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ORIGINAL ARTICLE

Developmental microbiome of the small carpenter bee, *Ceratina calcarata*

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Abstract

The importance of the holobiont has been studied across many bee species, but less is known about the changes in the microbiome throughout the course of development, particularly in subsocial bees. This study used 16S rRNA and ITS amplicon sequencing of pollen provisions and individuals of the small carpenter bee, *Ceratina calcarata*, across stages of development to characterize the composition and diversity of bacteria and fungi in the microbiome. Pollen provisions and larval stages showed similar beta diversity, but differences in taxa composition. There was no significant decrease in diversity during the transition between larval and pupal stages that was expected post defecation. However, there were unexpected and progressive declines in diversity as development progressed from the early to late pupal stages and again from the callow to adult stages. Bees across all stages lacked members of the *Lactobacillus* (now *Apilactobacillus)* genus, which has been shown in other studies to be part of the core bacterial community in *C. calcarata* and all bees. Three correlations between bacteria and fungi were found, suggesting common beneficial bacteria may protect the bees from prevalent fungal pathogens. Low alpha diversity, particularly in the later stages of development through adulthood, is concerning as the microbiome plays an important role in maintaining wild bee health.

KEYWORDS
16S, Apidae, bacteria, bee health, fungi, ITS, *Lactobacillus*, microbiome, pollen, wild bee

1 | INTRODUCTION

Research surrounding the microbiome and its role within the insect host has recently expanded over the past two decades (Engel & Moran, 2013). The holobiont describes the relationship between a host and any associated microorganisms on or within the individual, such as bacteria, fungi, and viruses (Rosenberg et al., 2007). These symbioses between the host and the symbiont may benefit the host in the form of mutualism, serve one of either the host or the microorganism through commensalism, or harm the host in the case that the microorganism is a pathogen (Lewis & Lizé, 2015; Rosenberg et al., 2007). The ways that the microbiome affects its host are widespread, ranging from physiological to behavioral to evolutionary processes (Lewis & Lizé, 2015). Conversely, microbial diversity varies with a host's habitat, diet, development, and phylogeny (Yun et al., 2014). Thus, in this complex relationship between the host and its microbiome, there are many different avenues in which symbionts can affect and be affected by their host's fitness and survival (Lewis & Lizé, 2015).

Compared to mammals, insects generally depend on fewer microbial species (Engel & Moran, 2013). However, insects of

different species, from different locations or in different developmental stages, can have significant variations in their microbiome diversity and composition (Nobles & Jackson, 2020; Yun et al., 2014). For instance, despite the fact that 13 dragonfly species predominantly featured a couple of bacterial phyla, life stage, location, and host species still accounted for strong variation in diversity (Nobles & Jackson, 2020). In addition, some insects have formed specialized associations with microorganisms (Engel & Moran, 2013). For example, there are distinct bacterial lineages in honey bees and bumble bees that are only found in these two social bee genera, and not in stingless bees or orchid bees (Engel et al., 2012; Koch et al., 2013). Thus, an insect's microbiome can be quite complex, with any number of different factors causing variances in holobiont composition among individuals and species.

There are different ways in which the microbiome is established in insects. While some endosymbionts are passed along through vertical transmission from mother to offspring, the microbiome of the insect gut tends to not be inherited and is rather acquired from the environment (Lewis & Lizé, 2015). In honey bee larvae, it was seen that as the brood developed, instars generally established more diverse compositions of bacterial species, likely due to the increased consumption of pollen (Vojvodic et al., 2013). In small carpenter bees, it has been suggested that differences in pollen collected across habitats result in varied microbiomes, with increased floral diversity correlated to increased microbial diversity (McFrederick & Rehan, 2019). Thus, a main source of microbes is likely a result of environmental exposure in the form of the pollen (Dew et al., 2020).

Questions have also been raised about how a host maintains its gut microbiome with beneficial symbionts while also preventing the establishment of pathogens. A study using Sonoran Desert turtle ants showed that the proventriculus filters out external bacteria and larger particles in order to maintain the core gut microbiome that is established prior to the development of the proventricular filter (Lanan et al., 2016). However, every time an insect molts or undergoes metamorphosis, their exoskeletal lining in the foregut and hindgut is shed and any microbes are disturbed, suggesting that the entire symbioses must be re-established (Engel & Moran, 2013). In a study using mosquitos, it was found that metamorphosis resulted in the elimination of most, if not all, gut bacteria such that adults did not have any bacteria upon emerging (Moll et al., 2001). As bees undergo defecation in between the fully grown larvae and prepupae stage, and as they molt to develop from a fully pigmented pupa into an adult, there may be changes in the microbiome composition, diversity, and abundance during these key transitions.

Studies of the microbiome in bees have revealed that a handful of bacterial groups are an important factor in bee health. Some have suggested that microbes could be playing a role in important processes such as pathogen defense, digestion, immunity, and nutrient use in the honey bee (Engel et al., 2012). On the contrary, in the microbiota of the solitary bee *Osmia bicornis*, the presence of some bacteria, such as *Paenibacillus* sp., *Sporosarcina* sp., and *Bacillus* sp., is indicative of larval mortality (Voulgari-Kokota et al., 2020). However, microbiome studies in the western honey bee, *Apis mellifera*, have shown that there is high genetic diversity in the gut microbiome, despite the presence of relatively few bacterial species (Engel et al., 2012). Bumble bees with microbiomes dominated by *Snodgrassella alvi* and *Lactobacillus* (now *Apilactobacillus) bombicola* experience increased survivorship against selenite by reducing metalloid toxicity (Rothman et al., 2019). Bumble bees have also revealed that high microbiome diversity and certain members of the microbiome, such as flower- and insect-associated yeasts, can inhibit growth of the *Crithidia* parasite (Mockler et al., 2018; Pozo et al., 2020). Thus, although some microbes can be detrimental to bee health, there are also beneficial relationships that allow bees to thrive with the aid of its microbiome.

While much of the research on bee microbiomes have focused on honey bees and bumble bees, factors such as social behavior and environmental exposure can heavily influence the microbiome such that highly social bee species can have unique microbiomes. The honey bee is a eusocial genus where diversity and compositions of the microbiome are established differently depending on the stage of development and whether or not the bee is a worker or a queen (Martinson et al., 2012; Tarpy et al., 2015). In a study comparing *A. mellifera*, *Bombus terrestris*, and *Osmia bicornis*, three species of bees with varying social behavior, it was shown that gut microbiota differed among species despite collecting pollen from the same plants (Mohr & Tebbe, 2006). However, sociality does not seem to always be a considerable factor, as a study of with the facultatively social bee species *Megalopta centralis* and *M. genalis* revealed that microbial diversity was driven by species and developmental stage, rather than sociality (McFrederick et al., 2014). As the study comparing three different social behaviors also revealed bacterial composition differed in larval and adult honey bees, this suggests that social behavior and environmental exposures are another factor affecting the microbiome that needs disentangling from the effects of life stage (Mohr & Tebbe, 2006).

The importance of the microbiome throughout development is variable among insects. Studies on butterflies have shown that there is an absence of strong host and microbiome associations throughout development, meaning that gut microbes did not influence butterfly or caterpillar fitness, growth, or survival (Hammer et al., 2017; Phalnikar et al., 2019). However, a study on honey bees showed that beginning larval instars were dominated by Acetobacteraceae, whereas later instars showed the dominant presence of *Lactobacillus* species, suggesting the bacterial communities could change during development (Vojvodic et al., 2013). Queen honey bee microbiomes also change from being dominated by enteric bacteria in early stages of development to alphaproteobacterial when mature (Tarpy et al., 2015). Changes in microbial composition may indicate significance in the role the microbiome has throughout development. In a study with larval mason bees, *Osmia ribifloris*, eliminating microbes from the pollen provisions resulted in larvae with decreased growth rates, biomass, and survivorship (Dharampal et al., 2019). Thus, there has been some indication that the microbiome could be directly involved in some insects' and especially bee survivorship.

Ceratina calcarata Robertson (Hymenoptera: Apidae) is a small carpenter bee that lives in the pith of dead, broken stems (Michener, 2007). Within the stem, a foundress mother will form a pollen mass using foraged pollen and nectar before laying an egg on the pollen provisions and closing the brood cell (Figure 1) (Michener, 2007; Rehan & Richards, 2010). The only source of food that each brood will receive before maturing will come from the provisioned pollen ball present when the egg was laid (Dew et al., 2020). Other potential access to the microbes from the environment would likely come from within the nest. This species of *Ceratina* is subsocial in which a solitary mothers guards and grooms offspring during development (Rehan, 2020). However, since subsocial *Ceratina* colonies are reduced to mother–offspring interaction, the sources of exposure to microbes from the social environment are more limited compared to a eusocial species with overlapping generations of adult bees and many individuals participating in cooperative brood care.

The core microbiome in adult *Ceratina calcarata* contains 13 core bacterial phylotypes and pollen provisions have 19 phylotypes (Graystock et al., 2017). Adult *C. calcarata* collect pollen from many different floral resources, but pollen provisions usually contain *Acinetobacter*, *Erwinia*, *Lactobacillus*, and *Sphingomonas* (Dew et al., 2020). Data suggest that microbes are brought back to brood by mothers after provisioning, with some specific plants acting as reservoirs for dominant bacteria and fungi (Graystock et al., 2017; McFrederick & Rehan, 2016). Therefore, the flowers that foraging bees collect pollen and nectar from play an important role in introducing microbes to developing brood.

Common fungal symbionts identified in bees include *Ascosphaera*, *Aspergillus*, *Penicillium*, *Bettsia*, and yeasts (Batra et al., 1973). Specifically in *C. calcarata*, two of the more abundant fungal amplicon sequence variants (ASVs) in the pollen provisions included the flower associated yeasts *Metschnikowia* and *Starmerella* (Graystock et al., 2017). *Metschnikowia gruessii* is one yeast that positively impacts bumble bee colony growth and can suppress a bumble bee pathogen (Pozo et al., 2020). However, this does not implicate that the same fungi will be present in *C. calcarata* in our study region. In *Ceratina australensis*, compositions of fungal communities in the microbiome differed across landscapes, suggesting regional differences (McFrederick & Rehan, 2019). Thus, even with previously described beneficial symbioses between bees and fungi, it is likely that the specific composition found in *C. calcarata* will differ from those in other regions because of exposures to different environments and floral sources.

This study characterizes how the microbiome of the small carpenter bee *C. calcarata* changes in composition and diversity over the course of brood development through its egg, larval, pupal, and adult stages. Bacterial and fungal communities from brood were analyzed using the 16S and ITS region for amplicon sequencing. While the microbiome has been characterized in adult *C. calcarata* and in pollen provisions provided to the brood, this study is the first to ask how and when during the stages of development is the holobiont established. It is predicted that brood microbiomes will increase in diversity over the course of development, with later stages reflecting the composition of microbes found in adult bees. This study provides important baseline data on how the microbiome is maintained throughout maturity, which has broader implications for wild bee health and conservation efforts.

2 | MATERIALS AND METHODS

2.1 | Bee samples

In total, 103 individuals of *Ceratina calcarata* were collected during June to August of 2020 from Toronto, Canada (43°46'26"N 79°30'14"W). Collected nests, mainly consisting of the pithy stems of raspberry cane and sumac, were split in half to gain access to pollen balls, adults, and brood. Tweezers and forceps were flame-sterilized using 95% ethanol in between each nest and brood cell. Pollen balls, adult females, and brood were individually stored in a −80°C freezer. For adult specimens, only lone females were collected from solitary nests and assumed to be the mother of the brood.

FIGURE 1 Overview of *Ceratina calcarata* developmental stages assayed in this study from left to right: egg on pollen provision, small larva 1/3rd the length of a pollen ball, medium larva twice the length of a pollen ball, prepupae, white-eyed pupa, red-eyed pupa, ¼ pigmented pupa, ¾ pigmented pupa, fully pigmented pupa. Photo credits: Sandra Rehan

Brood were individually removed from their nest and characterized by their current stage of development, as described by Rehan and Richards (2010). This study examined 10 pollen balls, 11 pooled small larvae, 10 medium larvae, 9 large larvae, 10 prepupae, 38 pupae, 9 newly emerged callows, and 10 mature adults (Figure 1).

2.2 | DNA extraction

DNA extraction was performed using Omega E.Z.N.A. Soil DNA Kits, following the manufacturer's protocol for 100–250 mg samples, including bead lysis, with some modifications. To increase DNA yields, the cHTR reagent recommended for soil extraction protocols was not used in this protocol, resulting in one less round of washing, centrifugation, and collection of the supernatant. Six blanks were included in all the DNA extraction steps and included in plate sample submission, library preparation, and sequence processing.

2.3 | Sequencing

Illumina MiSeq paired end amplicon sequencing was performed using the 16S rRNA region for bacteria and the ITS region for fungi. Specifically, the V5–V6 fragment was examined with the 799bF-CS1 forward primer (MGGATTAGATACCCKGG) and the 1115R-CS2 reverse primer (AGGGTTGCGCTCGTTG) for bacteria. The ITS1 fragment was used to examine fungi with the forward primer of ITS1F (CTTGGTCATTTAGAGGAAGTAA) and reverse primer of ITS2 (GCTGCGTTCTTCATCGATGC). Data were delivered as demultiplexed FASTQ files with adapters already trimmed. Library preparation and sequencing were performed by Génome Québec Centre D'Expertise et de Services.

2.4 | Statistical analysis

Qiime2 was used to conduct microbiome analysis (Bolyen et al., 2019). Demultiplexed sequences underwent sequence quality control using the DADA2 pipeline, which filters phiX reads, chimeric sequences, joins paired ends, denoises, and estimates amplicon sequence variants (Callahan et al., 2016). Sequences were trimmed to where quality scores dropped below 30, and read lengths fell below 283 bases for forward reads and 260 bases for reverse reads. Qiime2 was then used to create feature tables and to examine taxonomy (Bolyen et al., 2019; Katoh & Standley, 2013; McDonald et al., 2012; Price et al., 2010; Weiss et al., 2017).

Taxonomic analysis of the 5714 amplicon sequence variants (ASVs) was tested against the SILVA 128 classifier for 16S bacterial sequences and the UNITE (version 8.3) classifier for ITS sequences using the q2-feature-classifier and the classify-sklearn pipeline (Abarenkov et al., 2021; Bokulich et al., 2018; Pedregosa et al., 2011; Quast et al., 2013; Yilmaz et al., 2014). For 16S, imported sequences had reference reads extracted to increase the classification accuracy, which occurs when the naïve Bayes classifier is trained only on the regions of the target sequences that were sequenced (Werner et al., 2012). This does not apply to ITS sequences, and subsequently, full reference sequences were used to train the classifier. Taxa that were unidentified or unclassified were then individually identified using the best hit in NCBI BLAST (Johnson et al., 2008). Taxa were then exported to R (version 3.6.1) for further statistical analysis (Tables S1-S5) (R Core Team, 2019).

Using the "phyloseq" package in R, reads from blanks likely representing reagent or human-sourced contaminants were found using the "decontam" package and proportionally removed from the ASV tables imported from Qiime2 (Davis et al., 2017; McMurdie & Holmes, 2013). Any taxa of the genera *Wolbachia* and *Sodalis* were also excluded, as those are common intracellular endosymbionts often present due to contamination from mites or external transfer from a mother's crop to her brood (Graystock et al., 2017). This resulted in a remaining 1842 taxa from 80 samples in the phyloseq object using bacterial ASVs and 784 taxa from 103 samples using fungal ASVs (McMurdie & Holmes, 2013).

For alpha and beta diversity analyses, the decontaminated taxa counts were transformed into relative abundances in R (version 3.6.1) (R Core Team, 2019). Shannon diversity was calculated using the "phyloseq" package, and an ANOVA was performed with consideration for each sample's nest using the "lme4" package (Bates et al., 2015; McMurdie & Holmes, 2013). The adonis function in the "vegan" package was used to conduct permutational multivariate analyses (PERMANOVA) that test whether developmental stage significantly affected variations in composition (Oksanen et al., 2020). In confirming whether the PERMANOVA assumption that group dispersions were homogeneous was met, the betadisper function was used to generate ANOVA analyses and examine whether heterogeneous dispersions were confounding to the PERMANOVA results (Oksanen et al., 2020). This assumption was met for both fungal beta diversity and bacterial Bray Curtis dissimilarities but was not met for the bacterial Weighted Unifrac beta diversity. For measures where the assumption was met, a post hoc Tukey's test was performed. In addition, similarity percentage (SIMPER) was conducted within the PAST (version 4.07) program to identify taxa that were predominantly leading differences in diversity (Hammer et al., 2001).

Correlation analyses using both CoNet and SparCC were performed to determine whether the eight bacteria and eight fungi taxa with the highest read counts were commonly co-occurring. To be considered in the top eight taxa, taxa had to reach a threshold prevalence of at least three samples and abundance of at least 1% of reads. The package "CoNetinR" was used to create a network using Spearman, Bray, Pearson, and Kullback-Leibler edge scores (Faust & Raes, 2016). The package "SpiecEasi" was then used for its SparCC function and run with 100 bootstrap replicates (Friedman & Alm, 2012). All correlation analyses in both CoNet and SparCC were significant at a threshold p-value of 0.05.

3 | RESULTS

Illumina MiSeq sequencing resulted in a total of 6,312,114 reads with an average of 28,179 paired end reads per sample and average quality of 33.8. Across all the samples, the lowest number of reads was 6,470 and the greatest was 44,378 reads per sample. Samples were dominated by members of the Proteobacteria, Firmicutes, and Actinobacteria bacteria phyla (Tables S1-S2). At the genus level, *Melissococcus* was the most prevalent with the second most abundant genus, *Anaerotignum* (Figure 2a, Table S3). *Melissococcus*

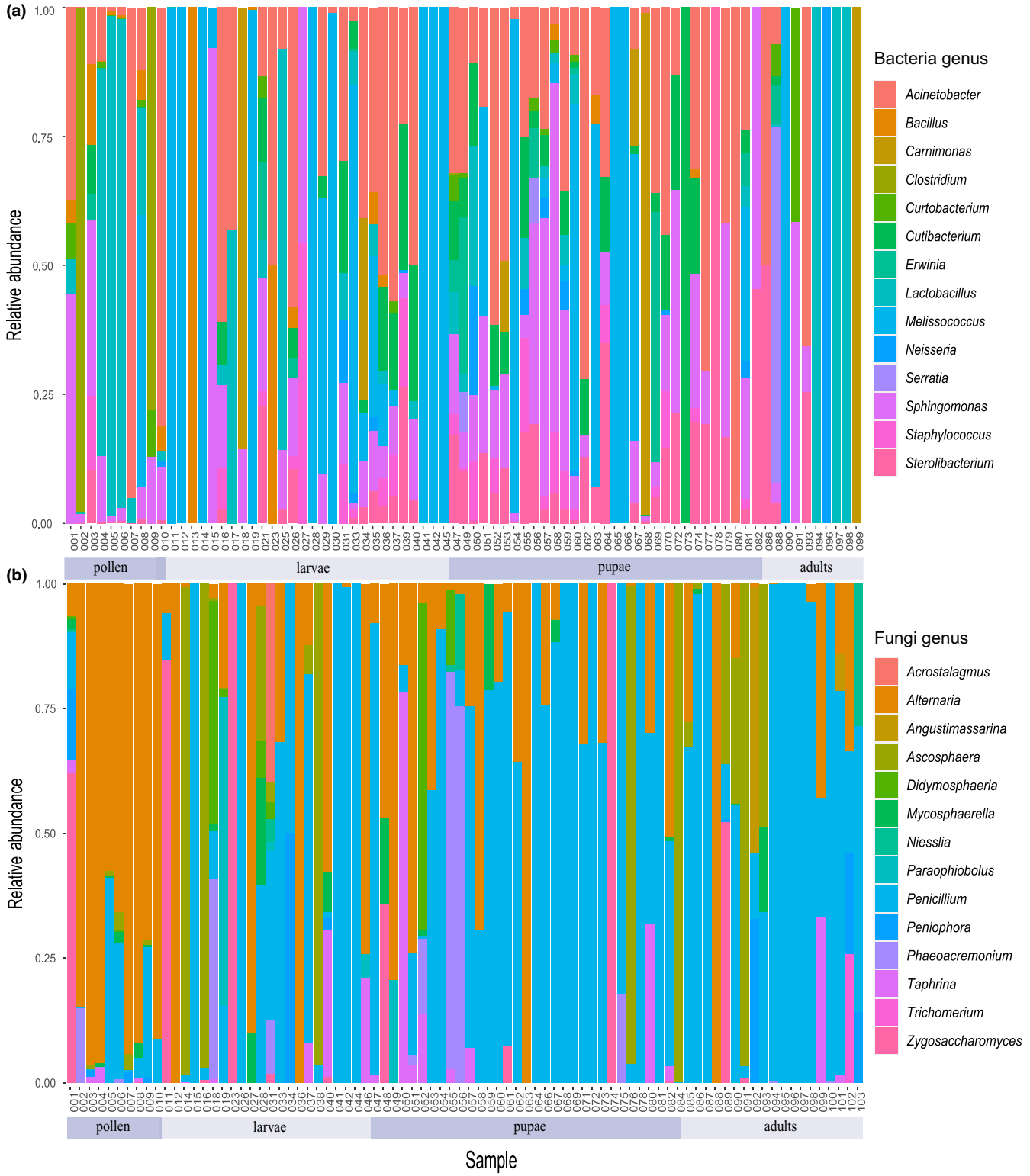

FIGURE 2 Relative abundances of the top 14 bacterial (a) and fungal (b) genera present in each of the pollen provisions, larval, pupal, and adult *Ceratina calcarata*. Detailed information for each sample ID can be found in Tables S1-S2 and S4-S5

was mostly found in earlier developmental stages, including most larval and prepupae samples, but was also relatively abundant in a pollen, white-eyed, and brown-eyed pupae samples (Table S2). *Anaerotignum* was most abundant in larval stages, but also present in brown-eyed and fully pigmented pupae (Table S2). The third most abundant bacteria genus, *Acinetobacter*, was found in pollen samples and throughout the range the developmental stages (Table S2). *Lactobacillus* was abundant in pollen samples, but with low relative abundance in larvae, pupae, and adult bees (Figure 2a, Table S2).

Other abundant bacteria genera, among the top eight found across developmental stages, were *Carnimonas*, *Sphingomonas*, *Cutibacterium*, and *Pantoea* (Figure 2a, Table S3). *Carnimonas* is a member of the Proteobacteria phyla that was found in bees from later developmental stages, including brown-eyed pupae and adults (Table S2). *Sphingomonas* was found across pollen samples, larval stages, and in early pupal stages (Table S2). *Cutibacterium* is relatively abundant in fully grown larvae, prepupae, and some early pupae (Table S2). The *Pantoea* genus was abundant in pollen samples, early pupae, and callow adults (Table S2).

Among fungal taxa, significant phyla include Ascomycota and Basidiomycota (Table S4). The two most abundant genera include *Ascosphaera* and *Penicillium*, accounting for over 57% of all reads from the ITS ASVs (Figure 2b, Table S4). *Ascosphaera* was found in all the developmental stages and in pollen samples (Table S4). *Penicillium* is highly prevalent in pollen samples and in later developmental stages, such as brown-eyed pupae, fully pigmented pupae, and adults (Table S5). *Alternaria* was seen with high relative abundances in pollen samples, early and some late pupal stages, but was notably absent in adults (Table S5). *Phaeoacremonium* was predominantly found in white-eyed pupae but was also present in pollen and medium larvae (Figure 2b). The other abundant fungi including *Zygosaccharomyces*, *Taphrina*, *Paraophiobolus*, *Didymosphaeriea*, and *Mycosphaerella* genera are relatively abundant in samples across the entire range of developmental stages (Table S5).

3.1 | Diversity analyses

Alpha diversity of bacteria, as calculated by Shannon's diversity index, was significantly lowest in adults (ANOVA, $F = 8.616$, $df = 3$, $p = 0.000037$; Figure 3a). Bacterial diversity remains steady as larvae mature into pupae, followed by a subsequent decline in diversity as the bees reach the late pupal stages and adulthood (Figure S1a). This is most evident in comparing early pupal stages to adults and even white-eyed pupae to fully pigmented pupae (Figure S1a). Among fungal ASVs, there was no significant difference among pollen, larvae, pupae, and adults (ANOVA, $F = 0.744$, $df = 3$, $p = 0.53$; Figure 3b, Figure S1b).

For both bacteria and fungi, the developmental stages were significantly different in overall ASVs (Bray Curtis bacteria, PERMANOVA, $R^2 = 0.080$, $df = 3$, $p = 0.001$; fungi, $R^2 = 0.060$, $df = 3$, $p = 0.001$; Figure 4, Figure S2). When comparing across all developmental stages, SIMPER tests using Bray Curtis dissimilarities revealed that *Melissococcus* and *Lactobacillus* were the bacteria with the greatest difference in read counts among stages (Table S6). *Lactobacillus* drove the most dissimilarity between pollen-adults and pollen-pupae, due to an overrepresentation in pollen. *Melissococcus* had the greatest impact in all other pairwise comparisons, with larvae and pupae having the greatest relative abundance. Another genus driving dissimilarities between stages was *Acinetobacter*, which resulted in about 14% dissimilarity between pollen-adults due to a high abundance in pollen and relatively little presence in adults (Table S6). For fungi, *Alternaria*, *Penicillium*, and *Ascosphaera* account for the most dissimilarity across all developmental stages (Table S7). At a pairwise level, dissimilarity was most driven by *Alternaria* in comparing pollen samples to bees due to an underrepresentation in all bees. *Penicillium* was important when comparing larvae–adults or pupae–adults, due to high abundance in adults. *Ascosphaera* was a key genus driving over 40% of the differences between larvae-pupae, but with some ASVs of the genus greater in larvae and others greater in pupae.

3.2 | Correlation analyses

There were ten correlations between bacteria and fungi genera using CoNet and ten correlations using SparCC (Tables S8, S9). Across both analyses, three positive bacteria to fungi correlations were found between *Lactobacillus* and *Phaeoacremonium*, *Anaerotignum*, and *Alternaria*, as well as *Pantoea* and *Didymosphaeria* (Table 1). A consistent significant correlation was also found between two bacterial genera: *Sphingomonas* and *Pantoea* (Table 1). Between fungi, two positive correlations across both analyses were found between *Alternaria* and *Taphrina* as well as *Phaeoacremonium* and *Taphrina*, in addition to a negative correlation between *Mycosphaerella* and *Alternaria* (Table 1).

4 | DISCUSSION

Here, we present the first characterization of the bacterial and fungal microbiome across 103 samples of pollen and *Ceratina calcarata* individuals including egg, larva, pupa, and adult stages to examine how the microbiome is acquired and changes throughout bee development. In examining taxonomy, we found that bacterial and fungal composition vary across pollen provisions, juvenile bees, and mature adults. Through diversity analyses, we found significant differences in bacterial and fungal beta diversity between pollen and bees of all life stages. Bacterial alpha diversity was lowest in adults, whereas fungal alpha diversity did not vary over the course of development.

4.1 | Microbial composition of pollen

In this study, the bacterial microbiome in pollen provisions consisted of a few common classes: Gammaproteobacteria, Alphaproteobacteria, Bacilli, and Actinobacteria (Table S3). At

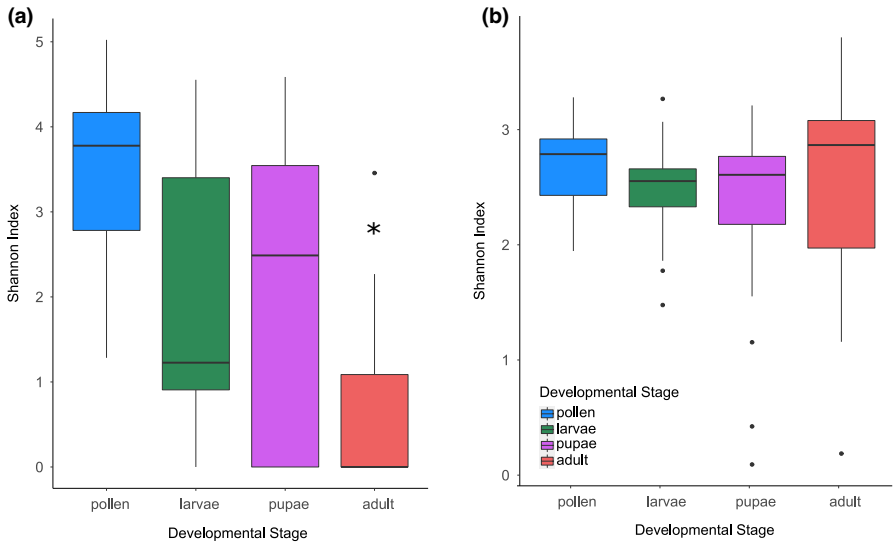

FIGURE 3 Shannon diversity indices using (a) bacterial ASVs and (b) fungal ASVs. Significant differences are noted with an asterisk ($p < 0.05$). Bacterial diversity is reduced in adults compared to juvenile stages, whereas fungal diversity does not vary significantly over the course of development

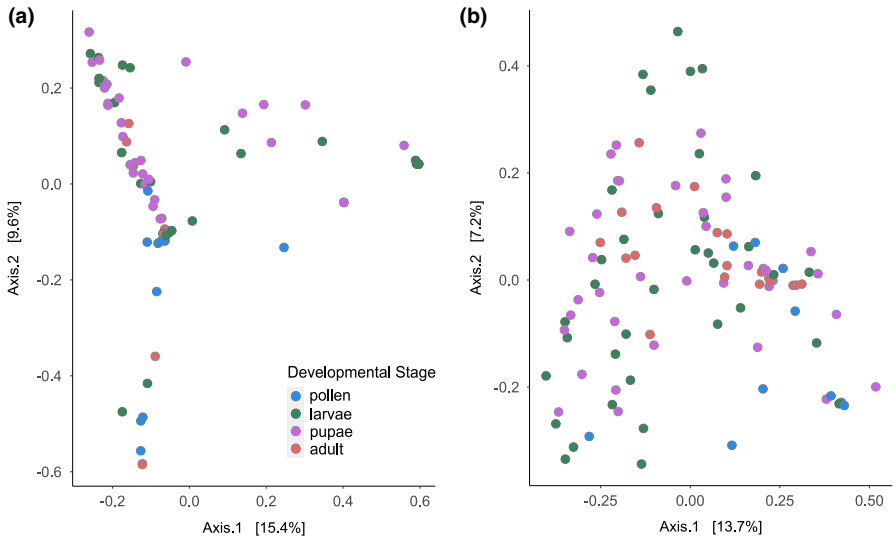

FIGURE 4 Principal coordinates analysis (PCoA) of Bray Curtis dissimilarity across developmental stages using (a) bacterial ASVs ($p < 0.001$) and (b) fungal ASVs ($p < 0.001$). Overall pollen samples were the most significantly different from juvenile and adult bee stages

TABLE 1 SparCC correlation scores after 100 bootstrap replicates comparing top eight bacterial and fungal genera. Scores are reported if significant ($p < 0.05$) and agree with CoNet results using Spearman, Bray, Pearson, and Kullback-Leibler for calculating edge scores ($p < 0.05$)

Relationship	Genera	Correlation	*p*-Value
Bacteria–Fungi	*Lactobacillus–Phaeoacremonium*	0.613	$p < 0.01$
	Alternaria–Anaerotignum	0.710	$p = 0.04$
	Pantoea–Didymosphaeria	0.506	$p < 0.01$
Bacteria–Bacteria	*Pantoea–Sphingomonas*	0.707	$p < 0.01$
Fungi–Fungi	*Alternaria–Taphrina*	0.669	$p = 0.04$
	Phaeoacremonium–Taphrina	0.533	$p = 0.04$
	Alternaria–Mycosphaerella	−0.269	$p = 0.02$

the genus level, pollen samples were dominated by *Acinetobacter* and *Lactobacillus* (now *Apilactobacillus*) (Figure 2a, Table S2). The presence of these bacteria is consistent with previous studies examining pollen microbiomes and indicates that pollen provisions may have a core bacterial composition, despite differences in geographic location (Dew et al., 2020; McFrederick & Rehan, 2016). For example, in a study with *C. calcarata* across eastern North America, despite diversity of floral resources, *Acinetobacter*, *Erwinia*, *Lactobacillus*, and *Sphingomonas* were consistently in the top ten bacterial genera found in pollen provisions (Dew et al., 2020). *Acinetobacter* has been shown to stimulate nutrient release from pollen through germination and bursting, potentially allowing consumers access to proteins (Christensen et al., 2021). The presence of *Lactobacillus* in pollen is particularly important because it is a bacteria that has been found in both core bee and pollen microbiomes in three *Ceratina* enterotypes (Graystock et al., 2017).

Not only is *Lactobacillus* commonly found in both bees and plants, this bacteria is also an important symbiont that helps regulate immune function in bees (Daisley et al., 2020; Parichehreh et al., 2018; Rothman et al., 2019). Thus, our results are consistent with other studies of pollen provisions for this species.

The conserved nature of bacteria found in pollen provisions may not be surprising because some pollen types act as reservoirs for certain bacteria, resulting in pollen and bacteria associations (McFrederick & Rehan, 2016). For example, *C. calcarata* mainly use the pithy stems and readily accessible pollen of raspberry (*Rubus)* and sumac (*Rhus)* for their nests and pollen provisions, respectively; further, *Rubus* plants have been shown to be correlated with *Acinetobacter* bacteria and this pollen and bacteria association explains the presence of this bacteria in provisions (Dew et al., 2020; McFrederick & Rehan, 2016). Nectar sources can also be an important determinant in the composition of bacteria in provisions and *Acinetobacter* is one species common in floral nectar (Alvarez-Perez, 2012). However, it has also been shown that plant–bacteria associations with the most common bacteria were often regional and differed between sites (Dew et al., 2020). Thus, differences in pollen bacterial composition are likely a result of access to a variety of floral resources that have varying plant–bacteria associations.

In addition to bacteria, fungi present in provisions were consistent with those in other bees. Pollen samples contained fungi that were almost exclusively from the Ascomycota phylum (Table S4). The most prominent species included *Alternaria angustiovoidea*, *Penicillium steckii*, and *Ascosphaera major* (Table S4). This result is similar to studies focusing on pollen from honey bees, where *Alternaria* spp and *Penicillium* spp were found in the vast majority of samples (De Jesus Inacio et al., 2021; González et al., 2005). Additionally, in this study, the *Zygosaccaromyces* genus had very high relative abundance in a pollen sample and was found in individuals across all developmental stages (Figure 2b, Table S5). This genus has been shown to be crucial in the development of the stingless bee *Scaptotrigona depilis*, which relies on the dietary steroids obtained from this brood cell fungus in order to pupate (Paludo et al., 2018). Furthermore, taxa of the family Didymellaceae had low relative abundance, but could be found across 90% of pollen samples in our study (Table S4). This family includes many species of plant pathogens known to be abundant in fruit pollen and is also known in *C. australensis* pollen provisions (Do et al., 2021; McFrederick & Rehan, 2019). Thus, the fungal taxa identified in pollen samples are concordant with those found in other bee species.

The pollen samples collected in this study showed slightly greater levels of alpha diversity in bacterial composition when compared to bee samples (Figure S1a). This differs when examining fungal composition, where pollen and bee samples had similar levels of diversity (Figure 2b). High levels of diversity were expected in pollen provisions, as even though there are usually a few core phyla present, there is generally great diversity at a species level in plant microhabitats (Ambika Manirajan et al., 2016). Studies of bumble bee pollen baskets have also shown that plant and bacterial alpha diversity are correlated (Sookhan et al., 2021). This correlation in diversities does not extend to bee microbial diversity, however; rather, it may be mainly the core bacteria from provisions that are acquired and maintained within bee hosts (McFrederick & Rehan, 2016). This is likely important in generalist bees, such as *C. calcarata*, as provisions often have a core bacterial community regardless of how many flowers a mother visited (McFrederick & Rehan, 2016). Pollen samples in this study had higher levels of bacterial diversity than bees, indicating that foraging mothers were making provisions with floral resources that had diverse bacterial compositions (Graystock et al., 2017). While much research is needed to understand the sources, succession, and functional role of bee fungal symbionts, the observed diversity in pollen was found to be comparable to and persistent in the microbiome of immature developmental stages and adult bees.

4.2 | Microbiome throughout development

Small and medium larvae were dominated by *Melissococcus* and *Anaerotignum*, which were present in the pollen samples at relatively high abundance but were not taxa seen in other larval bees (Figure 2a, Table S2). In the early stages of bee development, the composition of the gut microbiome is expected to be driven by the bacterial species present in pollen provisions (Dew et al., 2020; McFrederick & Rehan, 2019). This may explain why these bacterial genera were not abundant in a similar study in alkali bees (*Nomia melanderi*), where small larvae mainly hosted Lactobacillaceae, Moraxellaceae, Pseudomonadaceae, and some Enterobacteriaceae (Kapheim et al., 2021). These bacteria also differ from larval honey bee queens, as *Apis mellifera* were seen to have microbiomes dominated by *Escherichia*, *Gilliamella*, and *Bifidobacterium* (Tarpy et al., 2015). Former studies on honey bee larvae also surface sterilized samples removing any bacteria that may have been obtained from pollen residues in the nest (Vojvodic et al., 2013). Thus, the bacterial ASVs found within the *C. calcarata* larvae largely differ from other larval bees but have comparable composition to their own pollen provisions.

Many fungi found in bee larvae could also be found in pollen provisions, including *Alternaria*, *Penicillium*, *Zygosaccharomyces*, and *Ascosphaera* (Figure 2b, Table S4). Interestingly, *Ascosphaera xerophila* was a dominant fungal taxon found in two small larvae samples but not in pollen or more developed bee samples, putting into question the source of this fungi (Table S5). It is possible that *A. xerophila* may have been introduced through the nest substrate rather than the pollen provisions. The *Ascosphaera* genus contains both pathogenic and apathogenic fungi, and little is currently known about the *A. xerophila* species in particular (Klinger et al., 2013). Nonetheless, some species of *Ascosphaera* are known to cause chalkbrood disease in honey bees and the leaf-cutting bee *Megachile rotundata* (Aronstein & Murray, 2010; James, 2005). This potential fungal pathogen is of interest because its presence may have a negative effect on larval survival.

The stability of the microbiome diversity continued throughout development into early pupal stages, as alpha diversity of bacteria did not significantly increase or decrease as the prepupae developed

into white and pink eyed pupae (Figure S1a). After larval stages, *C. calcarata* defecates, metamorphosizes, and becomes a smaller sized prepupae (Rehan & Richards, 2010). We predicted that following defecation, bacterial composition and diversity would decrease, as is commonly seen in insects (Engel & Moran, 2013; Moll et al., 2001). However, the results of this study coincide with a study in *N. melanderi* where small larvae and prepupae had no significant difference in alpha diversity (Kapheim et al., 2021).

While the overall diversity may not have changed, the microbial composition was affected following metamorphosis. For example, *Ascosphaera duoformis* is a fungal species that was found exclusively in larvae and prepupae, indicating that there may have been a loss of some fungi in later pupal stages (Table S4). *A. duoformis* forms ascospores and is closely related to *A. subglobosa*, which has been found in pollen provisions and leaf lining in nests of leafcutter bees (Wynns et al., 2012). This suggests that this species may be prevalent when larval bees rely on pollen provisions in their brood cell but does not remain a part of the core microbiome as brood mature. Similarly, metamorphosis resulted in the decrease of Firmicutes members and an increase in Gammaproteobacteria when examined in honey bees (Hroncova et al., 2019). Although defecation does not seem to drastically change the core bacterial microbiome in alkali bees (Kapheim et al., 2021) and small carpenter bees (this study), these physiological changes may nevertheless impact the composition of the overall microbiome.

Considering mature bees, overall diversity was much lower than expected and a counterintuitive result given that these individuals can leave the nest to forage and interact with new environments. In a study with adult bumble bees, *Bombus lantschouensis*, changes in microbiome abundance and composition were independent of the act of mating, but were rather related to the age of the queen after eclosion (Wang et al., 2019). Former studies of *Ceratina* adults from rural New Hampshire revealed they were dominated by 13 core bacterial phylotypes (Graystock et al., 2017). While some of these taxa were present at some point during *C. calcarata's* development, such as *Acinetobacter* and *Sphingomonas*, those core phylotypes were largely absent in the adults in this urban Toronto based study (Table S2). This low diversity and difference in the microbiome may be indicative of regional and environmental stressors where adults may be depleted of healthy microbiomes in urban environments.

4.3 | Role of the microbiome

There is interest in the relationship between symbionts because some fungal pathogens can be inhibited by bacteria common to the honey bee microbiome (Khan et al., 2020; Parichehreh et al., 2018). This study revealed three positive correlations between bacterial and fungal genera: *Lactobacillus* & *Phaeoacrmonium*, *Alternaria* & *Anaerotigmum*, and *Pantoea* & *Didymosphaeria* (Table 1). It should be noted that these correlations should be treated with caution, as changes in abundance of any one taxon could significantly affect the relative abundance of other taxa. The fungi involved in bacteria–fungi and fungi–fungi correlations in this study, *Phaeoacremonium* and *Mycosphaerella*, are thought to be plant pathogens (Goodwin & Zismann, 2001; Mostert et al., 2005). The bacteria in these correlations often inhibit fungal pathogens. For example, *Lactobacillus (*now *Apilactobacillus)* is a common bacterial symbiont in bees inhibiting fungal pathogens, such as *Ascosphaera apis*, in honey bees (Daisley et al., 2020; Parichehreh et al., 2018). *Pantoea* is a bacteria that inhibits fire blight in plants and can commonly be found in the flowers visited by honey bees (Loncaric et al., 2009; Wright et al., 2001). While there were no negative correlations between bacteria and fungi, the positive correlation between *Lactobacillus* and *Phaeoacremonium* may indicate a relationship between a bee's beneficial bacterial symbiont and a plant pathogen. The negative correlation between *Mycosphaerella* and *Alternaria* may be a result of competition between the two fungi.

Many taxa present in pollen and bee microbiomes require further study to determine their effects on bee health. For example, *Melissococcus* is a pathogen for honey bees causing European foulbrood (Bailey & Collins, 1982) and was found in some *C. calcarata* larvae, prepupae, and pupae with no known effects on the small carpenter bee brood (Table S2). Similarly, many *Ascosphaera* species are pathogenic in causing chalkbrood disease in some bee species (Klinger et al., 2013), but no functional work has been conducted in the vast majority of wild bee species.

There was a notable absence of the *Lactobacillus (*now *Apilactobacillus)* genus in bees of this study (Figure 2a, Table S2). *Lactobacillus* is a common symbiont with wild bees (Graystock et al., 2017; McFrederick et al., 2017). Thus, it was hypothesized that *C. calcarata* individuals would have an abundance in *Lactobacillus*. The *Lactobacillus* genera is present and abundant in megachilid pollen provisions, larval and adult bee guts, creating an association between the flowers and bacteria that indicate the flowers may be acting as a transmission hub (McFrederick et al., 2017). In honey bees, *Lactobacillus* protects bees against bacterial brood diseases in a form of pathogen defense mechanism (Parichehreh et al., 2018). In past studies using *Ceratina* enterotypes, *Lactobacillus* was a bacteria consistently noted in pollen provisions and adult bee microbiomes (Graystock et al., 2017). Additional studies would be required to determine whether this species of bee does not consistently associate with this bacterial genus in this region or whether its relative abundance may vary due to some specific environmental stressors.

Relatively low levels of microbial diversity in the pupal phase and into adulthood are of potential concern because a rich microbiome can have positive implications on bee health (Dharampal et al., 2019; Mockler et al., 2018; Parichehreh et al., 2018; Rothman et al., 2019). In pollen provisions and earlier stages of development, *C. calcarata* likely benefits from having a diverse microbiome. *Anopheles stephensi* that were treated with antibiotics throughout development suffered from delayed development and asynchrony (Chouaia et al., 2012). Sterilizing pollen provisions in larval mason bees resulted in stunted growth rates and survivorship (Dharampal et al., 2019).

Therefore, the dependence of young developing larvae on having a diverse array of bacterial symbionts is common among different insects and seemingly important for survivorship and health.

Future studies should examine environmental variables, similar to what has been done in honey bees in comparing the microbiome of bees in agricultural versus pristine environments (Muñoz-Colmenero et al., 2020). While this study focused mainly on characterizing the developmental microbiome in small carpenter bees, there are many unstudied environmental contexts and wild bee species important to understanding the role the microbiome plays in determining bee health.

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CONFLICTS OF INTEREST

None.

AUTHOR CONTRIBUTIONS

PNN processed samples, conducted DNA extractions and data analyses, and wrote the manuscript; SMR conceived the study, provided funding, analyses, and interpretation of data, and edited the manuscript.

DATA AVAILABILITY STATEMENT

Sequence data are publicly available in NCBI SRA- BioProject: PRJNA805022; BioSamples: SAMN25891990-SAMN25892379.

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