genes are responsive to immunostimulation and are correlated with changes in cuticular hydrocarbon patterns (Richard et al., 2012).

Furthermore, in this study we observed increased abundance of Delta (Δ)-9 desaturase, which is involved in fatty acid metabolism, the PPAR signaling pathway, the pathway responsible for biosynthesis of unsaturated fatty acids, longevity, and AMPK signaling. Δ9-desaturases function as key enzymes in the biosynthesis of monounsaturated fatty acids. These fatty acids are vital substrates for producing various lipids including phospholipids, cholesteryl esters, and triglycerides. These enzymes desaturate fatty acid chains, which influence triglyceride melting temperature and the fluidity of biological membranes. Loss of function of Δ9-desaturases showed remarkable decreased in fatty acids and placed *C. elegans* in a catabolic state (Castro et al., 2012). The Δ (9)-desaturase was also upregulated in diapausing pharate larvae of *Aedes albopictus* (Reynolds et al., 2012). Thus, increased abundance of Δ9-desaturase plays active roles in generating unsaturated fatty acids at low temperatures. Conversely, the conservation of lipids during diapause is reflected in lowering the expression of transcripts involved in lipid catabolism including putative fatty acyl-CoA reductase, very-long-chain enoyl-

CoA reductase, 2-acylglycerol O-acyltransferase 2-A-like, Delta3-Delta2-enoyl-CoA isomerase, and transporter proteins like f*atty acid-binding protein 12-like* that transport fatty acids and other lipophilic substances between extra- and intracellular membranes. Together, these results point to substantial distinction in lipid metabolism as a consequence of diapause induction.

**Autophagy**

Diapausing insects may rely on autophagy to generate energy reserves for winter survival in the absence of food. Autophagy actively breaks down cytoplasmic components, including macromolecules and organelles in the lysosomes or vacuoles. This vital and conserved process respond dynamically to various physiological activities such as tissue remodeling, organelle quality control, and stress resistance. Nevertheless, autophagy has been shown to be involved in the diapause process of other organisms, including the crustacean *Artemia parthenogenetica* (Lin et al., 2016) and harvestmen *Amilenus aurantiacus* (Lipovšek et al., 2015). Proteins associated with gamma-aminobutyric acid have been observed to increase in *Riptortus pedestris* during late diapause in the absence of food, especially when other reserves were depleted (Tachibana et al., 2020). In present study we found one variant of mucin transcript (mucin-17, transcript variant X7) highly expressed in diapausing individuals. An RNAi study suggested that mucins are essential for growth and development of the locust *Locusta migratoria*, and are responsible for development of gastric caecum, formation of peritrophic membrane, structural integrity of the wings, and cuticle (Zhao et al., 2020). Moreover, transcriptomic and proteomic analysis showed *mucin* was one of the most upregulated genes, including heat shock protein, and other transcription initiation factors in diapausing *Locusta migratoria* (Tu et al., 2015). *Mucin-2* was transcriptionally upregulated in onion maggots (*Delia antiqua*) facing in summer diapause (Ren

et al., 2018). Hence, the findings in this study emphasizing the significance of autophagy and mucin-related mechanisms in developmental arrest.

This study generated an expanding list of mRNAs that were unchanged during diapause with 1,592 transcripts that were differently expressed between diapausing and non-diapausing individuals. This might be due to our limitations in predicting when the diapause decision is being made. There is no data that shows when this decision is being made and no phenotypic markers are available that can differentiate diapausing individuals from non-diapausing individuals. The only way to determine the diapause status of the sample was by inferring it through the siblings' diapause status. Although this study is informative, it is limited because only the mRNA and their levels are identified and hence the inference falls short of demonstrating function. Mutational studies like gene knockouts using RNA interference (RNAi) are required to confirm molecular functionality of the gene candidates. Spatial transcriptomics is another approach that can comprehensively track transcriptional activity within intact tissue sections, which could accurately resolve mRNA expression at the cellular level.

## Conclusion

In conclusion, our study provides a comprehensive understanding of the molecular mechanisms underlying diapause initiation in the alfalfa leafcutter bee, *M. rotundata.* Through transcriptomic analysis, GO enrichment and KEGG pathway studies, key pathways and processes that are significantly altered during diapause were elucidated, shedding light on the intricate interplay between metabolism, signaling pathways, and cellular processes in this crucial stage of insect development. This study reveals significant changes in transcript abundance between diapausing and non-diapausing individuals. Notably, the diapausing prepupae exhibited increased expression of transcripts associated with metabolic and lipid biosynthesis processes.

Conversely, transcripts related to cell cycle regulation and cellular communication showed decreased abundance, pointing to a state of cell cycle arrest, decreased signaling pathways, DNA replication and hormone synthesis arrest, and reduced metabolic activity during diapause.

Enrichment analysis studies highlighted the involvement of specific pathways in diapause regulation. The downregulation of the Wnt signaling pathway, coupled with decreased abundance of cell cycle activators, supports the notion of cell cycle arrest as a hallmark of diapause initiation. Additionally, the upregulation of components of diapause 'toolbox', such as FOXO and AMPK, suggests downregulation of insulin signaling and activation of energy-saving mechanisms to facilitate diapause maintenance. Furthermore, our findings with respect to lipid metabolism provide insights into how diapausing individuals prioritize energy storage for long-term survival. The increased expression of genes involved in lipid biosynthesis, coupled with the downregulation of lipid catabolism pathways, indicates a shift towards lipid accumulation and utilization as a primary energy source during diapause. This metabolic adaptation is crucial for sustaining vital cellular functions and enhancing tolerance to environmental stress during extended periods of dormancy. Overall, our study contributes to a deeper understanding of the molecular basis of differences between diapausing and non-diapausing individuals during diapause initiation in M. rotundata. This study contributes significant insights into the adaptive mechanisms utilized by insects to endure adverse environmental conditions through diapause. By identifying and elucidating different but important pathways between diapausing and non diapausing individuals, our findings lay the groundwork for future research endeavors aimed at deepening our understanding of insect dormancy. Leveraging advanced techniques such as spatial transcriptomics and functional analyses like RNAi promise for unraveling the molecular components governing diapause regulation. Understanding the functional roles of different

pathways not only enhances our comprehension of insect physiology but also carries broader implications such as to anticipate the impact of climate change on insect populations undergoing diapause.

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# CHAPTER 5: CONCLUSION

This study is a multifaceted approach to explore the ecological and physiological challenges in both social and solitary bee species, with a particular emphasis on the role of the gut microbiome and the molecular mechanisms underlying diapause initiation. The study underscores the significance of exploring these aspects to gain a comprehensive understanding of bee biology and to inform strategies for bee management and conservation in the face of environmental challenges. The study focuses on the microbiome of bees, which can play a crucial role in their health and development. The gut microbiome of honey bees, for example, influences its physiology, development, and behavior. Social bees, and particularly honey bees, have a core set of bacteria in their gut that is actively transmitted to the next generation through social contact. These bacteria aid in breaking down macromolecules, providing nutrients, and neutralizing dietary toxins. Therefore, the research delves into the overwintering physiology of honey bees. The composition of the honey bee gut microbiome is hypothesized to be impacted by overwintering temperature, in spite of whether the bees are housed indoors at a consistent temperature or subjected to natural temperature fluctuations outdoors. Solitary bees, in contrast, do not share food and gut microbiome with nest mates. The transfer of plant-associated bacteria to solitary bees occurs via pollen ingestion. The bacterial diversity found in the brood cell provisions of solitary bees, particularly the *Megachilidae* family, is highly diverse and yet fluctuating. One most abundant bacterial species of bacteria in the wild bees, *Apilactobacillus micheneri*, has unique adaptations for survival in wild bee guts. Another aim of this research was to study the function(s) of gut microbiome, with specific focus on *A. micheneri,* through microbial bioassays to gain experimental data on the functionality of this bacterial species. This research provides valuable insights into the role of the gut microbiome in solitary bee

development and survival. In the final section, this research, shifts its focus to diapause, a developmental phase that offers advantages to insects to ward off unfavorable conditions for their development, survival, and reproduction. *Megachile rotundata*, a bivoltine solitary bee undergoing facultative diapause, serves as a model to explore the molecular mechanisms during diapause initiation. Understanding the molecular differences between diapausing and non-diapausing individuals will enhance our knowledge of *M. rotundata's* physiology at the diapause initiation stage.

In chapter 2, the study investigated the gut microbiota of overwintering adult worker bees illustrating a complex interplay shaped by host genetics, bee type, storage conditions, and temporal variations across different months. Using 16S rRNA gene amplicon sequencing, the study delved into the microbial dynamics among the commercial Bolton bees and Mann Lake bees, revealing significant distinctions in their gut bacterial composition. The prevalence and dominance of *Lactobacillus* in overwintering bees, irrespective of bee type or storage conditions, underscores the stability of specific microbial communities during winter. The distinct bacterial profiles in different commercial cultivars like the Bolton and Mann Lake bees highlights the impact of genetic factors, with Bolton bees exhibiting higher abundances of *Bartonella, Bifidobacterium*, and *Wolbachia*, while the Mann Lake bees showed elevated levels of *Commensalibacter* and *Lactobacillus kosoi*, attributed to their breeding and genetic selection. The relationship between host genetics, microbiota composition, and environmental conditions underscores the need for comprehensive studies of these factors to promote bee colony health, especially in regions with harsh winters. As beekeepers navigate the complexities of hive management during the overwintering period, our findings provide reassurance by suggesting notable stability in the bee gut microbiota across different environmental conditions. This

stability implies that beekeepers may not need to overly concern themselves with potential microbiota-related impacts on overwintering health. In essence, the current study contributes valuable insights to the broader understanding of bee biology, emphasizing the connections between genetics, microbiota, and environmental resilience during the critical overwintering phase.

In chapter 3, the study highlights that the bee's dependency for the growth and survival on gut associated bacteria is not an universal phenomenon across bee species. Environmentally acquired non-host specific bacteria might not shape solitary bee fitness in all host species. More phenotypic traits like adult reproduction, flight performance, and overwintering survival should be included to better understand the functions performed by gut microbes in solitary bees. Furthermore, this research opens new avenues for understanding interactions between gut microbiomes, especially *A. micheneri* and endosymbionts like *Sodalis*. One of the future goals arising from the current study would be to assess the functional role of *Sodalis* bacteria in solitary bees using histological, immunological, and network analyses. This will help us to better understand the importance of environmentally gathered microbiomes for solitary bee survival, growth, and development.

In chapter 4, this study provides a comprehensive insight into the transcriptomic dynamics underlying diapause initiation in *M. rotundata*. The observed patterns in cell cycle arrest, insulin signaling, Wnt signaling, innate immunity, lipid biosynthesis, and autophagy contribute to our understanding of the molecular mechanisms governing diapause. The complex interplay between various signaling pathways highlights the complexity of diapause initiation and emphasizes the need for further research to uncover specific interactions and crosstalk between these pathways. Considering the ecological and agricultural importance of *M.*

*rotundata*, these findings are foundational for future studies to explore targeted interventions for pollinator management. In conclusion, this research comprehensively addresses the microbial and molecular aspects of both social and solitary bee species, contributing valuable insights to bee ecology and physiology.

## APPENDIX A. FIGURE FOR CHAPTER 4 - ALFALFA LEAFCUTTING BEE DIAPAUSE TRANSCRIPTOMICS

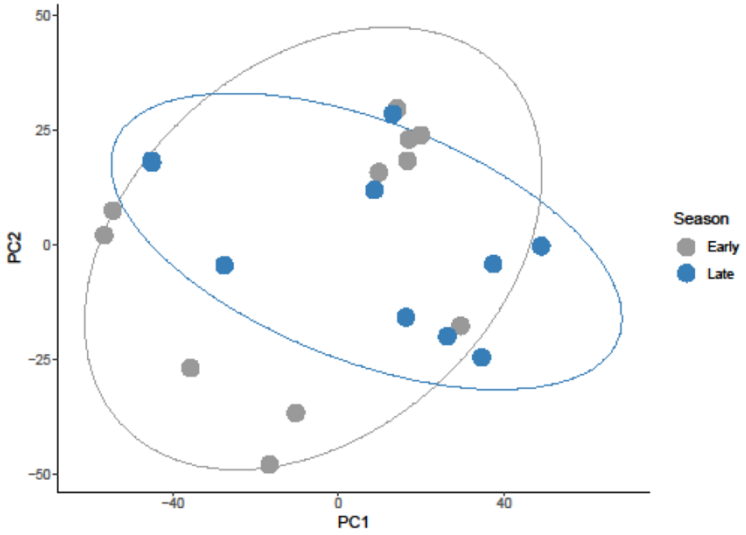


Figure A1. Principal Component Analysis (PCA) shows clustering of RNA-seq samples by seasonality. Ellipses on the plot indicate 95% confidence intervals.

## APPENDIX B. TABLES FOR CHAPTER 3 - ALFALAFA LEAFCUTTING BEE MICROBIOME

Table B1. Pairwise PERMANOVA comparisons show the significant difference between pollen with antibiotics (AC), sterile pollen (s), and sterile pollen with added *A. micheneri* (SAm) as compared to natural pollen (n)

| Group 1 | Group 2 | Permutations | pseudo-F | p-vlaue | q-value |
|---|---|---|---|---|---|
| AC | N | 999 | 2.10509071 | 0.004 | 0.0066667 |
| AC | NAm | 999 | 2.1050907 | 0.001 | 0.00333333 |
| AC | SAm | 999 | 2.5634931 | 0.001 | 0.0033333 |
| AC | S | 999 | 3.76797511 | 0.001 | 0.00333333 |
| N | NAm | 999 | 1.1878203 | 0.239 | 0.239 |
| N | SAm | 999 | 1.67940687 | 0.026 | 0.0325 |
| N | S | 999 | 2.63223888 | 0.002 | 0.004 |
| NAm | SAm | 999 | 1.74451873 | 0.008 | 0.0114286 |
| NAm | S | 999 | 2.3043381 | 0.002 | 0.004 |
| SAm | S | 999 | 1.44020666 | 0.056 | 0.0622222 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (NAm), sterile pollen (S), sterile pollen with *A. micheneri* (SAm), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads.

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C10D1 | SP | < 2.5% | 43.475 |
| C10D1 | SP | Apilactobacillus micheneri | 4.96 |
| C10D1 | SP | Sodalis | 10.965 |
| C10D1 | SP | Unclassified  Acinetobacter | 0 |
| C10D1 | SP | Unclassified Lachnospiraceae | 3.875 |
| C10D1 | SP | Unclassified Xanthobacteraceae | 33.11 |
| C10D1 | SP | Unclassified d__Bacteria | 3.615 |
| C10D2 | SP | < 2.5% | 91.03 |
| C10D2 | SP | Apilactobacillus micheneri | 0 |
| C10D2 | SP | Sodalis | 0.5 |
| C10D2 | SP | Unclassified  Acinetobacter | 1.98 |
| C10D2 | SP | Unclassified Lachnospiraceae | 3.34 |
| C10D2 | SP | Unclassified Xanthobacteraceae | 0.925 |
| C10D2 | SP | Unclassified d__Bacteria | 2.225 |
| C10D5 | SAm | < 2.5% | 82.485 |
| C10D5 | SAm | Apilactobacillus micheneri | 5.17 |
| C10D5 | SAm | Sodalis | 4.5 |
| C10D5 | SAm | Unclassified  Acinetobacter | 4.62 |
| C10D5 | SAm | Unclassified Lachnospiraceae | 0.66 |
| C10D5 | SAm | Unclassified Xanthobacteraceae | 2.185 |
| C10D5 | SAm | Unclassified d__Bacteria | 0.38 |
| C10D6 | SAm | < 2.5% | 49.715 |
| C10D6 | SAm | Apilactobacillus micheneri | 40.58 |
| C10D6 | SAm | Sodalis | 0 |
| C10D6 | SAm | Unclassified  Acinetobacter | 1.095 |
| C10D6 | SAm | Unclassified Lachnospiraceae | 2.965 |
| C10D6 | SAm | Unclassified Xanthobacteraceae | 4.185 |
| C10D6 | SAm | Unclassified d__Bacteria | 1.46 |
| C10D7 | SAm | < 2.5% | 63.145 |
| C10D7 | SAm | Apilactobacillus micheneri | 0 |
| C10D7 | SAm | Sodalis | 0 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C10D7 | SAm | Unclassified  Acinetobacter | 4.36 |
| C10D7 | SAm | Unclassified Lachnospiraceae | 3.665 |
| C10D7 | SAm | Unclassified Xanthobacteraceae | 14.235 |
| C10D7 | SAm | Unclassified d__Bacteria | 14.595 |
| C10D8 | SAm | < 2.5% | 68.8 |
| C10D8 | SAm | Apilactobacillus micheneri | 22.515 |
| C10D8 | SAm | Sodalis | 0 |
| C10D8 | SAm | Unclassified  Acinetobacter | 1.445 |
| C10D8 | SAm | Unclassified Lachnospiraceae | 0 |
| C10D8 | SAm | Unclassified Xanthobacteraceae | 5.315 |
| C10D8 | SAm | Unclassified d__Bacteria | 1.925 |
| C11D1 | SAm | < 2.5% | 77.295 |
| C11D1 | SAm | Apilactobacillus micheneri | 8.89 |
| C11D1 | SAm | Sodalis | 0 |
| C11D1 | SAm | Unclassified  Acinetobacter | 0 |
| C11D1 | SAm | Unclassified Lachnospiraceae | 0 |
| C11D1 | SAm | Unclassified Xanthobacteraceae | 9.755 |
| C11D1 | SAm | Unclassified d__Bacteria | 4.06 |
| C11D2 | SAm | < 2.5% | 92.985 |
| C11D2 | SAm | Apilactobacillus micheneri | 0 |
| C11D2 | SAm | Sodalis | 0 |
| C11D2 | SAm | Unclassified  Acinetobacter | 0 |
| C11D2 | SAm | Unclassified Lachnospiraceae | 0 |
| C11D2 | SAm | Unclassified Xanthobacteraceae | 7.015 |
| C11D2 | SAm | Unclassified d__Bacteria | 0 |
| C11D3 | SAm | < 2.5% | 68.655 |
| C11D3 | SAm | Apilactobacillus micheneri | 4.49 |
| C11D3 | SAm | Sodalis | 0 |
| C11D3 | SAm | Unclassified  Acinetobacter | 18.315 |
| C11D3 | SAm | Unclassified Lachnospiraceae | 2.86 |
| C11D3 | SAm | Unclassified Xanthobacteraceae | 5.68 |
| C11D3 | SAm | Unclassified d__Bacteria | 0 |
| C11D4 | SAm | < 2.5% | 43.31 |
| C11D4 | SAm | Apilactobacillus micheneri | 14.7 |
| C11D4 | SAm | Sodalis | 0 |
| C11D4 | SAm | Unclassified  Acinetobacter | 0 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C11D4 | SAm | Unclassified Lachnospiraceae | 32.555 |
| C11D4 | SAm | Unclassified Xanthobacteraceae | 4.33 |
| C11D4 | SAm | Unclassified d__Bacteria | 5.105 |
| C11D6 | SAm | < 2.5% | 90.265 |
| C11D6 | SAm | Apilactobacillus micheneri | 2.03 |
| C11D6 | SAm | Sodalis | 0 |
| C11D6 | SAm | Unclassified  Acinetobacter | 0 |
| C11D6 | SAm | Unclassified Lachnospiraceae | 1.51 |
| C11D6 | SAm | Unclassified Xanthobacteraceae | 3.57 |
| C11D6 | SAm | Unclassified d__Bacteria | 2.625 |
| C11D7 | SAm | < 2.5% | 61.41 |
| C11D7 | SAm | Apilactobacillus micheneri | 0 |
| C11D7 | SAm | Sodalis | 0 |
| C11D7 | SAm | Unclassified  Acinetobacter | 5.325 |
| C11D7 | SAm | Unclassified Lachnospiraceae | 0 |
| C11D7 | SAm | Unclassified Xanthobacteraceae | 33.265 |
| C11D7 | SAm | Unclassified d__Bacteria | 0 |
| C11D8 | SAm | < 2.5% | 80.875 |
| C11D8 | SAm | Apilactobacillus micheneri | 2.315 |
| C11D8 | SAm | Sodalis | 0 |
| C11D8 | SAm | Unclassified  Acinetobacter | 1.13 |
| C11D8 | SAm | Unclassified Lachnospiraceae | 2.345 |
| C11D8 | SAm | Unclassified Xanthobacteraceae | 13.045 |
| C11D8 | SAm | Unclassified d__Bacteria | 0.29 |
| C12D1 | SAm | < 2.5% | 12.485 |
| C12D1 | SAm | Apilactobacillus micheneri | 23.4 |
| C12D1 | SAm | Sodalis | 53.855 |
| C12D1 | SAm | Unclassified  Acinetobacter | 3.62 |
| C12D1 | SAm | Unclassified Lachnospiraceae | 0 |
| C12D1 | SAm | Unclassified Xanthobacteraceae | 2.33 |
| C12D1 | SAm | Unclassified d__Bacteria | 4.31 |
| C12D2 | SAm | < 2.5% | 51.76 |
| C12D2 | SAm | Apilactobacillus micheneri | 22.13 |
| C12D2 | SAm | Sodalis | 0 |
| C12D2 | SAm | Unclassified  Acinetobacter | 13.34 |
| C12D2 | SAm | Unclassified Lachnospiraceae | 3.035 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C12D2 | SAm | Unclassified Xanthobacteraceae | 9.64 |
| C12D2 | SAm | Unclassified d__Bacteria | 0.095 |
| C12D3 | SAm | < 2.5% | 33.475 |
| C12D3 | SAm | Apilactobacillus micheneri | 0.1 |
| C12D3 | SAm | Sodalis | 0 |
| C12D3 | SAm | Unclassified  Acinetobacter | 25.495 |
| C12D3 | SAm | Unclassified Lachnospiraceae | 0 |
| C12D3 | SAm | Unclassified Xanthobacteraceae | 24.475 |
| C12D3 | SAm | Unclassified d__Bacteria | 16.455 |
| C12D4 | SAm | < 2.5% | 87.61 |
| C12D4 | SAm | Apilactobacillus micheneri | 2.39 |
| C12D4 | SAm | Sodalis | 0 |
| C12D4 | SAm | Unclassified  Acinetobacter | 4.425 |
| C12D4 | SAm | Unclassified Lachnospiraceae | 0 |
| C12D4 | SAm | Unclassified Xanthobacteraceae | 3.215 |
| C12D4 | SAm | Unclassified d__Bacteria | 2.36 |
| C12D5 | SAm | < 2.5% | 81.57 |
| C12D5 | SAm | Apilactobacillus micheneri | 0.14 |
| C12D5 | SAm | Sodalis | 0 |
| C12D5 | SAm | Unclassified  Acinetobacter | 6.92 |
| C12D5 | SAm | Unclassified Lachnospiraceae | 0 |
| C12D5 | SAm | Unclassified Xanthobacteraceae | 6.865 |
| C12D5 | SAm | Unclassified d__Bacteria | 4.505 |
| C12D6 | SAm | < 2.5% | 43.9 |
| C12D6 | SAm | Apilactobacillus micheneri | 42.36 |
| C12D6 | SAm | Sodalis | 0 |
| C12D6 | SAm | Unclassified  Acinetobacter | 8.38 |
| C12D6 | SAm | Unclassified Lachnospiraceae | 1.875 |
| C12D6 | SAm | Unclassified Xanthobacteraceae | 2.31 |
| C12D6 | SAm | Unclassified d__Bacteria | 1.175 |
| C12D7 | SAm | < 2.5% | 5.235 |
| C12D7 | SAm | Apilactobacillus micheneri | 0.075 |
| C12D7 | SAm | Sodalis | 0 |
| C12D7 | SAm | Unclassified  Acinetobacter | 0.865 |
| C12D7 | SAm | Unclassified Lachnospiraceae | 93.5 |
| C12D7 | SAm | Unclassified Xanthobacteraceae | 0.11 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C12D7 | SAm | Unclassified d__Bacteria | 0.215 |
| C12D8 | SAm | < 2.5% | 76.12 |
| C12D8 | SAm | Apilactobacillus micheneri | 5.345 |
| C12D8 | SAm | Sodalis | 0 |
| C12D8 | SAm | Unclassified  Acinetobacter | 16.645 |
| C12D8 | SAm | Unclassified Lachnospiraceae | 0.05 |
| C12D8 | SAm | Unclassified Xanthobacteraceae | 1.195 |
| C12D8 | SAm | Unclassified d__Bacteria | 0.645 |
| C1D1 | N | < 2.5% | 4.085 |
| C1D1 | N | Apilactobacillus micheneri | 2.875 |
| C1D1 | N | Sodalis | 92.24 |
| C1D1 | N | Unclassified  Acinetobacter | 0.06 |
| C1D1 | N | Unclassified Lachnospiraceae | 0.49 |
| C1D1 | N | Unclassified Xanthobacteraceae | 0.03 |
| C1D1 | N | Unclassified d__Bacteria | 0.22 |
| C1D2 | N | < 2.5% | 0.115 |
| C1D2 | N | Apilactobacillus micheneri | 0.12 |
| C1D2 | N | Sodalis | 0.575 |
| C1D2 | N | Unclassified  Acinetobacter | 0 |
| C1D2 | N | Unclassified Lachnospiraceae | 99.175 |
| C1D2 | N | Unclassified Xanthobacteraceae | 0 |
| C1D2 | N | Unclassified d__Bacteria | 0.015 |
| C1D3 | N | < 2.5% | 0.365 |
| C1D3 | N | Apilactobacillus micheneri | 2.85 |
| C1D3 | N | Sodalis | 5.485 |
| C1D3 | N | Unclassified  Acinetobacter | 0 |
| C1D3 | N | Unclassified Lachnospiraceae | 91.3 |
| C1D3 | N | Unclassified Xanthobacteraceae | 0 |
| C1D3 | N | Unclassified d__Bacteria | 0 |
| C1D4 | N | < 2.5% | 0.745 |
| C1D4 | N | Apilactobacillus micheneri | 0.61 |
| C1D4 | N | Sodalis | 29.43 |
| C1D4 | N | Unclassified  Acinetobacter | 0 |
| C1D4 | N | Unclassified Lachnospiraceae | 69.185 |
| C1D4 | N | Unclassified Xanthobacteraceae | 0 |
| C1D4 | N | Unclassified d__Bacteria | 0.03 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C1D5 | N | < 2.5% | 30.115 |
| C1D5 | N | Apilactobacillus micheneri | 43.905 |
| C1D5 | N | Sodalis | 2.11 |
| C1D5 | N | Unclassified  Acinetobacter | 22.765 |
| C1D5 | N | Unclassified Lachnospiraceae | 0.305 |
| C1D5 | N | Unclassified Xanthobacteraceae | 0.13 |
| C1D5 | N | Unclassified d__Bacteria | 0.67 |
| C1D6 | N | < 2.5% | 37.68 |
| C1D6 | N | Apilactobacillus micheneri | 55.415 |
| C1D6 | N | Sodalis | 0.72 |
| C1D6 | N | Unclassified  Acinetobacter | 1.61 |
| C1D6 | N | Unclassified Lachnospiraceae | 1.35 |
| C1D6 | N | Unclassified Xanthobacteraceae | 3.225 |
| C1D6 | N | Unclassified d__Bacteria | 0 |
| C1D7 | N | < 2.5% | 0.5 |
| C1D7 | N | Apilactobacillus micheneri | 0.065 |
| C1D7 | N | Sodalis | 0 |
| C1D7 | N | Unclassified  Acinetobacter | 0.01 |
| C1D7 | N | Unclassified Lachnospiraceae | 99.37 |
| C1D7 | N | Unclassified Xanthobacteraceae | 0 |
| C1D7 | N | Unclassified d__Bacteria | 0.055 |
| C1D8 | N | < 2.5% | 0.775 |
| C1D8 | N | Apilactobacillus micheneri | 0.055 |
| C1D8 | N | Sodalis | 0.255 |
| C1D8 | N | Unclassified  Acinetobacter | 0.135 |
| C1D8 | N | Unclassified Lachnospiraceae | 98.755 |
| C1D8 | N | Unclassified Xanthobacteraceae | 0 |
| C1D8 | N | Unclassified d__Bacteria | 0.025 |
| C1E1 | N | < 2.5% | 3.66 |
| C1E1 | N | Apilactobacillus micheneri | 69.7 |
| C1E1 | N | Sodalis | 1.52 |
| C1E1 | N | Unclassified  Acinetobacter | 0.225 |
| C1E1 | N | Unclassified Lachnospiraceae | 24.83 |
| C1E1 | N | Unclassified Xanthobacteraceae | 0.065 |
| C1E1 | N | Unclassified d__Bacteria | 0 |
| C2D1 | N | < 2.5% | 4.14 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C2D1 | N | Apilactobacillus micheneri | 19.04 |
| C2D1 | N | Sodalis | 74.715 |
| C2D1 | N | Unclassified  Acinetobacter | 0.03 |
| C2D1 | N | Unclassified Lachnospiraceae | 1.54 |
| C2D1 | N | Unclassified Xanthobacteraceae | 0.175 |
| C2D1 | N | Unclassified d__Bacteria | 0.36 |
| C2D2 | N | < 2.5% | 0.445 |
| C2D2 | N | Apilactobacillus micheneri | 0.115 |
| C2D2 | N | Sodalis | 0.04 |
| C2D2 | N | Unclassified  Acinetobacter | 0 |
| C2D2 | N | Unclassified Lachnospiraceae | 99.32 |
| C2D2 | N | Unclassified Xanthobacteraceae | 0.06 |
| C2D2 | N | Unclassified d__Bacteria | 0.02 |
| C2D3 | N | < 2.5% | 35.72 |
| C2D3 | N | Apilactobacillus micheneri | 4.405 |
| C2D3 | N | Sodalis | 0 |
| C2D3 | N | Unclassified  Acinetobacter | 0.225 |
| C2D3 | N | Unclassified Lachnospiraceae | 59.48 |
| C2D3 | N | Unclassified Xanthobacteraceae | 0 |
| C2D3 | N | Unclassified d__Bacteria | 0.17 |
| C2D4 | N | < 2.5% | 4.425 |
| C2D4 | N | Apilactobacillus micheneri | 91.68 |
| C2D4 | N | Sodalis | 0.615 |
| C2D4 | N | Unclassified  Acinetobacter | 0.59 |
| C2D4 | N | Unclassified Lachnospiraceae | 1.665 |
| C2D4 | N | Unclassified Xanthobacteraceae | 0.53 |
| C2D4 | N | Unclassified d__Bacteria | 0.495 |
| C2D5 | N | < 2.5% | 0.585 |
| C2D5 | N | Apilactobacillus micheneri | 0.08 |
| C2D5 | N | Sodalis | 12.11 |
| C2D5 | N | Unclassified  Acinetobacter | 0 |
| C2D5 | N | Unclassified Lachnospiraceae | 87.185 |
| C2D5 | N | Unclassified Xanthobacteraceae | 0.02 |
| C2D5 | N | Unclassified d__Bacteria | 0.02 |
| C2D6 | N | < 2.5% | 0.835 |
| C2D6 | N | Apilactobacillus micheneri | 13.39 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C2D6 | N | Sodalis | 31.37 |
| C2D6 | N | Unclassified  Acinetobacter | 0.025 |
| C2D6 | N | Unclassified Lachnospiraceae | 54.38 |
| C2D6 | N | Unclassified Xanthobacteraceae | 0 |
| C2D6 | N | Unclassified d__Bacteria | 0 |
| C2D8 | AC | < 2.5% | 23.155 |
| C2D8 | AC | Apilactobacillus micheneri | 68.515 |
| C2D8 | AC | Sodalis | 0 |
| C2D8 | AC | Unclassified  Acinetobacter | 2.495 |
| C2D8 | AC | Unclassified Lachnospiraceae | 0.76 |
| C2D8 | AC | Unclassified Xanthobacteraceae | 0.625 |
| C2D8 | AC | Unclassified d__Bacteria | 4.45 |
| C2E1 | N | < 2.5% | 0.37 |
| C2E1 | N | Apilactobacillus micheneri | 0.27 |
| C2E1 | N | Sodalis | 0.715 |
| C2E1 | N | Unclassified  Acinetobacter | 0 |
| C2E1 | N | Unclassified Lachnospiraceae | 98.645 |
| C2E1 | N | Unclassified Xanthobacteraceae | 0 |
| C2E1 | N | Unclassified d__Bacteria | 0 |
| C3D1 | AC | < 2.5% | 33.94 |
| C3D1 | AC | Apilactobacillus micheneri | 52.945 |
| C3D1 | AC | Sodalis | 0.94 |
| C3D1 | AC | Unclassified  Acinetobacter | 1.625 |
| C3D1 | AC | Unclassified Lachnospiraceae | 8.335 |
| C3D1 | AC | Unclassified Xanthobacteraceae | 2.045 |
| C3D1 | AC | Unclassified d__Bacteria | 0.17 |
| C3D2 | AC | < 2.5% | 24.29 |
| C3D2 | AC | Apilactobacillus micheneri | 49.34 |
| C3D2 | AC | Sodalis | 1.235 |
| C3D2 | AC | Unclassified  Acinetobacter | 0.48 |
| C3D2 | AC | Unclassified Lachnospiraceae | 22.005 |
| C3D2 | AC | Unclassified Xanthobacteraceae | 2.55 |
| C3D2 | AC | Unclassified d__Bacteria | 0.1 |
| C3D3 | AC | < 2.5% | 70.065 |
| C3D3 | AC | Apilactobacillus micheneri | 6.5 |
| C3D3 | AC | Sodalis | 2.785 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C3D3 | AC | Unclassified  Acinetobacter | 2.065 |
| C3D3 | AC | Unclassified Lachnospiraceae | 13.71 |
| C3D3 | AC | Unclassified Xanthobacteraceae | 3.84 |
| C3D3 | AC | Unclassified d__Bacteria | 1.035 |
| C3D4 | AC | < 2.5% | 7.76 |
| C3D4 | AC | Apilactobacillus micheneri | 87.68 |
| C3D4 | AC | Sodalis | 0.285 |
| C3D4 | AC | Unclassified  Acinetobacter | 0.395 |
| C3D4 | AC | Unclassified Lachnospiraceae | 2.46 |
| C3D4 | AC | Unclassified Xanthobacteraceae | 1.415 |
| C3D4 | AC | Unclassified d__Bacteria | 0.005 |
| C3D5 | AC | < 2.5% | 8.36 |
| C3D5 | AC | Apilactobacillus micheneri | 87.07 |
| C3D5 | AC | Sodalis | 0.915 |
| C3D5 | AC | Unclassified  Acinetobacter | 0 |
| C3D5 | AC | Unclassified Lachnospiraceae | 2.73 |
| C3D5 | AC | Unclassified Xanthobacteraceae | 0.925 |
| C3D5 | AC | Unclassified d__Bacteria | 0 |
| C3D6 | AC | < 2.5% | 13.98 |
| C3D6 | AC | Apilactobacillus micheneri | 77.4 |
| C3D6 | AC | Sodalis | 0.315 |
| C3D6 | AC | Unclassified  Acinetobacter | 0.035 |
| C3D6 | AC | Unclassified Lachnospiraceae | 6.65 |
| C3D6 | AC | Unclassified Xanthobacteraceae | 1.62 |
| C3D6 | AC | Unclassified d__Bacteria | 0 |
| C3D7 | AC | < 2.5% | 37.65 |
| C3D7 | AC | Apilactobacillus micheneri | 20.93 |
| C3D7 | AC | Sodalis | 3.325 |
| C3D7 | AC | Unclassified  Acinetobacter | 0.32 |
| C3D7 | AC | Unclassified Lachnospiraceae | 21.315 |
| C3D7 | AC | Unclassified Xanthobacteraceae | 15.52 |
| C3D7 | AC | Unclassified d__Bacteria | 0.94 |
| C3D8 | AC | < 2.5% | 6.21 |
| C3D8 | AC | Apilactobacillus micheneri | 87.7 |
| C3D8 | AC | Sodalis | 1.17 |
| C3D8 | AC | Unclassified  Acinetobacter | 0.345 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C3D8 | AC | Unclassified Lachnospiraceae | 3.045 |
| C3D8 | AC | Unclassified Xanthobacteraceae | 1.53 |
| C3D8 | AC | Unclassified d__Bacteria | 0 |
| C4D1 | AC | < 2.5% | 4.2 |
| C4D1 | AC | Apilactobacillus micheneri | 95.195 |
| C4D1 | AC | Sodalis | 0.035 |
| C4D1 | AC | Unclassified  Acinetobacter | 0.14 |
| C4D1 | AC | Unclassified Lachnospiraceae | 0.145 |
| C4D1 | AC | Unclassified Xanthobacteraceae | 0.285 |
| C4D1 | AC | Unclassified d__Bacteria | 0 |
| C4D2 | AC | < 2.5% | 54.655 |
| C4D2 | AC | Apilactobacillus micheneri | 40.37 |
| C4D2 | AC | Sodalis | 0 |
| C4D2 | AC | Unclassified  Acinetobacter | 1.435 |
| C4D2 | AC | Unclassified Lachnospiraceae | 0.485 |
| C4D2 | AC | Unclassified Xanthobacteraceae | 2.93 |
| C4D2 | AC | Unclassified d__Bacteria | 0.125 |
| C4D3 | AC | < 2.5% | 5.685 |
| C4D3 | AC | Apilactobacillus micheneri | 93.19 |
| C4D3 | AC | Sodalis | 0 |
| C4D3 | AC | Unclassified  Acinetobacter | 0 |
| C4D3 | AC | Unclassified Lachnospiraceae | 0.215 |
| C4D3 | AC | Unclassified Xanthobacteraceae | 0.91 |
| C4D3 | AC | Unclassified d__Bacteria | 0 |
| C4D4 | AC | < 2.5% | 35.36 |
| C4D4 | AC | Apilactobacillus micheneri | 58.135 |
| C4D4 | AC | Sodalis | 3.82 |
| C4D4 | AC | Unclassified  Acinetobacter | 1.44 |
| C4D4 | AC | Unclassified Lachnospiraceae | 0.405 |
| C4D4 | AC | Unclassified Xanthobacteraceae | 0.82 |
| C4D4 | AC | Unclassified d__Bacteria | 0.02 |
| C4D5 | AC | < 2.5% | 37.895 |
| C4D5 | AC | Apilactobacillus micheneri | 32.64 |
| C4D5 | AC | Sodalis | 23.43 |
| C4D5 | AC | Unclassified  Acinetobacter | 1.7 |
| C4D5 | AC | Unclassified Lachnospiraceae | 0.185 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C4D5 | AC | Unclassified Xanthobacteraceae | 3.175 |
| C4D5 | AC | Unclassified d__Bacteria | 0.975 |
| C4D6 | AC | < 2.5% | 27.695 |
| C4D6 | AC | Apilactobacillus micheneri | 71.075 |
| C4D6 | AC | Sodalis | 0.305 |
| C4D6 | AC | Unclassified  Acinetobacter | 0.54 |
| C4D6 | AC | Unclassified Lachnospiraceae | 0 |
| C4D6 | AC | Unclassified Xanthobacteraceae | 0.385 |
| C4D6 | AC | Unclassified d__Bacteria | 0 |
| C4D7 | AC | < 2.5% | 6.5 |
| C4D7 | AC | Apilactobacillus micheneri | 91.16 |
| C4D7 | AC | Sodalis | 0 |
| C4D7 | AC | Unclassified  Acinetobacter | 0.7 |
| C4D7 | AC | Unclassified Lachnospiraceae | 0 |
| C4D7 | AC | Unclassified Xanthobacteraceae | 0.265 |
| C4D7 | AC | Unclassified d__Bacteria | 1.375 |
| C4D8 | AC | < 2.5% | 15.51 |
| C4D8 | AC | Apilactobacillus micheneri | 80.945 |
| C4D8 | AC | Sodalis | 0 |
| C4D8 | AC | Unclassified  Acinetobacter | 0 |
| C4D8 | AC | Unclassified Lachnospiraceae | 0.345 |
| C4D8 | AC | Unclassified Xanthobacteraceae | 2.65 |
| C4D8 | AC | Unclassified d__Bacteria | 0.55 |
| C4E1 | N | < 2.5% | 6.775 |
| C4E1 | N | Apilactobacillus micheneri | 90.225 |
| C4E1 | N | Sodalis | 2.445 |
| C4E1 | N | Unclassified  Acinetobacter | 0.12 |
| C4E1 | N | Unclassified Lachnospiraceae | 0.25 |
| C4E1 | N | Unclassified Xanthobacteraceae | 0.085 |
| C4E1 | N | Unclassified d__Bacteria | 0.1 |
| C5C1 | N | < 2.5% | 0.16 |
| C5C1 | N | Apilactobacillus micheneri | 0.23 |
| C5C1 | N | Sodalis | 0 |
| C5C1 | N | Unclassified  Acinetobacter | 0 |
| C5C1 | N | Unclassified Lachnospiraceae | 99.6 |
| C5C1 | N | Unclassified Xanthobacteraceae | 0.01 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C5C1 | N | Unclassified d__Bacteria | 0 |
| C5D1 | AC | < 2.5% | 50.9 |
| C5D1 | AC | Apilactobacillus micheneri | 41.725 |
| C5D1 | AC | Sodalis | 0.09 |
| C5D1 | AC | Unclassified  Acinetobacter | 2.585 |
| C5D1 | AC | Unclassified Lachnospiraceae | 2.57 |
| C5D1 | AC | Unclassified Xanthobacteraceae | 2.05 |
| C5D1 | AC | Unclassified d__Bacteria | 0.08 |
| C5D2 | AC | < 2.5% | 6.32 |
| C5D2 | AC | Apilactobacillus micheneri | 85.185 |
| C5D2 | AC | Sodalis | 0.15 |
| C5D2 | AC | Unclassified  Acinetobacter | 5.12 |
| C5D2 | AC | Unclassified Lachnospiraceae | 0.785 |
| C5D2 | AC | Unclassified Xanthobacteraceae | 2.44 |
| C5D2 | AC | Unclassified d__Bacteria | 0 |
| C5D3 | AC | < 2.5% | 31.555 |
| C5D3 | AC | Apilactobacillus micheneri | 51.295 |
| C5D3 | AC | Sodalis | 6.375 |
| C5D3 | AC | Unclassified  Acinetobacter | 7.32 |
| C5D3 | AC | Unclassified Lachnospiraceae | 1.275 |
| C5D3 | AC | Unclassified Xanthobacteraceae | 2.17 |
| C5D3 | AC | Unclassified d__Bacteria | 0.01 |
| C5D4 | NAm | < 2.5% | 2.235 |
| C5D4 | NAm | Apilactobacillus micheneri | 1.93 |
| C5D4 | NAm | Sodalis | 95.82 |
| C5D4 | NAm | Unclassified  Acinetobacter | 0 |
| C5D4 | NAm | Unclassified Lachnospiraceae | 0 |
| C5D4 | NAm | Unclassified Xanthobacteraceae | 0.015 |
| C5D4 | NAm | Unclassified d__Bacteria | 0 |
| C5D5 | NAm | < 2.5% | 3.15 |
| C5D5 | NAm | Apilactobacillus micheneri | 3.335 |
| C5D5 | NAm | Sodalis | 93.3 |
| C5D5 | NAm | Unclassified  Acinetobacter | 0.035 |
| C5D5 | NAm | Unclassified Lachnospiraceae | 0.01 |
| C5D5 | NAm | Unclassified Xanthobacteraceae | 0.09 |
| C5D5 | NAm | Unclassified d__Bacteria | 0.08 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C5D6 | NAm | < 2.5% | 2.76 |
| C5D6 | NAm | Apilactobacillus micheneri | 2.935 |
| C5D6 | NAm | Sodalis | 94.22 |
| C5D6 | NAm | Unclassified  Acinetobacter | 0 |
| C5D6 | NAm | Unclassified Lachnospiraceae | 0.015 |
| C5D6 | NAm | Unclassified Xanthobacteraceae | 0.04 |
| C5D6 | NAm | Unclassified d__Bacteria | 0.03 |
| C5D7 | NAm | < 2.5% | 2.655 |
| C5D7 | NAm | Apilactobacillus micheneri | 1.69 |
| C5D7 | NAm | Sodalis | 95.52 |
| C5D7 | NAm | Unclassified  Acinetobacter | 0 |
| C5D7 | NAm | Unclassified Lachnospiraceae | 0 |
| C5D7 | NAm | Unclassified Xanthobacteraceae | 0 |
| C5D7 | NAm | Unclassified d__Bacteria | 0.135 |
| C5D8 | NAm | < 2.5% | 4.595 |
| C5D8 | NAm | Apilactobacillus micheneri | 19.08 |
| C5D8 | NAm | Sodalis | 74.67 |
| C5D8 | NAm | Unclassified  Acinetobacter | 0 |
| C5D8 | NAm | Unclassified Lachnospiraceae | 0.08 |
| C5D8 | NAm | Unclassified Xanthobacteraceae | 0.025 |
| C5D8 | NAm | Unclassified d__Bacteria | 1.55 |
| C6D1 | NAm | < 2.5% | 34.675 |
| C6D1 | NAm | Apilactobacillus micheneri | 8.48 |
| C6D1 | NAm | Sodalis | 11.28 |
| C6D1 | NAm | Unclassified  Acinetobacter | 0 |
| C6D1 | NAm | Unclassified Lachnospiraceae | 45.565 |
| C6D1 | NAm | Unclassified Xanthobacteraceae | 0 |
| C6D1 | NAm | Unclassified d__Bacteria | 0 |
| C6D2 | NAm | < 2.5% | 4.235 |
| C6D2 | NAm | Apilactobacillus micheneri | 10.665 |
| C6D2 | NAm | Sodalis | 84.995 |
| C6D2 | NAm | Unclassified  Acinetobacter | 0.035 |
| C6D2 | NAm | Unclassified Lachnospiraceae | 0.05 |
| C6D2 | NAm | Unclassified Xanthobacteraceae | 0.02 |
| C6D2 | NAm | Unclassified d__Bacteria | 0 |
| C6D3 | NAm | < 2.5% | 4.135 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C6D3 | NAm | Apilactobacillus micheneri | 2.28 |
| C6D3 | NAm | Sodalis | 93.585 |
| C6D3 | NAm | Unclassified  Acinetobacter | 0 |
| C6D3 | NAm | Unclassified Lachnospiraceae | 0 |
| C6D3 | NAm | Unclassified Xanthobacteraceae | 0 |
| C6D3 | NAm | Unclassified d__Bacteria | 0 |
| C6D4 | NAm | < 2.5% | 9.975 |
| C6D4 | NAm | Apilactobacillus micheneri | 51.365 |
| C6D4 | NAm | Sodalis | 36.62 |
| C6D4 | NAm | Unclassified  Acinetobacter | 1.375 |
| C6D4 | NAm | Unclassified Lachnospiraceae | 0.02 |
| C6D4 | NAm | Unclassified Xanthobacteraceae | 0.16 |
| C6D4 | NAm | Unclassified d__Bacteria | 0.485 |
| C6D5 | NAm | < 2.5% | 2.525 |
| C6D5 | NAm | Apilactobacillus micheneri | 1.24 |
| C6D5 | NAm | Sodalis | 96.225 |
| C6D5 | NAm | Unclassified  Acinetobacter | 0 |
| C6D5 | NAm | Unclassified Lachnospiraceae | 0 |
| C6D5 | NAm | Unclassified Xanthobacteraceae | 0.005 |
| C6D5 | NAm | Unclassified d__Bacteria | 0.005 |
| C6D6 | NAm | < 2.5% | 1.615 |
| C6D6 | NAm | Apilactobacillus micheneri | 62.07 |
| C6D6 | NAm | Sodalis | 36.29 |
| C6D6 | NAm | Unclassified  Acinetobacter | 0 |
| C6D6 | NAm | Unclassified Lachnospiraceae | 0 |
| C6D6 | NAm | Unclassified Xanthobacteraceae | 0.02 |
| C6D6 | NAm | Unclassified d__Bacteria | 0.005 |
| C6D7 | NAm | < 2.5% | 2.485 |
| C6D7 | NAm | Apilactobacillus micheneri | 6.645 |
| C6D7 | NAm | Sodalis | 90.77 |
| C6D7 | NAm | Unclassified  Acinetobacter | 0 |
| C6D7 | NAm | Unclassified Lachnospiraceae | 0 |
| C6D7 | NAm | Unclassified Xanthobacteraceae | 0.01 |
| C6D7 | NAm | Unclassified d__Bacteria | 0.09 |
| C6D8 | NAm | < 2.5% | 2.125 |
| C6D8 | NAm | Apilactobacillus micheneri | 30.91 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C6D8 | NAm | Sodalis | 66.84 |
| C6D8 | NAm | Unclassified  Acinetobacter | 0.015 |
| C6D8 | NAm | Unclassified Lachnospiraceae | 0.035 |
| C6D8 | NAm | Unclassified Xanthobacteraceae | 0.005 |
| C6D8 | NAm | Unclassified d__Bacteria | 0.07 |
| C6E1 | N | < 2.5% | 0.56 |
| C6E1 | N | Apilactobacillus micheneri | 0.33 |
| C6E1 | N | Sodalis | 0.15 |
| C6E1 | N | Unclassified  Acinetobacter | 0 |
| C6E1 | N | Unclassified Lachnospiraceae | 98.945 |
| C6E1 | N | Unclassified Xanthobacteraceae | 0.015 |
| C6E1 | N | Unclassified d__Bacteria | 0 |
| C7D1 | NAm | < 2.5% | 35.115 |
| C7D1 | NAm | Apilactobacillus micheneri | 9.67 |
| C7D1 | NAm | Sodalis | 4.785 |
| C7D1 | NAm | Unclassified  Acinetobacter | 0 |
| C7D1 | NAm | Unclassified Lachnospiraceae | 50.42 |
| C7D1 | NAm | Unclassified Xanthobacteraceae | 0 |
| C7D1 | NAm | Unclassified d__Bacteria | 0.01 |
| C7D2 | NAm | < 2.5% | 4.14 |
| C7D2 | NAm | Apilactobacillus micheneri | 76.36 |
| C7D2 | NAm | Sodalis | 17.775 |
| C7D2 | NAm | Unclassified  Acinetobacter | 0.125 |
| C7D2 | NAm | Unclassified Lachnospiraceae | 0.07 |
| C7D2 | NAm | Unclassified Xanthobacteraceae | 0.125 |
| C7D2 | NAm | Unclassified d__Bacteria | 1.405 |
| C7D3 | NAm | < 2.5% | 10.1 |
| C7D3 | NAm | Apilactobacillus micheneri | 48.655 |
| C7D3 | NAm | Sodalis | 40.66 |
| C7D3 | NAm | Unclassified  Acinetobacter | 0 |
| C7D3 | NAm | Unclassified Lachnospiraceae | 0.21 |
| C7D3 | NAm | Unclassified Xanthobacteraceae | 0.265 |
| C7D3 | NAm | Unclassified d__Bacteria | 0.11 |
| C7D4 | NAm | < 2.5% | 2.925 |
| C7D4 | NAm | Apilactobacillus micheneri | 3.14 |
| C7D4 | NAm | Sodalis | 93.845 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C7D4 | NAm | Unclassified  Acinetobacter | 0 |
| C7D4 | NAm | Unclassified Lachnospiraceae | 0 |
| C7D4 | NAm | Unclassified Xanthobacteraceae | 0.015 |
| C7D4 | NAm | Unclassified d__Bacteria | 0.075 |
| C7D5 | NAm | < 2.5% | 2.125 |
| C7D5 | NAm | Apilactobacillus micheneri | 29.125 |
| C7D5 | NAm | Sodalis | 68.6 |
| C7D5 | NAm | Unclassified  Acinetobacter | 0 |
| C7D5 | NAm | Unclassified Lachnospiraceae | 0.025 |
| C7D5 | NAm | Unclassified Xanthobacteraceae | 0.01 |
| C7D5 | NAm | Unclassified d__Bacteria | 0.115 |
| C7D6 | NAm | < 2.5% | 2.175 |
| C7D6 | NAm | Apilactobacillus micheneri | 1.5 |
| C7D6 | NAm | Sodalis | 96.28 |
| C7D6 | NAm | Unclassified  Acinetobacter | 0 |
| C7D6 | NAm | Unclassified Lachnospiraceae | 0 |
| C7D6 | NAm | Unclassified Xanthobacteraceae | 0 |
| C7D6 | NAm | Unclassified d__Bacteria | 0.045 |
| C7D7 | NAm | < 2.5% | 6.555 |
| C7D7 | NAm | Apilactobacillus micheneri | 85.32 |
| C7D7 | NAm | Sodalis | 4.25 |
| C7D7 | NAm | Unclassified  Acinetobacter | 0 |
| C7D7 | NAm | Unclassified Lachnospiraceae | 0.01 |
| C7D7 | NAm | Unclassified Xanthobacteraceae | 0.225 |
| C7D7 | NAm | Unclassified d__Bacteria | 3.64 |
| C7D8 | SP | < 2.5% | 68.08 |
| C7D8 | SP | Apilactobacillus micheneri | 3.35 |
| C7D8 | SP | Sodalis | 0 |
| C7D8 | SP | Unclassified  Acinetobacter | 1.46 |
| C7D8 | SP | Unclassified Lachnospiraceae | 0.565 |
| C7D8 | SP | Unclassified Xanthobacteraceae | 7.685 |
| C7D8 | SP | Unclassified d__Bacteria | 18.86 |
| C7E1 | N | < 2.5% | 20.555 |
| C7E1 | N | Apilactobacillus micheneri | 77.39 |
| C7E1 | N | Sodalis | 1.22 |
| C7E1 | N | Unclassified  Acinetobacter | 0.11 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C7E1 | N | Unclassified Lachnospiraceae | 0.625 |
| C7E1 | N | Unclassified Xanthobacteraceae | 0.055 |
| C7E1 | N | Unclassified d__Bacteria | 0.045 |
| C8D1 | SP | < 2.5% | 59.73 |
| C8D1 | SP | Apilactobacillus micheneri | 2.56 |
| C8D1 | SP | Sodalis | 0 |
| C8D1 | SP | Unclassified  Acinetobacter | 31.675 |
| C8D1 | SP | Unclassified Lachnospiraceae | 0 |
| C8D1 | SP | Unclassified Xanthobacteraceae | 4.125 |
| C8D1 | SP | Unclassified d__Bacteria | 1.91 |
| C8D2 | SP | < 2.5% | 63.41 |
| C8D2 | SP | Apilactobacillus micheneri | 0 |
| C8D2 | SP | Sodalis | 0 |
| C8D2 | SP | Unclassified  Acinetobacter | 0 |
| C8D2 | SP | Unclassified Lachnospiraceae | 0 |
| C8D2 | SP | Unclassified Xanthobacteraceae | 33.02 |
| C8D2 | SP | Unclassified d__Bacteria | 3.57 |
| C8D3 | SP | < 2.5% | 82.4 |
| C8D3 | SP | Apilactobacillus micheneri | 0 |
| C8D3 | SP | Sodalis | 0 |
| C8D3 | SP | Unclassified  Acinetobacter | 0.895 |
| C8D3 | SP | Unclassified Lachnospiraceae | 0.275 |
| C8D3 | SP | Unclassified Xanthobacteraceae | 13.81 |
| C8D3 | SP | Unclassified d__Bacteria | 2.62 |
| C8D4 | SP | < 2.5% | 88.03 |
| C8D4 | SP | Apilactobacillus micheneri | 0 |
| C8D4 | SP | Sodalis | 0 |
| C8D4 | SP | Unclassified  Acinetobacter | 1.2 |
| C8D4 | SP | Unclassified Lachnospiraceae | 2.06 |
| C8D4 | SP | Unclassified Xanthobacteraceae | 2.3 |
| C8D4 | SP | Unclassified d__Bacteria | 6.41 |
| C8D5 | SP | < 2.5% | 47.775 |
| C8D5 | SP | Apilactobacillus micheneri | 0 |
| C8D5 | SP | Sodalis | 0 |
| C8D5 | SP | Unclassified  Acinetobacter | 37.26 |
| C8D5 | SP | Unclassified Lachnospiraceae | 0 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C8D5 | SP | Unclassified Xanthobacteraceae | 8.24 |
| C8D5 | SP | Unclassified d__Bacteria | 6.725 |
| C8D6 | SP | < 2.5% | 91.19 |
| C8D6 | SP | Apilactobacillus micheneri | 0 |
| C8D6 | SP | Sodalis | 0 |
| C8D6 | SP | Unclassified  Acinetobacter | 3.19 |
| C8D6 | SP | Unclassified Lachnospiraceae | 0.475 |
| C8D6 | SP | Unclassified Xanthobacteraceae | 4.01 |
| C8D6 | SP | Unclassified d__Bacteria | 1.135 |
| C8D7 | SP | < 2.5% | 65.5 |
| C8D7 | SP | Apilactobacillus micheneri | 0 |
| C8D7 | SP | Sodalis | 2.05 |
| C8D7 | SP | Unclassified  Acinetobacter | 0 |
| C8D7 | SP | Unclassified Lachnospiraceae | 2.685 |
| C8D7 | SP | Unclassified Xanthobacteraceae | 19.29 |
| C8D7 | SP | Unclassified d__Bacteria | 10.475 |
| C9D2 | SP | < 2.5% | 96.17 |
| C9D2 | SP | Apilactobacillus micheneri | 0.69 |
| C9D2 | SP | Sodalis | 0.8 |
| C9D2 | SP | Unclassified  Acinetobacter | 0.23 |
| C9D2 | SP | Unclassified Lachnospiraceae | 0.945 |
| C9D2 | SP | Unclassified Xanthobacteraceae | 1.155 |
| C9D2 | SP | Unclassified d__Bacteria | 0.01 |
| C9D3 | SP | < 2.5% | 36.995 |
| C9D3 | SP | Apilactobacillus micheneri | 0.565 |
| C9D3 | SP | Sodalis | 57.785 |
| C9D3 | SP | Unclassified  Acinetobacter | 0.19 |
| C9D3 | SP | Unclassified Lachnospiraceae | 0 |
| C9D3 | SP | Unclassified Xanthobacteraceae | 1.41 |
| C9D3 | SP | Unclassified d__Bacteria | 3.055 |
| C9D5 | SP | < 2.5% | 68.91 |
| C9D5 | SP | Apilactobacillus micheneri | 4.225 |
| C9D5 | SP | Sodalis | 0 |
| C9D5 | SP | Unclassified  Acinetobacter | 15.97 |
| C9D5 | SP | Unclassified Lachnospiraceae | 1.075 |
| C9D5 | SP | Unclassified Xanthobacteraceae | 5.79 |

Table B2. Relative abundances of the top 6 bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. treatments were: natural pollen (N), natural pollen with added *A. micheneri* (nam), sterile pollen (S), sterile pollen with *A. micheneri* (sam), and natural pollen with an antibacterial cocktail (AC). less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. (continued)

| Sample | Treatment | Species | Abundance |
|---|---|---|---|
| C9D5 | SP | Unclassified d__Bacteria | 4.03 |
| C9D7 | SP | < 2.5% | 67.255 |
| C9D7 | SP | Apilactobacillus micheneri | 0 |
| C9D7 | SP | Sodalis | 0 |
| C9D7 | SP | Unclassified  Acinetobacter | 1.205 |
| C9D7 | SP | Unclassified Lachnospiraceae | 2.105 |
| C9D7 | SP | Unclassified Xanthobacteraceae | 25.645 |
| C9D7 | SP | Unclassified d__Bacteria | 3.79 |
| C9D8 | SP | < 2.5% | 77.07 |
| C9D8 | SP | Apilactobacillus micheneri | 0.72 |
| C9D8 | SP | Sodalis | 0 |
| C9D8 | SP | Unclassified  Acinetobacter | 2.745 |
| C9D8 | SP | Unclassified Lachnospiraceae | 0.035 |
| C9D8 | SP | Unclassified Xanthobacteraceae | 1.205 |
| C9D8 | SP | Unclassified d__Bacteria | 18.225 |