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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

Microbiome and floral associations of a wild bee using biodiversity survey collections

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Abstract

The health of bees can be assessed through their microbiome, which serves as a biomarker indicating the presence of both beneficial and harmful microorganisms within a bee community. This study presents the characterisation of the bacterial, fungal, and plant composition on the cuticle of adult bicoloured sweat bees (*Agapostemon virescens*). These bees were collected using various methods such as pan traps, blue vane traps and sweep netting across the northern extent of their habitat range. Non-destructive methods were employed to extract DNA from the whole pinned specimens of these wild bees. Metabarcoding of the 16S rRNA, ITS and rbcL regions was then performed. The study found that the method of collection influenced the detection of certain microbial and plant taxa. Among the collection methods, sweep net samples showed the lowest fungal alpha diversity. However, minor differences in bacterial or fungal beta diversity suggest that no single method is significantly superior to others. Therefore, a combination of techniques can cater to a broader spectrum of microbial detection. The study also revealed regional variations in bacterial, fungal and plant diversity. The core microbiome of *A. virescens* comprises two bacteria, three fungi and a plant association, all of which are commonly detected in other wild bees. These core microbes remained consistent across different collection methods and locations. Further extensive studies of wild bee microbiomes across various species and landscapes will help uncover crucial relationships between pollinator health and their environment.

INTRODUCTION

The global decline of wild bees is alarming, given their crucial role in providing pollination services (Goulson et al., 2015; Kleijn et al., 2015; Mathiasson & Rehan, 2020; Winfree, 2010). Wild bees are not often given the same amount of recognition as the European honey bee, *Apis mellifera*, despite being economically and ecologically important pollinators in natural, agricultural and urban contexts (Garantonakis et al., 2016; Kleijn et al., 2015; Kremen et al., 2002; Mallinger & Gratton, 2015; Winfree, 2010). As bee populations are forced to change in response to increased anthropogenic activity such as industrial agriculture and urbanization (Brain & Anderson, 2020; Marshman et al., 2019; Theodorou, Herbst, et al., 2020; Theodorou, Radzevičiūtė, et al., 2020; Winfree et al., 2009), there is increasing interest in understanding the different contributors to protecting and maintaining bee health. For example, the intersection between genetic, physiological, and microbial biomarkers can indicate bee health status across species and populations (Lopez-Uribe et al., 2020).

The microbiome, consisting of microorganisms such as bacteria and fungi that reside within or on a host

(Belizário & Faintuch, 2018; Turnbaugh & Gordon, 2009), offers a unique perspective for exploring bee health. Microbiota can sometimes be harmful to bees, as with certain pathogens like *Ascosphaera apis* that cause chalkbrood in honey bees and *Melissococcus plutonius* that lead to European foulbrood (Aronstein & Murray, 2010; Floyd et al., 2020; Foley et al., 2014; Fünfhaus et al., 2018; Ye et al., 2021). However, many microbes develop mutualisms which can support bees' immune system (Daisley, Chmiel, et al., 2020; Li et al., 2019; Rothman et al., 2019; Tauber et al., 2019), metabolism (Christensen et al., 2021; Dharampal et al., 2020; Li et al., 2019) and memory (Li et al., 2021; Zhang et al., 2022). Removing these symbionts can lead to dysbiosis, the lack of beneficial relationships between microbes and their host (Belizário & Faintuch, 2018; Ye et al., 2021). With their absence potentially resulting in declines in growth rate, survivorship, and fitness (Dharampal et al., 2019, 2020, 2022), fostering beneficial microbial communities is one method to support bee health.

The core microbiome represents the most common microbes found within a population at a certain abundance and frequency (Custer et al., 2023; Neu et al., 2021; Risely, 2020). Obligately eusocial bees, such as *Apis* or *Bombus*, typically rely on social interactions within colonies to obtain and maintain their microbiome (Kwong & Moran, 2016; Martinson et al., 2011, 2012; Powell et al., 2018). This allows them to foster very stable core microbiomes composed of eight bacterial phylotypes that contribute up to 95% of the overall gut microbial load (Engel et al., 2012; Kwong & Moran, 2016; Martinson et al., 2011). In comparison, solitary and facultatively social bees gain more of their microbiomes from their environment (Cohen et al., 2022; Dew et al., 2020; Graystock et al., 2017; Keller et al., 2021; McFrederick et al., 2012, 2017), bolstering more variable microbiota (Dew et al., 2020; McFrederick et al., 2017; Sookhan et al., 2021). Studies focusing on wild bee microbiomes are currently limited yet remain ongoing (Chau et al., 2023; Cohen et al., 2020; Handy et al., 2022; Kapheim et al., 2021; Nguyen & Rehan, 2023a; Shell & Rehan, 2022). Therefore, characterising the microbial ecology of additional bee species is necessary to compare differences in symbioses underpinning bee health.

Collection methods for bee biodiversity surveys can include pan traps, blue vane traps and sweep nets, among other methods. While each presents its benefits, incorporating several techniques simultaneously can offer a more comprehensive collection effort that more accurately reflects the sampled region's bee diversity (McCravy, 2018; Prendergast et al., 2020; Roulston et al., 2007). Studies of the microbiome have predominantly utilised bees caught on the wing and immediately flash frozen (Liberti et al., 2022; Nguyen & Rehan, 2022a; Tarpy et al., 2015; Thamm et al., 2023), but this can be expensive in time and materials spent collecting and freezing whereas passive methods can capture a wider snapshot of the bee community with fewer hours of labour. Non-destructive DNA extraction has allowed for the use of pinned specimens to characterise microbiota, and microbial detection has been shown to withstand lengthy periods spent in storage (Chalifour et al., 2022; Madison et al., 2023; Martoni et al., 2021; Santos et al., 2018). While studies of the microbiome often focus on the gut, whole-body specimens and the cuticular microbiome have also been shown to harbour distinct microbes (Reiß et al., 2023; Thamm et al., 2023). In using these alternative methods, microbial characterisations are increasingly performable on a wider variety of collections, including pinned museum specimens (Chalifour et al., 2022; Hammer et al., 2015; Madison et al., 2023; Martoni et al., 2021).

The bicoloured sweat bee, *Agapostemon virescens* Fabricius (Hymenoptera: Halictidae), is a common ground-nesting bee widespread throughout North America (Eickwort, 1981; Roberts, 1973). Many females share a single nest entrance and live in communal tunnel systems below the ground (Abrams & Eickwort, 1981; Roberts, 1973). *A. virescens* are polylectic or are generalists, visiting and providing important pollination services to a variety of flowers (Gardiner et al., 2010; Sivakoff et al., 2018), but with prominent associations with asters such as *Silphium* cup plants and silflowers (Butters et al., 2022). In a comparison between honey bee and non-corbiculate microbiomes, Martinson et al. (2011) noted that an individual *A. virescens* lacked the bacterial phylotypes common to the honey bee, but was abundant instead in the bacteria *Burkholderia*. To our knowledge, no other studies have characterised the bacterial or fungal microbiome in *A. virescens*. Limited studies in Halictinae and Megachilinae offer preliminary characterisations of bacterial communities in native, non-eusocial bees (McFrederick et al., 2012, 2017; Voulgari-Kokota et al., 2019), but none have reported a core or fungal microbiome.

While this has not yet been studied using *A. virescens*, metabarcoding techniques have aided in determining dietary breadth and host plant associations in other bees. In honey bees, DNA barcoding has been used alongside melissopalynology to describe specific plants involved in important interactions despite honey bees being commonly classified as supergeneralists (Hawkins et al., 2015; Wizenberg et al., 2023). In generalist small carpenter bees, targeting the rbcL locus has provided insights into the plants commonly found in pollen provisions and on adult bees, offering the ability to find differences in plant associations across environments (Dew et al., 2020; McFrederick & Rehan, 2016, 2019; Nguyen & Rehan, 2023b). As the developing field of environmental DNA continues to explore the microbiome of an individual's surroundings (Bovo et al., 2018;

Johnson et al., 2021; Tremblay et al., 2019), examining the traces of plant, pollen and nectar that are in bee nests or directly on bees themselves is an easier method of characterising plant associations than traditional monitoring of plant-pollinator interactions.

Here, we characterise the microbiome and plant associations of the bicoloured sweat bee *A. virescens* across the northern extent of its range and across common wild bee biodiversity survey collection methods. The four aims of this study are to: first, assess the utility of using museum specimens for determining microbiome composition; second, compare the bacterial, fungal and plant associations of bees across collection methods; third, to contrast the microbiome of bees collected across sampling locations; and fourth, to determine the core microbiome and common floral associations for this wild pollinator. This research offers the first description of the *A. virescens* core microbiome and supports expanded methods for using pinned specimens from common biodiversity surveys.

MATERIALS AND METHODS

Sample collection and DNA extractions

In total, we processed 86 *A. virescens* samples across four provinces (Ontario, Manitoba, Nova Scotia and Quebec) and three collection methods (pan trap, sweep net and blue vane) (Figure 1; Table S1). All samples were pinned specimens collected for ongoing biodiversity surveys for use in various projects between 2017 and 2021, as described in Samad-zada and Rehan (2023). Ontario blue vane traps were unbaited, while Manitoba utilised dish detergent solution. All pan trap samples contained dish detergent solution for a deployment duration of 5.0–6.5 h. DNA was extracted via a non-destructive method using the Qiagen Blood & Tissue kit (catalogue #69506), as described in Martoni et al. (2021). Following overnight incubation at 56°C in 200 μL ATL butter and 20 μL proteinase K, DNA extraction was conducted according to manufacturer instructions using the soaking solution the whole bee body was in during incubation.

Sequencing

We employed amplicon sequencing techniques in triplicate before combining replicates to characterise bacterial, fungal and plant communities using 16S rRNA, ITS and rbcL, respectively. For 16S rRNA, we sequenced the V5-V6 region using the 799bF-CS1 forward primer (MGGATTAGATACCCKGG) and the 1115R-CS2 reverse primer (AGGGTTGCGCTCGTTG). To sequence ITS, we used the forward primer of ITS1F (CTTGGTCATTTAGAGGAAGTAA) and the reverse primer of ITS2 (GCTGCGTTCTTCATCGATGC). For plant metabarcoding, we used the RBCL7 forward primer (CTCCTGAMTAYGAAACCAAAGA) and the RBCL8 reverse primer (GTAGCAGCGCCCTTTGTAAC). All primers were described in McFrederick and Rehan (2016). PCR amplification, library preparation and sequencing were performed at the Centre for Biodiversity Genomics at the University of Guelph in Guelph, Canada. Adapter removal and demultiplexing were also performed at the sequencing facility.

Data analysis

We used QIIME2 (Bolyen et al., 2019) to process the resulting FASTQ files. After files were imported into QIIME2, quality control, including denoising and

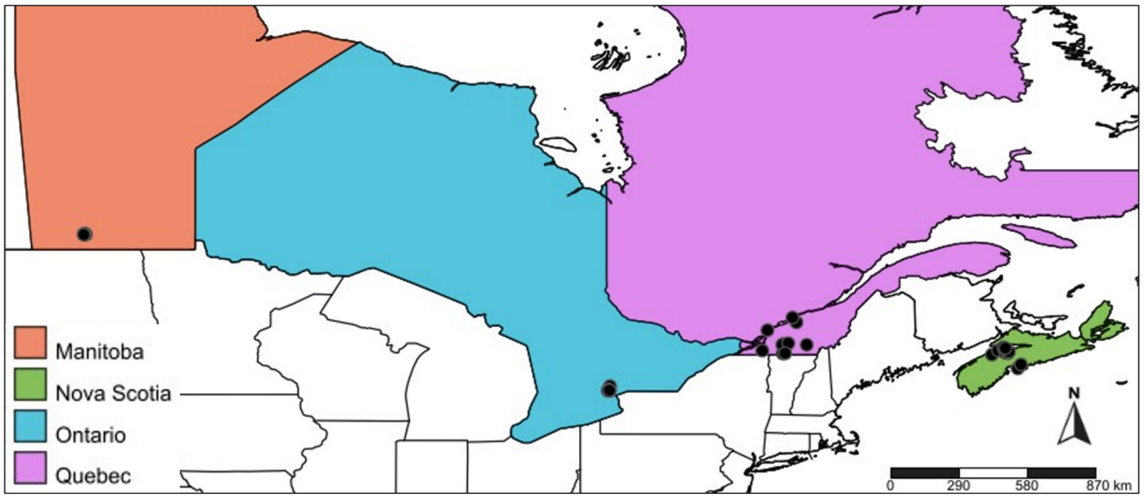

FIGURE 1 Map of *Agapostemon virescens* collection sites in Ontario (ON, $n = 28$), Quebec (QC, $n = 25$), Manitoba (MB, $n = 16$) and Nova Scotia (NS, $n = 16$). This map was created using SimpleMappr.

chimaera removal, was performed using DADA2 (Callahan et al., 2016). For 16S rRNA, we removed the first 18 base pairs (–p-trim-left 18) and trimmed the sequence length to 270 base pairs (–p-trunc-len 270) based on an approximate visual cutoff in quality. For rbcL, we removed the first 18 base pairs and trimmed the sequence length to 150 base pairs. Given the length variation of the ITS gene and the possibility of read-through, we processed ITS sequencing using *cutadapt* (Martin, 2011), first removing the forward and then the reverse primer. Once trimmed, sequences were processed through the DADA2 plugin in QIIME2, with the –p-trim-left parameter set to 22 and the –p-trunc-len parameter set to 220. The resulting tables of amplicon sequence variants (ASVs), along with their corresponding sequences, were output by QIIME2 and used for downstream analysis.

For each of the three amplicons, taxonomic classification was performed using two methods. First, we employed a Naïve Bayes Classifier approach as implemented in QIIME2, using the q2-feature-classifier and the classify-sklearn pipeline (Bokulich et al., 2018). For 16S and rbcL, input sequences were compared against extracted reference sequences that matched the target regions to increase classification accuracy, but for ITS, a comparison was performed against the full length of the gene. The following databases were used for classification: SILVA 138 at 99% sequence similarity for 16S (Quast et al., 2013; Yilmaz et al., 2014), UNITE at 99% sequence similarity for ITS (Abarenkov et al., 2021) and a non-dereplicated database for rbcL (Dubois et al., 2022). Second, we used BLAST+ (2.13.0, April 2023) to compare ASV sequences against the *nt* database and extracted taxonomic identification for each hit (Johnson et al., 2008). In cases where there was a discrepancy in taxonomic classification among the produced hits, we used the lowest common ancestor approach to assign the taxonomic rank. Finally, we compared the results of the Naïve Bayes classification and BLAST analysis, and for each ASV kept the more specific classification. For rbcL sequences, we cross-examined the resulting hits against the USDA plant database to ensure that no sequences were assigned to plants that do not occur in North America (USDA, 2023). The resulting taxonomy files were used for downstream analyses.

We then exported the feature and taxonomy tables for each amplicon to R using the qiime2 R package (Bisanz, 2018) to further filter the dataset. We identified and removed contaminant ASVs using the prevalence method as implemented in the R package *decontam* (Davis et al., 2018), after which the blank samples were removed from the dataset. For the 16S rRNA dataset specifically, we removed contaminant ASVs that were classified as *Wolbachia*, *Sodalis*, mitochondria and chloroplast (Graystock et al., 2017). *Wolbachia* and *Sodalis* are common intracellular endosymbionts that can skew microbial characterisations (Dew et al., 2020; Graystock et al., 2017). We removed any taxa not identified at the genus level. Next, for ASVs that, in each sample, had a relative abundance below 0.01%, we converted the absolute values to 0 and removed ASVs that had 0 reads across the entire dataset. Finally, we removed samples that had fewer than 500 reads from the feature table and exported this dataset to use in all downstream analyses.

Comparisons

For all the analyses, we divided the datasets into two subsets that separately addressed the effects of collection methods and geographic location. First, we compared only samples from Ontario across the three collection methods (pan trap, blue vane, sweep net; $n = 29$) and second, we compared only the pan trap samples across the four provinces (Manitoba, Ontario, Quebec, Nova Scotia; $n = 64$). We identified the top 10 genera of each subset using relative abundance. We also identified core taxa using thresholds in Graystock et al. (2017), where taxa with a mean relative abundance above 1% and prevalence in at least 50% of the examined samples were considered core.

Similarity percentage (SIMPER) calculations were performed using the PAST software (Hammer et al., 2001). After rarefying ASVs to 1000, alpha and beta diversity were measured with Shannon indices and Bray–Curtis dissimilarities, respectively, using the R package *phyloseq* package to generate the box plots and PCoAs (McMurdie & Holmes, 2013). ANOVAs and pairwise Wilcoxon rank-sum exact tests were used to confirm differences between alpha diversity across provinces and across collection methods. The *adonis* and *betadisper* functions validated differences in beta diversity in the R package *vegan* (Oksanen et al., 2020), which also was used to correct for multiple pairwise comparisons using Benjamini–Hochberg.

RESULTS

Across all 86 bee whole-body extractions, the average read count of ASVs after filtering was 18,138 for bacteria, 16,112 for fungi, and 21,734 for plant taxa (Tables S2–S4). Across all ASVs, the average read count was 18,475 for pan trap samples, 18,719 for blue vane samples, and 19,794 for sweep net samples, indicating no differences in ASV read counts in samples obtained using different collection methods (ANOVA; $F = 0.10$, $df = 2$, $p = 0.91$; Tables S2–S4). The top three bacterial genera found in the *A. virescens* microbiome from all collection methods and provinces were *Apilactobacillus*, *Acinetobacter* and *Arsenophonus* (Table S2). The most relatively abundant fungal taxa

were *Cladosporium*, *Alternaria* and *Renatobasidium* (Table S3), while the most common plants were *Trifolium*, *Hypericum* and *Rosa* (Table S4).

Microbial and plant composition across collection methods

Examining *A. virescens* collected in Ontario across different collection methods, sweep net individuals had lower fungal alpha diversity than blue vane samples (Tukey test; blue vane-sweep net, adj $p = 0.035$; blue vane-pan trap, adj $p = 0.93$; pan trap-sweep net, adj $p = 0.143$; Figure 2B). While bacterial alpha diversity did not differ across collection methods (Tukey test, all pairwise comparisons, all adj $p = 0.76$, Figure 2A), differences in bacterial beta diversity were significant (ANOVA; $R^2 = 0.10$, df = 2, $p = 0.018$; Figure S1A). On the contrary, fungal beta diversity did not differ among collection methods ($R^2 = 0.11$, df = 2, $p = 0.075$; Figure S1B), suggesting minimal variation in microbial diversity was associated with the collection method.

Ontario samples were most relatively abundant in *Enterobacter*, *Acinetobacter* and *Escherichia* bacteria and the *Alternaria*, *Cladosporium* and *Candida* fungi (Figure 3A,B), deviating slightly from the most relatively abundant microbes found across all provinces (Tables S2 and S3). Within the province, the bacterial genera contributing most to dissimilarities between collection methods were *Apilactobacillus*, *Acinetobacter* and *Chryseobacterium* (Table S5). Notably, *Apilactobacillus* was completely absent in individuals collected from blue vane traps, and *Acinetobacter* was overrepresented in sweep net collected samples (Figure 3A; Table S5). Fungi detected in Ontario samples differed mostly in *Alternaria*, *Cladosporium* and *Fusarium* genera, with the first two underrepresented in blue vane samples and *Fusarium* found in lowest abundance in pan trap samples (Figure 3B; Table S5).

Plant alpha diversity in Ontario did not differ across collection methods (Wilcoxon rank-sum test; pan trap-blue vane, adj $p = 0.53$; pan trap-sweep net, adj $p = 0.53$; blue vane-sweep net, adj $p = 0.97$; Figure 2C). Beta diversity was significantly different across methods (ANOVA; $R^2 = 0.13$, df = 2, $p = 0.001$; Figure S1C), but pairwise tests were not significant after corrections for multiple comparisons (Tukey test; pan trap-blue vane, adj $p = 0.73$; pan trap-sweep net, adj $p = 0.99$; blue vane-sweep net, adj $p = 0.76$). Plants most abundantly found in Ontario were *Ailanthus*, *Rosa* and *Trifolium* (Figure 3C). There was an absence of the *Ailanthus* genus in sweep nets that contributed to 10% of all dissimilarities in plant composition using SIMPER (Table S5). Read counts for the plants of the *Rosa* genus were overrepresented in blue vane collected samples, contributing to 8% of the total differences in plant taxa across collection methods (Table S5). *Hypericum* was mostly absent in blue vane traps and contributed to 8% of all dissimilarities in plant composition (Figure 3C; Table S5).

Microbial and plant composition across provinces

From pan trap collected bees, microbial diversity differed among provinces. Ontario samples had a greater bacterial alpha diversity than bees from other provinces (Figure 4A). Manitoba bees had lower fungal alpha diversity than those from Quebec and Nova Scotia (Figure 3B). These differences in microbial diversity were supported by variations in Bray–Curtis dissimilarities (ANOVA; bacteria, $R^2 = 0.15$, df = 3, $p = 0.004$; fungi, $R^2 = 0.21$, df = 3, $p = 0.001$; Figure S2A,B). Pairwise comparisons indicated that Ontario bacterial beta diversity differed from Manitoba, Nova Scotia and Quebec samples (Tukey test; all pairwise comparisons, adj $p = 0.004$; Figure S2A), but all other combinations were insignificant ($p = 0.447$; Figure S2A). As for fungal beta diversity, all pairwise comparisons were significantly different except for Nova Scotia and Quebec (Tukey test; NS-QC, adj $p = 0.22$; NS-ON, adj $p = 0.0084$; NS-MB, ON-MB, ON-QC, QC-MB, adj $p = 0.0015$; Figure S2B).

The microbial composition of *A. virescens* collected from pan traps in Ontario harbours a more diverse array of bacterial communities that are overrepresented by *Acinetobacter* and *Enterobacter* (Table S5), whereas all other provinces were most abundant in *Apilactobacillus* (Figure 5A). *Apilactobacillus* contributed upwards of 40% of the differences in bacteria by province, being underrepresented in Ontario and Nova Scotia, but overrepresented in Manitoba and Quebec (Table S5). *Acinetobacter* was present in very low counts in Manitoba and Nova Scotia samples, but overrepresented in Quebec and Ontario samples (Table S5). The three most dissimilar fungal genera across provinces were *Cladosporium*, *Alternaria* and *Renatobasidium* (Figure 5B), with *Cladosporium* overrepresented in Manitoba and Nova Scotia, *Renatobasidium* overrepresented in Manitoba and *Alternaria* overrepresented in Ontario (Table S5). Quebec samples were overrepresented in the *Cladonia* genus (Table S5).

Despite having a lower fungal diversity than other provinces, Manitoba has a significantly higher plant alpha diversity than Nova Scotia and Quebec (Wilcoxon rank-sum exact test; MN-NS, adj $p = 0.0001$; MN-QC, adj $p = 0.0001$; all other combinations, adj $p > 0.05$; Figure 4C). Across the regions, plant beta diversity differed (ANOVA; $R^2 = 0.012$, df = 3, $p = 0.001$), where Manitoba was different from Nova Scotia and Quebec (Tukey test; MB-NS, adj $p = 0.012$; MN-QC, adj $p = 0.004$; all other combinations, adj $p > 0.05$). Plant genera were usually associated with certain provinces (Figure 5C). For example, *Hypericum* was absent in Manitoba, *Rubus* was not

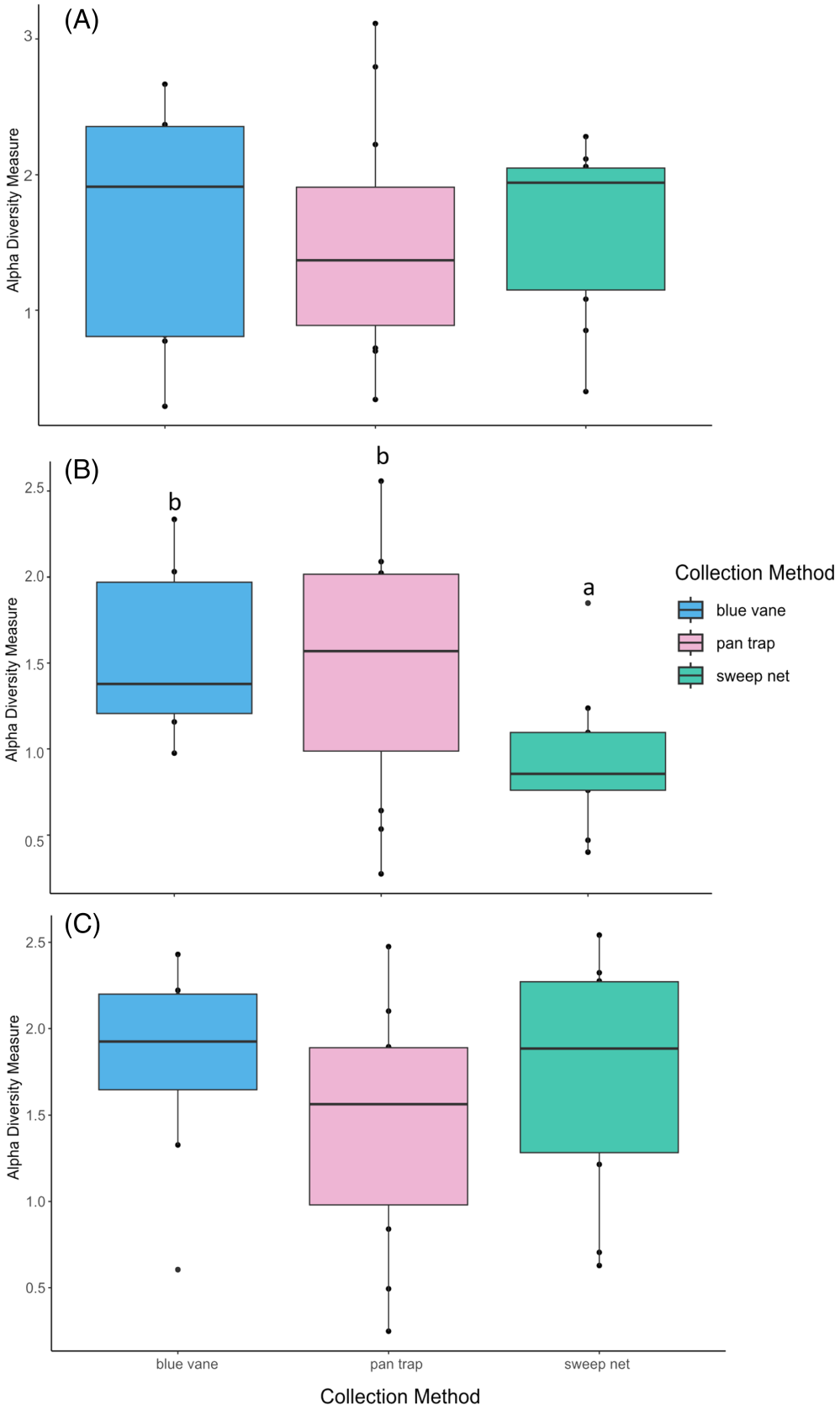

FIGURE 2 Alpha diversity of (A) bacteria, (B) fungi and (C) plants in Ontario using Shannon diversity across three collection methods. Sweep net-caught bees had a lower fungal diversity than blue vane and pan-trap-caught bees.

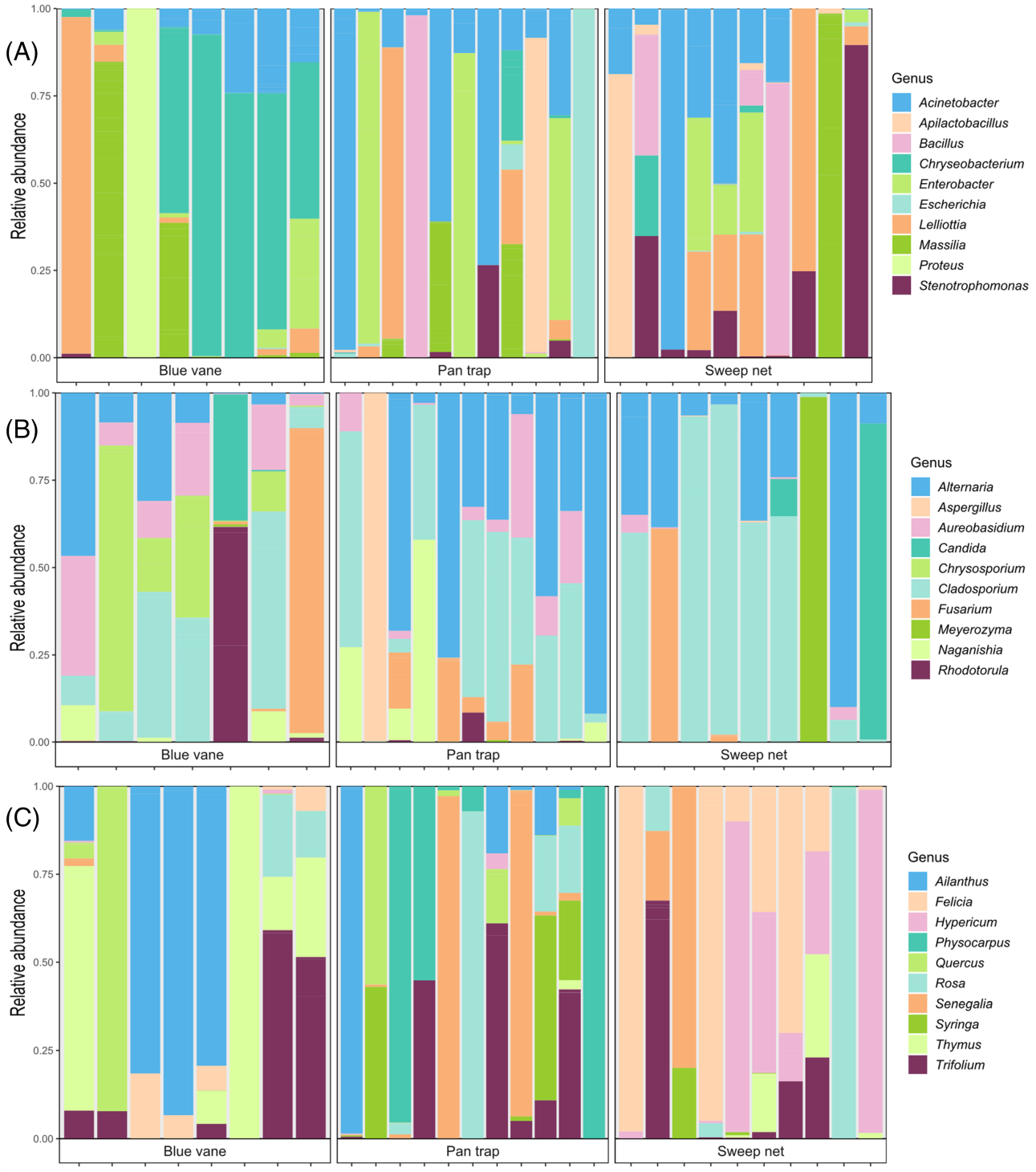

FIGURE 3 Taxonomic bar plot of the top 10 (A) bacterial, (B) fungal and (C) plant genera in Ontario across individuals collected using the different collection methods of blue vane traps, pan traps and sweep net samples.

found in Ontario and was underrepresented in Quebec, and *Ailanthus* was exclusive to Ontario individuals (Figure 5C). Contributing to 43% of the dissimilarities in plant composition were the genera *Trifolium*, *Rosa*, *Rubus* and *Hypericum* (Table S5). Quebec and Nova Scotia were overrepresented in *Trifolium*, while *Rosa* was overrepresented in Ontario (Figure 4C; Table S5).

The core microbiome

Across all samples, we detected two core bacteria, three core fungi and one plant found at a prevalence of at least 50% of samples and frequency of 1% of read counts (Table 1). Examining only samples collected in pan traps also resulted in the same core microbiome

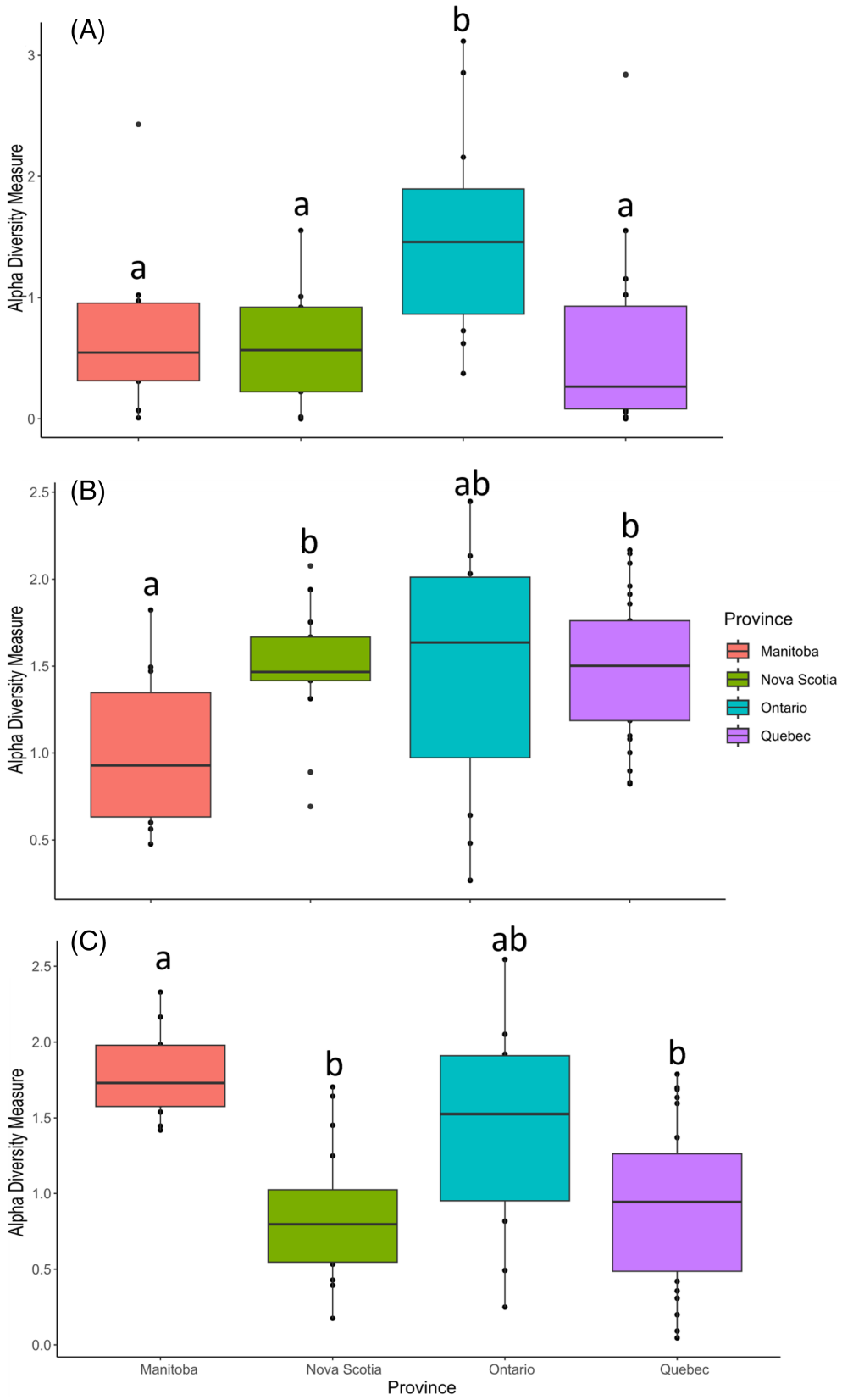

FIGURE 4 Alpha diversity of (A) bacteria, (B) fungi and (C) plants across four provinces using Shannon indices for bees collected from pan traps. Ontario samples harbour the greatest bacterial diversity, while Manitoba samples have lower fungal and highest plant diversity than Quebec and Nova Scotia.

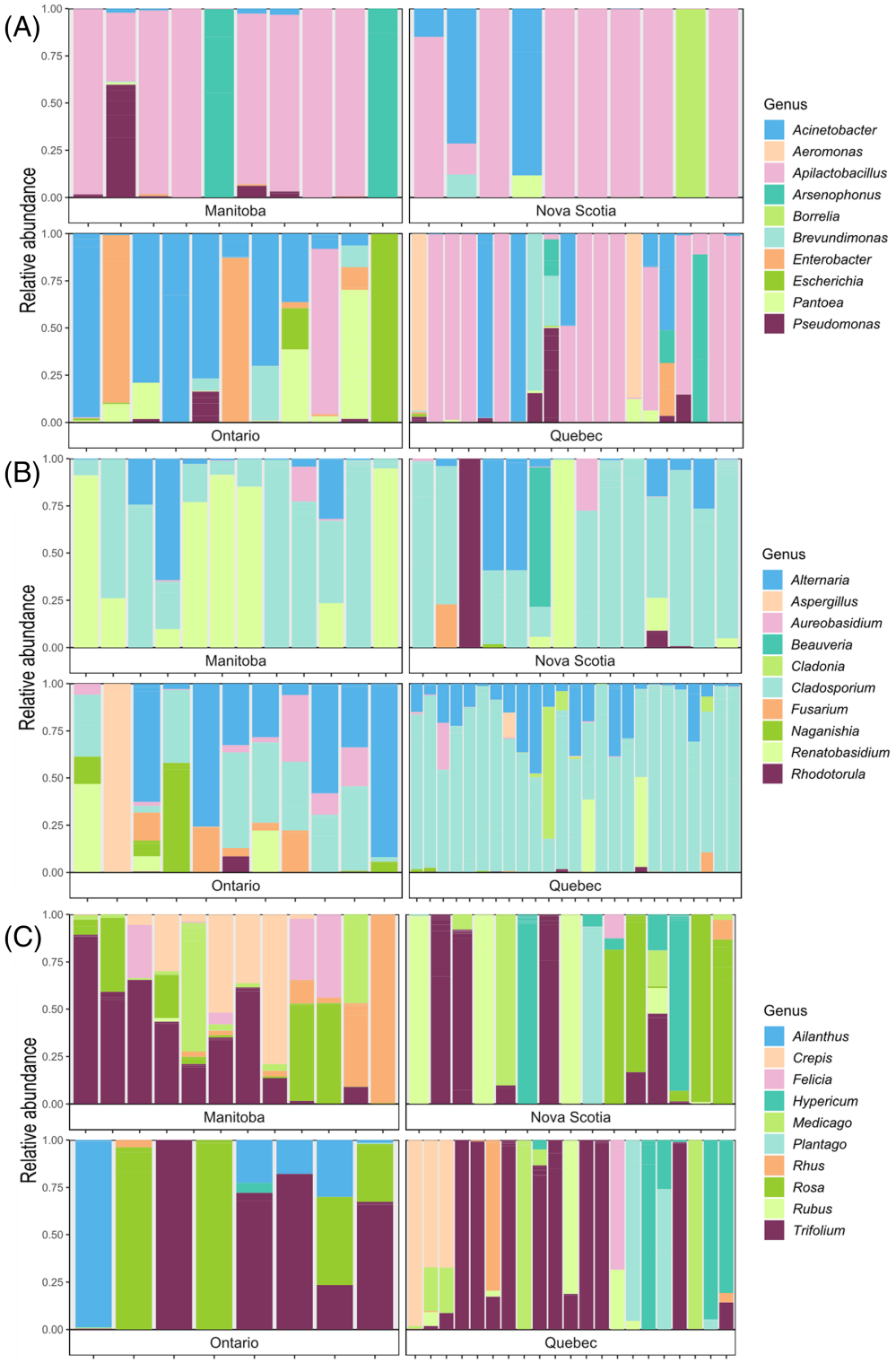

FIGURE 5 Taxonomic bar plot of the top 10 (A) bacterial, (B) fungal and (C) plant genera in *Agapostemon virescens* individuals collected in pan traps from four provinces.

(Table S6). Characterising the core microbiome of *A. virescens* in Ontario led to four additional bacteria genera meeting the threshold, although *Apilactobacillus* did not make the cutoff (Table S6). This indicates that while *Acinetobacter* is ubiquitous across all individuals of *A. virescens* across the four provinces and different collection methods, the expected core bacteria *Apilactobacillus* is less relatively abundant in this species in Ontario. Martinson et al. (2011) described a high relative abundance of *Burkholderia* bacteria in their

TABLE 1 The core microbiome of *Agapostemon virescens* found at a frequency of at least 1% and detected in at least 50% of the samples, across all provinces and collection methods.

	Phylum	Family	Genus
Bacteria	Firmicutes	Lactobacillaceae	*Apilactobacillus*
	Proteobacteria	Moraxellaceae	*Acinetobacter*
Fungi	Ascomycota	Cladosporiaceae	*Cladosporium*
	Ascomycota	Pleosporaceae	*Alternaria*
	Ascomycota	Saccothecaceae	*Aureobasidium*
Plant	Streptophyta	Fabaceae	*Trifolium*

A. virescens, which were not found in this study. At the class level, the former study found a low relative abundance of Actinobacteria, Bacilli, Alphaproteobacteria and Gammaproteobacteria, all of which comprise the vast majority of bacteria found in this study.

DISCUSSION

This study provides the first detailed characterisation of the *A. virescens* microbiome, comparing the bacterial, fungal and plant composition detected on bees obtained using three collection methods and across the northern extent of the species' range. By using non-destructive DNA extraction methods on pinned bee specimens collected during surveys, we were able to examine the microbiome of an understudied, native solitary bee. We found that collection methods can affect the detection of certain microbes and plants, perhaps depending on handling times, preservation methods or other external factors. Across the four provinces examined, Ontario bees supported the greatest bacterial diversity, while Manitoba had lower fungal and higher plant diversity than Quebec and Nova Scotia. Regardless of collection method or location, the core microbiome of the bicoloured sweat bee consists of two bacteria and three fungi ASVs, in addition to core associations with one plant that has been described in other wild bees. The high relative abundances of these known bee-associated microorganisms suggest that these methods can effectively characterise the microbiome of pinned specimens.

Collection method effects

We detected differences in fungal alpha diversity (Figure 2B) and bacterial beta diversity (Figure S1A) associated with collection method, particularly noting that sweep net samples harboured the lowest fungal diversity (Figure 2B). This may be because sweep nets involve the least amount of active collection time (Prendergast et al., 2020), leaving less opportunity for environmental fungi to be introduced passively as they could in pan traps or blue vane traps, which in Ontario were left for 6.5 h and 14 days, respectively (Table S1). In particular, the fungus *Alternaria alternata* can be detected through passive air samplers for allergens, relying on gravitational settling over time (Yamamoto et al., 2011). Fungal genera driving microbial differences included many plant pathogens, including *Alternaria*, *Cladosporium* and *Fusarium* (Figure 3B; Table S5), and suggest that passive, open-air collection methods like pan traps may detect more of these environmental pathogens. Similarly, studies on herbarium collections have identified *A. alternata* to be a probable contaminant colonised on plants during mounting and storage (Bieker et al., 2020). There were initial concerns about the underrepresentation of *Alternaria* and *Cladosporium* in blue vane traps reflecting moulds not thriving during extended periods in enclosed environments containing detergents, unlike trap nests, where moulds can be of particular concern (McCravy, 2018). On the contrary, Ontario blue vane traps lacked detergents, suggesting that their physical structure may inhibit undesirable fungal growth for surveyors and reduce the relative abundances of certain fungal characterisations in bee microbiomes.

In terms of bacteria, *Apilactobacillus* was present in Ontario individuals collected using sweep netting and pan traps, contrasted with its absence in unbaited blue vane traps (Figure 3A; Table S5). This raises concerns about the detection of important facultatively anaerobic bee symbionts after exposure to enclosed blue vane traps. This bacteria's growth can be resilient but involves the formation of slimy biofilms that grow to reach a detachment phase from its initial attachment place after about 16 h (Martinez et al., 2020; Pachla et al., 2021; Simsek et al., 2022). Thus, extended periods within blue vane traps and the subsequent process of cleaning specimens for pinning may inhibit the detection of this bacteria when the collector easily notices and removes slimy growth. Across all samples, we did not expect any potential impact of ethanol storage or washing on microbiome composition, as Hammer et al. (2015) discovered that there were limited effects of surface sterilisation on microbiome characterisations and storage in 95% ethanol when compared to other storage methods. However, *Apilactobacillus* are found in practically all studied bees (Kwong & Moran, 2016; Martinson et al., 2011; Nguyen & Rehan, 2023a; Shell & Rehan, 2022), playing important roles in building immunity (Daisley et al., 2017; Daisley, Pitek, et al., 2020) and aiding in host learning and memory behaviour (Zhang et al., 2022). Although potentially correlated to patterns of low relative abundances of *Apilactobacillus* in urban Ontario (Nguyen & Rehan, 2022b) and in agreement with Martinson et al. (2011), which found the absence of Firm-4 and Firm-5 bacterial phylotypes in *A. virescens*, considerations should be made regarding the effectiveness of certain

bacterial characterisation depending on collection method.

While overall plant diversity did not differ across collection methods, plants on bees in Ontario had the most variance in relative abundance among collection methods (Figure 2C; Table S5). There was an absence of the plant *Ailanthus* (tree of heaven) in sweep net samples and an overrepresentation of the genus *Rosa* (rose) in blue vane samples (Figure 3C; Table S5). The rapidly growing *Ailanthus* branches can grow as much as 1.8 m in height after one season (Feret, 1985), making them more easily avoided during sweep netting. Furthermore, the overrepresentation of *Rosa* rose bushes in blue vane traps, which are perched higher at about 1.5 m above the ground (Acharya et al., 2022), may suggest that the height of the collection method impacts plant characterisations. Actively flying bees may be more likely to be caught during foraging, resulting in an overrepresentation of the flowers they recently visited. In this instance, the plant composition detected would be associated with the bee's foraging preferences (Cook et al., 2003; Essenberg, 2012; Hagbery & Nieh, 2012), as opposed to bias towards a certain collection method. However, as *Ailanthus* and *Rosa* plants have been known to be visited by bees, particularly of the Apidae family (Aldrich et al., 2008; Jesse et al., 2006), their underrepresentation in certain collection methods raises concerns about their effectiveness in reducing sampling bias and collecting bees that have recently been foraging from a variety of plants.

The microbiome across a geographical range

In agreement with differences in beta diversity (Figure S2A,B), Ontario bees yielded the greatest bacterial alpha diversity (Figure 4A), while Manitoba bees had the lowest fungal diversity (Figure 4B). There were no differences in diversity in the eastern provinces of Nova Scotia and Quebec, despite these sampling locations being situated in saltmarsh and mixed urban and agricultural sites, respectively (Figure 4; Table S1). The diverse array of bacterial communities in Ontario included *Acinetobacter* and *Enterobacter*, but notably lacked *Apilactobacillus* (Figure 5A). This is consistent with patterns noted in the small carpenter bee *Ceratina calcarata*, where the normally ubiquitous *Apilactobacillus* was present in low relative abundances in Ontario (Nguyen & Rehan, 2022b), despite being previously characterised as a core bacterium and being found in almost all other bees (Graystock et al., 2017; Martinson et al., 2011; Nguyen & Rehan, 2022b, 2023a; Shell & Rehan, 2022). Martinson et al. (2011) similarly found the absence of the bacterial phylotypes associated with the genus *Lactobacillus*. The underrepresentation of *Apilactobacillus* may also be attributed to a variety of factors including different storage methods or collection techniques (Hammer et al., 2015). However, *Acinetobacter*, which is particularly overrepresented in Ontario, may act as a replacement beneficial symbiont in regions where *Apilactobacillus* are in low abundance (Christensen et al., 2021; Crowley & Russell, 2021), allowing bees to benefit from having a diverse range of bacterial symbionts. *Acinetobacter* is common in floral nectar and could improve the metabolism of nutrients for pollinators due to its ability to induce germination and pollen bursting (Christensen et al., 2021; Rering et al., 2021). Thus, while there are differences in microbial composition across provinces, the overall effects of how these different microbes interact, co-exist and adjust in the absence of others to determine bee health are still widely unknown and require future studies.

The implications of Manitoba yielding the lowest fungal diversity are still unknown and dependent on the interactions between each fungal genus and *A. virescens* (Figure 4B). The mutualistic/antagonistic switching that occurs with the opportunistic *Aspergillus* can cause disease in some wild and managed bees, while the fungus beneficially supports others by detoxifying xenobiotics and out-competing other pathogens within the bee (Becchimanzi & Nicoletti, 2022). Interestingly, our results indicate that Manitoba also yields the highest levels of plant diversity (Figure 4C). *Alternaria* moulds being underrepresented in Manitoba may be indicative of lower levels of plant pathogens in this province (Figure 5B), despite exposure to more diverse plants for the bees. While this would support the idea of the Manitoba landscape or environment being most ideal for *A. virescens*, the plant genera identified were typically unique to each province and the effects of each association between bees, plants, and fungi remain unclear (Figure 5C). For example, Ontario samples harboured plants not common elsewhere and were underrepresented in the most common plants of other provinces, such as *Trifolium* and *Rubus* (Figure 5C; Table S5). Particularly in highly urbanised areas such as those in Ontario, gardens and city parks host a diverse selection of plants that can result in more unique taxa present (Ayers & Rehan, 2021, 2023; Baldock et al., 2019; Seitz et al., 2022) (Table S1). While these Ontario *A. virescens* may harbour increased amounts of fungal plant pathogens, they may also benefit from the mutualistic relationships that plant pathogens, such as *Alternaria*, have with bees (Becchimanzi & Nicoletti, 2022; Dalinova et al., 2020; Ye et al., 2021).

Determining the core microbiome

The core microbiome of *A. virescens* across provinces consists of the bacteria *Apilactobacillus* and *Acinetobacter*, as well as the fungi *Cladosporium*, *Alternaria*

and *Aureobasidium* (Table 1). These two bacteria are abundant and beneficial in other wild bees, although not previously detected in *A. virescens* (Martinson et al., 2011; Nguyen & Rehan, 2023a; Shell & Rehan, 2022). The three core fungal genera maintain close relationships with plants: *Alternaria* is a plant pathogen that is found in healthy honey bee larvae due to its antagonistic relationship with brood diseases (Ye et al., 2021), while *Cladosporium* and *Aureobasidium* support pollen preservation through degradation and fermentation, ultimately aiding in nutrient utilisation for honey bees (Disayathanoowat et al., 2020; Hsu et al., 2021; Parish et al., 2020; Rutkowski et al., 2023). Characterised as part of the core microbiome in *A. virescens* across the country, these fungi may prove to be mutualists, or the very least, commensalists.

Plants of the *Trifolium* genus also met the criteria for "core" (Table 1). This suggests that *A. virescens* from across Canada may form important associations with clovers, regardless of location or collection method. Bicoloured sweat bees are generalists, visiting a wide variety of different plants with noted preferences for asters (Butters et al., 2022; Gardiner et al., 2010; Roberts, 1973; Sivakoff et al., 2018). However, *Trifolium* is a primary pollen source for *Ceratina* small carpenter bees that produce high-quality diets supporting development and survival (Lawson et al., 2017, 2020). Drawing parallels to *A. virescens*, clover may act as nutritionally healthy components of wild bee pollen, resulting in higher relative abundances of these plant genera found in bicoloured sweat bees across provinces.

CONCLUSION

While we found that using a combination of collection methods and geographic locations offered diverse characterisations of the microbiome, we caution that microbial descriptions of pinned specimens face limitations and may not be entirely representative of the larger bee population. We cannot rule out the possibility of human-introduced contamination, external storage or environmental contamination, and cross-contamination between bee species or individuals that are stored in proximity after collection. While introduced taxa remain an ongoing concern in all microbiome studies (Bieker et al., 2020; Hammer et al., 2015; Piro & Renard, 2023), this is particularly difficult in unstudied bees with no previous microbial characterisations as reference. Similarly, these microbial descriptions may be skewed to disproportionately detect microbes that are more likely to survive the drying and pinning processes involved for collections or that may have thrived postmortem. For example, if the optimised growth conditions for *Escherichia coli* or *A. alternata* are at room temperature, this may increase the relative abundance of these contaminants in the bee, postmortem and in storage (Bieker et al., 2020; Curran et al., 2020). While we can offer a preliminary description of the core taxa for these pinned *A. virescens*, microbiomes are still ideally studied using individuals collected across consistent and standard methods, particularly those optimised to preserve the microbiome, such as flash freezing (Hammer et al., 2015).

This study highlights the importance of characterising the microbiome of different wild bee species, as each bee harbours a unique community of microbiota that can be extrapolated to the population or species level. We found that utilising non-destructive methods for pinned specimens still allows for a robust microbiome to be described, although current methods favour flash-frozen specimens. While this study characterises a common core microbiome for this bee, many factors contribute to intraspecific and interspecific variances in microbial composition, including geographic location and bee collection method. Because both collection method and province were associated with differences in microbial and plant composition, using several methods in tandem presents a more comprehensive depiction of a bee population's microbiome. This study provides an initial characterisation of the *A. virescens* microbiome using pinned specimens but calls for further comparisons using immediately flash-frozen sweat bees or museum specimens of this and other bee genera. In addition, specific experiments targeting the role of these microbes and plant associations in bee health will help identify the benefits or detriments of these bacteria and fungi. As these relationships are clarified, proactive measures supporting wild bees, their microbial communities, and their floral resources can be considered for use in conservation efforts.

AUTHOR CONTRIBUTIONS

Phuong N. Nguyen: Investigation; writing – original draft; methodology; validation; visualization; formal analysis. **Farida Samad-zada:** Investigation; methodology; writing – review and editing; data curation. **Katherine D. Chau:** Investigation; writing – review and editing; validation; formal analysis; data curation. **Sandra M. Rehan:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision; data curation; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1068188.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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