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Ecological drivers of gut microbiota diversity and evolution in Brazilian stingless bees

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Abstract

The gut microbiota play a fundamental role in bee physiology and may contribute to their adaptation, ecological specialization, and evolutionary diversification. Here, we studied the gut microbiota of 29 species of Brazilian stingless bees from different states, encompassing a broad range of ecological traits. Gut bacterial communities were characterized by 16S rRNA gene sequencing. Taxonomic analyses identified a conserved bacterial core dominated by *Lactobacillus*, *Commensalibacter*, *Bombella*, *Snodgrassella*, *Bifidobacterium*, and *Weissella*, and their relative abundances varied markedly among bee species. Alpha diversity differed significantly among bees, whereas beta diversity analyses demonstrated that bee species was the primary factor structuring gut bacterial communities, followed by bee genus, while state explained only a small fraction of the observed variation, and geographic location had no significant effect. Geographic distance did not associate with microbiota dissimilarity, whereas host phylogeny correlated significantly with bacterial community composition, supporting a moderate pattern of phylosymbiosis. Phylogenetic analyses of dominant bacterial genera revealed contrasting patterns of amplicon sequence variants (ASVs) diversification, with *Lactobacillus* exhibiting the highest diversity, followed by *Bifidobacterium*, whereas *Snodgrassella*, *Floricoccus*, and Orbaceae displayed more restricted distributions among hosts. Microbial functional prediction indicated that amino acid biosynthesis, carbohydrate metabolism, energy production, and cofactor biosynthesis represented the predominant metabolic potential across stingless bee genera. Overall, our findings show that stingless bee gut microbiota are shaped primarily by host identity and evolutionary history, providing new insights into the evolution of host-associated microbial communities.

Keywords: Bee microbiota; Microbiota diversity; Coevolution; Geographic variation; Metagenomics.

1. Introduction

The gut microbiota plays a pivotal role in shaping the symbiotic relationships that sustain bee health and function. Its composition, richness, and stability reflect the adaptability of bees to diverse environmental and dietary conditions (Hall et al., 2021; Kwong et al., 2017). This plasticity not only influences the host's health and physiology but also drives the coevolution between bees and their microbiota, shaped by factors such as spatial distribution and resource availability (Kwong et al., 2017; McFrederick et al., 2012; Sarton-Lohéac et al., 2023). These coevolved microbiota underpin key physiological functions, as the bacteria residing in the bee gut mediate nutrient conversion and uptake via bacterial metabolism, fermentation, and food preservation, and provide crucial protection against pathogens and xenobiotics (Raymann and Moran, 2018; Todorov et al., 2024).

A significant portion of the bee gut bacteria is dedicated to carbohydrate metabolism, with different taxa performing specialized roles (Zheng et al., 2019). In addition to regulating the microbiota, these bacteria stimulate the innate immune system, promoting the persistence of core microbiota members and contributing to protection against pathogens (Daisley et al., 2020). Beyond their ecological role, some of these bacteria have probiotic potential. Their biotechnological properties also stand out, offering promising applications for developing next-generation antimicrobial agents and biopreservation strategies (Todorov et al., 2024).

Although the gut microbiota of social bees is characterized by a relatively limited number of core bacterial phylotypes at higher taxonomic levels, its composition can vary among host species, with dominant taxa typically belonging to Proteobacteria, Firmicutes, and Actinobacteria., predominantly from Proteobacteria, Firmicutes, and Actinobacteria (Cariveau et al., 2014; Cox-Foster et al., 2007; Hall et al., 2021). Five genera—*Snodgrassella*, *Gilliamella*, *Bombilactobacillus* (Firm-4), *Lactobacillus* (Firm-5), and *Bifidobacterium*—are recognized as the core gut microbiota of social bees (Kwong et al., 2017). Stingless bees (Meliponini) and bumblebees (Bombini) form a monophyletic clade, the sister group of honey bees (Apini), with a common ancestor estimated

between 80 and 100 million years ago (Danforth et al., 2013; Michener, 2007). This evolutionary relationship and a shared lifestyle, colony living, nectar and pollen consumption most likely explain the similarity and shared core microbiota among these bee groups.

Stingless bees (Meliponini) belong to the group of corbiculate bees, alongside bumblebees and honey bees, distinguished by the presence of a corbicula, an external tibial adaptation specialized for pollen, resin, and resource transport (Grüter, 2020). They are predominantly tropical pollinators, with greater diversity in the Neotropics, comprising 605 described species in 45 genera, of which 257 species in 29 genera occur in Brazil (Ascher and Pickering, 2026; Engel et al., 2023; Pedro, 2014).

Despite the megadiversity of stingless bee species, studies on their gut microbiota remain scarce. Therefore, the existence of a universal "core" microbiota shared across all stingless bees could so far not be confirmed. Recent research suggests that the gut microbiota composition varies considerably among stingless bee species, highlighting the possibility of the absence of a universal core. For example, *Scaptotrigona polysticta* of Amazon lacks the typical core microbiota found in other corbiculate bees, possibly due to specific adaptations to local environmental or dietary conditions (Pinto, 2022). Similarly, *Melipona bicolor*, *Melipona mondury*, and *Melipona quadrifasciata anthidioides* showed low or absent levels of *Snodgrassella* sp**.** and *Gilliamella* sp. (Cerqueira et al., 2024).

The sociality of stingless bees is key to the development and maintenance of specialized microbiomes. Gut microbiota acquisition primarily occurs through vertical transmission, where workers transfer microbes to new generations via feeding (trophalaxis) and contact with the colony’s internal environment, including feces. However, horizontal transmission can also occur through interactions with materials outside the nest and the collection of floral resources, facilitating the incorporation of new microbes (Cabirol et al., 2024; Cerqueira et al., 2024; Kwong et al., 2017; Kwong and Moran, 2016; Su et al., 2021). Colony size varies markedly among stingless bee species, from relatively small colonies in *Melipona*, such as *Melipona fulva* and *M. quadrifasciata*, with average colony

sizes of approximately 400 and 900 individuals, respectively, to much larger colonies in *Trigona spinipes*, averaging approximately 92,500 individuals, whereas *Plebeia droryana* has an intermediate colony size of approximately 2,400 individuals (Michener, 1974; Roldão-Sbordoni et al., 2018; Roubik, 1982). Such variation in colony size may influence opportunities for social contact and microbial transmission within colonies.

The interaction between stingless bees and floral resources extends beyond nectar and pollen collection, also representing an important route for microbiota acquisition. Studies show that stingless bees visit a wide variety of plants, with Fabaceae (161 genera) and Asteraceae (136 genera) being the most visited families, followed by Rubiaceae (65 genera), Malvaceae (56 genera), and Lamiaceae (43 genera) (Bueno et al., 2023). This diversity of plant interactions highlights not only the importance of foraging bees for pollination but also their potential influence on gut microbiota composition and the preservation of ecosystem functions in both natural and agricultural environments (Bueno et al., 2023; Cerqueira et al., 2024; Roubik, 2023).

Stingless bees also exhibit remarkable diversity in the structure and location of their nests (Bueno et al., 2023; Grüter, 2020; Roubik, 2023). Depending on the species, they may nest inside tree trunks, in the soil, or even occupy cavities left by other animals such as ants and birds. Many species, including some of the genera *Partamona*, *Trigona*, *Nannotrigona*, and *Tetragonisca*, have successfully adapted to urban environments, building their nests in cavities or holes in walls. In contrast, others are highly vulnerable, such as *Melipona* and *Cephalotrigona*, which depend on large hollow trees and are therefore at risk. Beyond nesting sites, there is also notable diversity in the materials used for nest construction, which may include wax, propolis, mud, or mixtures such as geopropolis (a blend of resin and mud) and even feces (Engel et al., 2023; Roubik, 2023). Body size also varies widely among species: there are tiny bees, such as *Plebeia* (the smallest highly eusocial bees in the world), and larger ones, like *Melipona scutellaris*, which is similar in size to the honey bee, *Apis mellifera*. With more than 255 species recorded in Brazil, it is expected

that a highly diverse microbiota exists within this ecologically rich context (Ascher and Pickering, 2026) .

Beyond taxonomic composition, characterizing the functional potential of the gut microbiota is important for understanding how bacterial communities may contribute to host nutrition, metabolism, and gut homeostasis (Bonilla-Rosso and Engel, 2018). Differences in bacterial community composition do not necessarily imply equivalent functional differences, as distinct bacterial taxa may share metabolic capabilities, whereas closely related taxa may differ in their functional potential. Functional profiling can therefore provide an additional perspective on microbiota variation by revealing conserved metabolic capacities as well as potential differences among host-associated communities (Prasad et al., 2025; Sarton-Lohéac et al., 2023; Su et al., 2022). In studies based on 16S rRNA sequencing, tools such as PICRUSt2 enable the inference of bacterial functional potential from taxonomic profiles, providing insights into metabolic pathways that complement taxonomic analyses and generate hypotheses for future metagenomic and experimental studies (Douglas et al., 2020).

This study studied the gut microbiota of 29 stingless bee species collected from different regions of Brazil, encompassing distinct biomes and environmental conditions and thereby capturing substantial ecological and geographic diversity. The sampled taxa encompassed a broad range of body sizes, nesting behaviors, and ecological strategies. Small-sized meliponines were represented by *Friesella schrottkyi*, *Frieseomelitta varia*, and species of *Plebeia*, whereas the genus *Melipona*, of considerable ecological and economic importance, was represented by several species. Additional genera included *Cephalotrigona*, *Nannotrigona*, *Partamona*, and *Scaptotrigona*, which exhibit diverse nesting habits, patterns of floral resource use, and defense strategies. Species from the genera *Tetragona* (*T. clavipes*), and *Trigona* were also examined, representing taxa recognized for their defensive behavior and relatively broad geographic distributions. In contrast, the sampling also included species with restricted or highly endemic distributions, such as *Melipona capixaba*. The dataset further encompassed species with particularly distinctive ecological and defensive traits, including *Lestrimelitta limao*, a cleptoparasitic species that obtains resources by raiding colonies of other

stingless bees, and *Oxytrigona cagafogo*, which possesses a chemical defense mechanism. Collectively, these taxa, drawn from multiple regions and encompassing contrasting ecological, behavioral, and biological traits, provide an opportunity to investigate variation in gut microbiota across stingless bees and its potential associations with host ecology, geography, and adaptation. Additionally, our results provide insights into the existence of a core microbiome in stingless bees. Beyond characterizing bacterial community composition and diversity, we assessed the predicted functional potential of the gut microbiota to identify conserved functional features and host-associated differences in metabolic profiles across stingless bee species.

2. Materials and methods

2.1. Bee collections

The present study was conducted with the appropriate authorization from the Chico Mendes Institute for Biodiversity Conservation (SISBIO No. 46746-1) and registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGen No. A7D5898), under the Brazilian Ministry of the Environment, and in accordance with Brazilian legislation. Sample collections were carried out in areas located in the states of Minas Gerais (MG), Espírito Santo (ES), Amazonas (AM) and Pernambuco (PE) (**Table 1**).

Adult bees identified as foragers from each species were captured at the entrance of the colonies using 15 mL centrifuge tubes or with tweezers at the entrance of the nest. After capture, the tubes were filled with 70% ethanol for sample preservation. Each colony was treated as an independent sampling unit, with the number of sampled colonies and processed guts varying by species availability. Standard sampling consisted of five guts per colony across three colonies. Exceptions included *L. limao* (1 colony, 20 guts), *O. cagafogo* (1 colony, 15 guts), *Cephalotrigona capitata* (2 colonies, 6 guts/colony), *Plebeia phrynostoma* (1 colony, 6 guts), *Scaptotrigona depilis* (1 colony, 7 guts), and two-colony samples with five guts each for *M. fulva, Schwarziana quadripunctata*, and *Trigona hyalinata*. Detailed sampling metadata for all species are provided in

Table 1 and Supplementary Table S1. The collected bees were then stored at -20°C until processing.

Table 1. Stingless bee species included in the gut microbiota characterization. AM: Amazonas; ES: Espírito Santo; MG: Minas Gerais; PE. Pernambuco

N°	Stingless bee species*	State	Number of samples (colonies) and guts per sample
1	*Cephalotrigona capitata*	MG	2 samples; 6 guts/sample
2	*Friesella schrottkyi*	MG	3 samples; 5 guts/sample
3	*Frieseomelitta varia*	MG	3 samples; 5 guts/sample
4	*Lestrimelitta limao*	MG	1 sample; 20 guts/sample
5	*Melipona asilvai*	MG	3 samples; 5 guts/sample
6	*Melipona bicolor*	ES	3 samples; 5 guts/sample
7	*Melipona capixaba*	ES	3 samples; 5 guts/sample
8	*Melipona marginata*	ES	3 samples; 5 guts/sample
9	*Melipona mondury*	MG	3 samples; 5 guts/sample
10	*Melipona quadrifasciata*	MG	3 samples; 5 guts/sample
11	*Melipona captiosa*	AM	3 samples; 5 guts/sample
12	*Melipona fulva*	AM	2 samples; 5 guts/sample
13	*Melipona scutellaris*	MG	3 samples; 5 guts/sample
14	*Melipona mandacaia*	ES	3 samples; 5 guts/sample
15	*Nannotrigona testaceicornis*	MG	3 samples; 5 guts/sample
16	*Oxytrigona cagafogo*	MG	1 sample; 5 guts/sample
17	*Partamona helleri*	MG	3 samples; 5 guts/sample
18	*Plebeia droryana*	MG	3 samples; 5 guts/sample
19	*Plebeia lucii*	MG	3 samples; 5 guts/sample
20	*Plebeia phrynostoma*	MG	1 sample; 6 guts/sample
21	*Scaptotrigona bipunctata*	MG	3 samples; 5 guts/sample
22	*Scaptotrigona depilis*	PE	1 sample; 7 guts/sample
23	*Scaptotrigona polysticta*	ES	3 samples; 5 guts/sample
24	*Scaptotrigona xanthotricha*	MG	3 samples; 5 guts/sample
25	*Schwarziana quadripunctata*	ES	2 samples; 5 guts/sample
26	*Tetragona clavipes*	MG	3 samples; 5 guts/sample
27	*Tetragonisca angustula*	MG	3 samples; 5 guts/sample
28	*Trigona hyalinata*	MG	2 samples; 5 guts/sample
29	*Trigona spinipes*	MG	3 samples; 5 guts/sample

*Details of the samples are provided in the Supplementary Table S1

2.2. Gut microbiota assessment

2.2.1. Dissection of the digestive tract

For the dissection, each bee was undergoing surface clearance through sequential washes in increasing concentrations of ethanol (80%, 90% and 100%) for 30 seconds each. This was followed by multiple washes in phosphate-buffered

saline (PBS; 0.1M; pH 7.6) and distilled water. After clearance, the midgut and hindgut of each individual was dissected from the abdomen via a ventral cut in the cuticle using micro scissors and forceps. Guts were then transferred to sterile 2 mL tubes to form species-specific pools per colony. The dissection tools were previously sterilized with 70% ethanol, and the entire procedure was performed under aseptic conditions. Samples were stored at -20 °C until DNA extraction (Botina et al., 2023).

2.2.2. DNA Extraction

DNA was extracted from the pool (**Table 1)**. DNA extraction was performed using the NucleoSpin Soil, Mini kit for DNA from soil, following the manufacturer's protocol. DNA quantification was carried out using a Qubit 2.0 fluorometer and the dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). DNA samples were stored at -20 °C.

2.2.3. PCR

The extracted DNA was used for PCR reactions targeting the 16S rRNA gene to quantify the gut microbiota. PCR reactions included 1.0 µL of total DNA, 2.5 µL of PCR buffer, 0.75 µL of $MgCl_2$, 1 µL each of primers 005F (TGGAGAGTTTGATCCTGGCTCA; 10 µM) and 1492R (TACIGITACTTTGTTACGACTT; 10 µM) (Weisburg et al., 1991), 0.5 µL of dNTPs (10 mM each), 0.2 µL of Taq DNA polymerase, and 18.05 µL of H_2O, for a total reaction volume of 25 µL (Botina et al., 2023). PCR products were evaluated by electrophoresis on a 1% (w/v) agarose gel stained with GelRed in 1× TBE buffer. Following confirmation of bacterial DNA amplification, the samples were stored at −20 °C until they were sent to BioSeq (Belo Horizonte, MG) for sequencing.

2.2.4. Metagenomics

Pooled DNA samples were sequenced by a commercial service provider to characterize bacterial community composition. The V4–V5 region of the bacterial 16S rRNA gene was amplified using the primers515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT) (Walters et al., 2016). The bacterial 16S rRNA genes (V4 region) were amplified and sequenced on the Illumina MiSeq platform.

Raw sequences obtained by amplicon sequencing were submitted to the National Center for Biotechnology Information (NCBI) under the accession BioProject PRJNA1440914 and Submission ID SUB16074325.

2.3. Statistical analysis

2.3.1. Bioinformatic

The bioinformatic workflow, including sequence processing, taxonomic assignment, microbiota diversity analyses, phylogenetic analyses, and functional prediction, followed the method described by Vidigal et al. (2026), with adaptations according to the objectives and dataset of the present study.

Raw sequence data was processed using the DADA2 pipeline (Callahan et al., 2016) implemented in R. The initial step involved quality assessment of paired-end Illumina reads through DADA2's built-in functions. Quality profiles of both forward and reverse reads were visualized to guide the selection of optimal parameters for trimming and filtering. Low-quality bases and sequencing adapters were removed by trimming, followed by filtering based on stringent criteria: a maximum of two expected errors per read (maxEE = 2), a truncation quality score threshold (truncQ = 2), and exclusion of reads containing ambiguous bases (maxN = 0). Following these preprocessing steps, the error rates for forward and reverse reads were learned independently, allowing the construction of error models specific to the sequencing run.

Amplicon sequence variants (ASVs) were inferred from the filtered reads using DADA2's denoising algorithm, which resolves sequencing errors without clustering. Subsequently, paired-end reads were merged, requiring sufficient overlap and the absence of mismatches. Chimeric sequences were detected and removed using the consensus method implemented in DADA2. The resulting ASVs were assembled into an abundance table, which served as the basis for downstream analyses. Taxonomic classification was performed using DADA2's native implementation of the naïve Bayesian classifier trained on the SILVA database version 138.2 (Quast et al., 2013). Taxonomy was assigned from the phylum to genera level, when possible, based on a minimum confidence threshold.

2.3.2. Taxonomic composition analysis

The relative abundance of microbiota taxa associated with bees' genera and species was visualized through taxonomic bar plots generated using the phyloseq package (McMurdie and Holmes, 2012) in R. These visualizations focused on the most abundant family and genera, cumulatively accounting for up to 80% or 90% of the total community composition, to highlight patterns of bacterial prevalence among groups. Heatmaps were constructed using ggplot2, with color gradients scaled to abundance values and numerical labels added for interpretability. Heatmaps were created using *ggplot2*, with color gradients scaled to abundance values and numerical labels included to enhance interpretability. Genera were ordered based on ecological or phylogenetic relevance, and sample groups were arranged to emphasize contrasts between *Melipona* and non-*Melipona* bees. To further explore group-specific microbiota profiles, additional heatmaps were generated separately for *Melipona* and non-*Melipona* species.

Assessment of microbiota diversity included both alpha (within-sample) and beta (between-sample) analyses. Alpha diversity was estimated using indices such as Shannon, Simpson, and Observed ASVs, calculated through the phyloseq package. Group comparisons (e.g., bee genera, or bee species) were evaluated statistically using Gamma Generalized Linear Model (GLM) with a log link function to account for non-normal continuous data, incorporating sequencing depth (Log_LibrarySize) as a covariate. Main effects were assessed using Type III Analysis of Deviance. Beta diversity was assessed using both non-phylogenetic (Bray–Curtis) and phylogeny-aware (e.g., UniFrac) distance metrics. Ordination techniques, including Principal Coordinates Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS), were applied to visualize clustering patterns in microbiota communities across samples. Statistical testing for differences in community composition among groups was performed using PERMANOVA (Permutational Multivariate Analysis of Variance), implemented in the vegan package. The analysis evaluated the influence of host identity (bee species and genus) and geographic location (state) on the gut microbiota composition of stingless bees.

Complementary analyses included species-by-species distance matrices for both host phylogeny and microbiota composition, which were visualized as clustered heatmaps. Both heatmaps used the same species ordering to facilitate

direct comparison. To quantify similarity between phylogenetic and microbiota distances, matrices were normalized using min-max scaling, and pairwise agreement scores were calculated as 1 minus the absolute difference between corresponding matrix entries. These scores were displayed using a gradient heatmap.

2.3.3. Phylogenetic and microbiota composition dissimilarity tree among bee species

Patterns of host-microbiome co-diversification were investigated by comparing phylogenetic relationships of host species and microbiota community. A phylogenetic tree of the bee species was constructed using publicly available molecular data (Henríquez-Piskulich et al., 2024) and formatted in Newick format. Because *O. cagafogo*, the species included in our sampling, was not represented in the reference phylogenetic dataset, *O. tataira* was used as a phylogenetic proxy. This substitution was based on the previous taxonomic treatment of *O. cagafogo* as a junior synonym of *O. tataira*, although the former was subsequently revalidated as a distinct species (Ramos et al., 2025). This tree was processed and visualized in R using the *ape* (Paradis and Schliep, 2019) and *ggtree* (Yu et al., 2017) packages, including tip annotations, node identifiers, and standardized branch lengths for visual clarity. The relationship between host phylogeny and microbiota composition was statistically evaluated through Mantel tests using the *vegan* package (Oksanen et al., 2025). These tests measured the correlation between the host phylogenetic distance matrix and the microbiota dissimilarity matrix. Microbiota community dissimilarity was calculated using Bray-Curtis distances between samples, as implemented in *phyloseq*. The average pairwise Bray-Curtis dissimilarity between species was derived from all inter-sample comparisons, resulting in a species-level dissimilarity matrix. This matrix was then used to construct a dendrogram via hierarchical clustering (*hclust*, method = "average"), which was converted to a tree object (*as.phylo*) for comparative purposes. Visual concordance between host and microbiota trees was examined using tanglegrams. Dendrograms were generated with the *dendextend* package (Galili, 2015), and tip alignment was optimized using the *untangle* function (method = "step2side").

2.3.4. Phylogenetic analysis of *Lactobacillus*, *Snodgrassella*, *Gilliamella*, and *Bifidobacterium*

Microbial community data was imported and managed using the phyloseq package in R. To resolve unclassified taxonomy within specific bacterial lineages, manual curation was performed; specifically, ASVs belonging to the family Orbaceae that had not reached genus resolution were explicitly reclassified at the genus level as "Orbaceae_NA" to facilitate targeted downstream analysis. The dataset was subsetted to analyze the phylogenies of specific target genera associated with the bee microbiome (e.g., *Bifidobacterium*, *Lactobacillus*, *Snodgrassella*, Orbaceae_NA, *Floricoccus*, *Bombilactobacillus*, *Bombella*, *Acinetobacter*, and *Commensalibacter*). Data wrangling was performed using dplyr and tidyr in R. For each target genus, an ASV-to-host mapping strategy was applied where ASVs with an abundance greater than zero were linked to their respective host bee species (extracted from sample metadata using stringr). Phylogenetic trees for each focal bacterial genus were constructed and visualized using the ggtree package in R. Genera containing fewer than two ASVs were automatically excluded from the tree rendering. Each resulting tree displays calculated branch lengths and customized tip labels, which consist of identifiers of all bee host genera sharing that specific ASV.

3. Results

From the analysis of 6,207,890 high-quality sequences obtained from 75 stingless bee gut samples, a total of 2,530 ASVs were identified after quality filtering and denoising, with an average sequencing depth of 78,580 reads per sample. Taxonomic assignment using the SILVA reference database revealed high resolution at broad taxonomic ranks, with nearly all reads classified at the phylum, class, and order levels. However, taxonomic resolution dropped significantly at finer ranks. Particularly, 10.8% of ASVs (representing 3.3% of total reads) could not be assigned to any known bacterial family, and 42.9% of ASVs (accounting for 38.9% of total reads) remained unclassified at the genus level (**Supplementary Figure S1**). Furthermore, 96.5% of ASVs (97.7% of reads) lacked species-level resolution. This high proportion of unassigned taxa, mainly at the genus and family levels, indicates that a substantial portion of the stingless

bee gut microbiota remains poorly represented in current reference databases, highlighting a large potential for previously uncharacterized bacterial diversity.

Taxonomic classification assigned the sequences to 16 bacterial phyla, more than 20 bacterial classes, and over 60 bacterial genera. The gut microbiota was dominated by the phyla Firmicutes (48.9%) and Proteobacteria (46.4%), whereas Bacteroidota (2.5%) and Actinobacteriota (2.3%) occurred at substantially lower abundances (**Supplementary Figure S2**). At the genus level, *Lactobacillus* was by far the most abundant taxon, followed by *Snodgrassella*, *Commensalibacter*, *Bombella*, *Pectinatus*, *Weissella*, *Floricoccus*, and *Bifidobacterium*, whereas the remaining genera were detected at comparatively low relative abundances (**Figure 1**). Each bee species exhibited a distinct gut microbiota profile, with specific composition and relative abundance of bacterial genera.

Taxonomic classification and relative abundance analyses revealed pronounced host-specific structuring of gut microbiota communities in stingless bees while also identifying a conserved bacterial core shared across phylogenetically distinct hosts. *Lactobacillus* was detected in all bee species and represented the most consistently distributed bacterial genus, highlighting its widespread association with stingless bees. However, it was absent from *F. schrottkyi* and *Plebeia lucii*. Other recurrent bacterial taxa, including *Commensalibacter*, *Bombella*, *Bifidobacterium*, *Weissella*, *Snodgrassella*, *Floricoccus*, and members of the family Orbaceae, were detected across multiple host species, although their relative abundances varied markedly among hosts (**Figure 2 Supplementary Table S4**). The occurrence of Orbaceae may include lineages closely related to *Gilliamella*, but their taxonomic identity could not be resolved at the genus level using the 16S rRNA V4–V5 region. In contrast, several bacterial genera exhibited strong host-specific associations. *Floricoccus* reached its highest relative abundances in *Melipona* (viz. *M. fulva*, 59.12% relative abundance), whereas *Pectinatus* predominated in *O. cagafogo* (87.27%), *Apibacter* in *Partamona helleri* (30.14%), *Bombella* in *P. phrynostoma* (68.11%) and *P. droryana* (39.87%), *Commensalibacter* in *S. quadripunctata* (63.71%) and *T. clavipes* (56.53%), *Snodgrassella* in *F. varia* (66.19%) and *Tetragonisca angustula* (87.09%), and *Swingsia* in *M. quadrifasciata* (64.12%) and *M. captiosa*

(80.68%). These contrasting distribution patterns indicate that, despite the existence of a conserved bacterial core, the dominance of specific bacterial taxa is strongly associated with host identity, supporting species-specific microbiota assembly in stingless bees.

Bacterial alpha diversity, measured using the Shannon diversity index, differed significantly among the bee species (**Figure 3; Supplementary Table S5**). The Shannon index accounts for both taxonomic richness and the distribution of relative abundances among bacterial taxa, providing an integrated measure of community diversity. Across all bee species, the observed Shannon values varied substantially among hosts (Gamma GLM, Type III Analysis of Deviance: LR chi^2 = 109.16, Df = 28, $p < 0.001$), with relatively high diversity observed in *M. bicolor* (average Shannon value = 2.177), *M. capixaba* (average Shannon value = 2.738), *M. quadrifasciata* (average Shannon value = 2.79), *M. scutellaris* (average Shannon value = 2.488), and *P. helleri* (average Shannon value = 2.982), whereas lower values were observed in *L. limao* (average Shannon value = 0.915), *F. schrottkyi* (average Shannon value = 1.305), *M. marginata* (average Shannon value = 1.234), and *T. angustula* (average Shannon value = 1.354). However, differences in Shannon diversity were not uniform across host groups. In *Melipona*, Shannon diversity did not differ significantly among the ten species examined, despite variation in their observed diversity values (LR chi^2 = 10.16, Df = 9, $p = 0.06$; **Figure 3A**). In contrast, significant differences were detected among non-*Melipona* species (LR chi^2 = 127.69, Df = 18, $p < 0.001$; **Figure 3B**). *Partamona helleri* exhibited the highest Shannon diversity among the non-*Melipona* species, whereas *L. limao* and *F. schrottkyi* showed comparatively lower values. Overall, these results indicate that bacterial alpha diversity varies among bee species, but this variation was more pronounced among non-*Melipona* species, whereas Shannon diversity was comparatively homogeneous among the *Melipona* species examined.

Weighted UniFrac analysis revealed that bacterial communities were primarily structured according to species rather than geographic origin (**Figure 4)**. Principal coordinate analysis (PCoA) showed clear clustering of samples according to host species, whereas no evident grouping was observed according to Brazilian state or collection locality (**Figure 4A–B; Supplementary Table S6**).

PERMANOVA models confirmed that host explained most of the variation in microbiota composition (R^2 = 0.53-0.699, P = 0.001), whereas neither state (R^2 < 0.001, P = NA) nor locality (R^2 = 0.001, P = 0.645) had a significant effect (**Supplementary Table S7)**. This indicates that even when environmental factors are shared, the microbiota communities differ, suggesting that host identity plays the most important role in shaping the composition of the gut microbiota in stingless bees. These results highlight the complex interplay between host-related factors (such as phylogeny or physiology) and environmental influences and suggest that gut microbiota assembly is primarily based on phylogenetic and taxonomic background and then determined by external conditions.

Mantel tests demonstrated that geographic distance was not associated with microbiota composition, either using Bray-Curtis dissimilarity (r = −0.033, P = 0.776) or weighted UniFrac distances (r = 0.010, P = 0.420) (**Figure 4C–D; Supplementary Table S7**). These results indicate that geographic separation does not explain the observed variation in gut bacterial communities and reinforce host species as the primary determinant of microbiota.

The relationship between host phylogeny and gut microbiota was evaluated, we compared a phylogenetic tree with a Bray-Curtis dissimilarity tree of gut microbiota composition for 26 bee species (**Figure 5; Supplementary Table S8**). *Melipona mondury*, *P. phrynostoma* and *P. lucii* were not included in this comparison due to the lack of data necessary to construct their phylogenetic placement.

We observed that closely related bee species generally harbored more similar gut microbiota, particularly within the genus *Melipona*, which formed a distinct cluster in both trees. For example, species such as *M. captiosa*, *M. capixaba*, and *M. scutellaris* exhibited very low microbiota dissimilarity values (e.g., 0.016–0.033), indicating high similarity in their microbiota communities. The Mantel test revealed a moderate but significant correlation between host phylogeny and microbiota composition (r = 0.47, P = 0.001), suggesting a phylogenetic signal that is, evolutionary history partially shapes gut microbiota composition (**Figure 5B**). Nonetheless, this relationship was not consistent across all taxa. Some closely related species, particularly in the non-*Melipona* cluster, exhibited notable differences in microbiota. For example, *T. spinipes* and *T.*

clavipes, despite being phylogenetically close, showed relatively high dissimilarity in gut microbiota composition (e.g., 0.122). Overall, these findings support the hypothesis that evolutionary history influences gut microbiota composition but also highlight the role of ecological and environmental factors, such as diet, nesting behavior, and habitat, in shaping microbiota communities among stingless bees. For example, *Partamona* and *Trigona* species construct nests using mud and other materials, whereas *T. angustula* can adapt to urban environments by nesting in cavities and holes in walls. In contrast, *M. capixaba* has a restricted geographic distribution in the mountainous regions of Espírito Santo, Brazil, and depends on large hollow trees for nesting. Furthermore, feeding strategies vary considerably among stingless bees. While most species rely on nectar and pollen, some exploit alternative food sources, such as honeydew or decaying organic matter. *Lestrimelitta* species exhibit cleptobiotic behavior, obtaining food by raiding the nests of other bee species rather than collecting floral resources directly (Cham et al., 2018; Grüter, 2020). Differences in dietary resources, nesting substrates, construction materials, and habitat use may therefore contribute to variation in microbial exposure and gut microbiota composition among stingless bee species.

We identified three main patterns when comparing bee phylogeny and gut microbiota composition. The *Melipona* cluster formed a well-supported group of closely related species that also shared highly similar gut microbiota communities, indicating a strong phylogenetic signal. The intermediate cluster included species such as *F. varia* and *T. angustula*, which showed moderate microbiota similarity and phylogenetic proximity. In contrast, *P. helleri* appeared as a distinct lineage, forming a separate branch in both the phylogenetic and microbiota trees, suggesting a unique evolutionary and microbiota profile. Finally, the non-*Melipona* cluster, composed of species from genera such as *Scaptotrigona*, *Cephalotrigona*, and *Trigona*, displayed greater variation in microbiota composition despite phylogenetic relatedness, highlighting the possible influence of ecological or environmental factors in shaping their gut communities.

The phylogenetic reconstruction of the dominant bacterial genera based on their 16S sequence revealed distinct patterns of ASV diversity among bees

(**Figure 6**). *Lactobacillus* exhibited the highest phylogenetic diversity, comprising 39 ASVs distributed across multiple phylogenetic clades (**Figure 6A**). Several ASVs were shared among different bee species and genera, whereas others were detected only in one or a few hosts. ASVs associated with *Melipona* were distributed throughout the phylogeny rather than forming a single monophyletic lineage, indicating that closely related bacterial lineages occur in multiple stingless bee taxa. *Bifidobacterium* exhibited lower phylogenetic diversity than *Lactobacillus*, comprising 25 ASVs organized into several distinct clades (**Figure 6B**). Multiple ASVs were shared among different stingless bee species, while others showed a more restricted distribution. Several lineages were detected in *P. helleri*.

When ASV distribution was examined at the bee genus level, similar phylogenetic patterns were observed (**Supplementary Figure S3 and S4**). For both bacterial genera, several ASVs were shared among multiple stingless bee genera, whereas others were restricted to a limited number of host genera, demonstrating heterogeneous distribution patterns of bacterial lineages across Meliponini.

Snodgrassella exhibited relatively low phylogenetic diversity, comprising five ASVs distributed across multiple bee species (**Figure 7A**). Most ASVs were associated with non-*Melipona*, particularly *F. varia* and *T. angustula*, whereas only a single ASV was detected in *Melipona*. Although few ASVs were identified, their distribution across different host taxa suggests that *Snodgrassella* is shared among several stingless bee lineages while remaining absent from many others.

Floricoccus was represented by six ASVs, with most lineages associated with species of *Melipona* (**Figure 7B**). However, some ASVs were also detected in *T. hyalinata*, *Scaptotrigona bipunctata*, *P. helleri*, and *C. capitata*, indicating that while *Floricoccus* is strongly associated with *Melipona*, certain lineages are shared among distantly related taxa.

Members of the family Orbaceae exhibited the highest phylogenetic diversity among these bacterial groups, comprising 16 ASVs distributed across multiple species (**Figure 7C**). Several ASVs were shared among different species, whereas others were detected in only one or a few hosts. Collectively,

these results demonstrate substantial variation in the distribution of bacterial lineages among bees, with some ASVs showing broad host occurrence and others exhibiting more restricted host associations.

Functional prediction using PICRUSt2 identified a broad diversity of MetaCyc pathways associated with the gut microbiota (**Figure 8A; Supplementary Table S9 – S11**). The largest proportion of predicted functions was related to amino acid biosynthesis, followed by pathways involved in nucleoside and nucleotide biosynthesis, cofactor, prosthetic group, electron carrier, and vitamin biosynthesis, carbohydrate biosynthesis, and energy metabolism. Additional functional categories included nutrient acquisition and degradation, cellular maintenance and information processing, stress resistance and detoxification, and metabolic flexibility, although these represented a smaller proportion of the predicted functional repertoire.

Functional enrichment analysis revealed that the predicted metabolic potential differed among bee genera (**Figure 8B**). Pathways associated with amino acid biosynthesis, carbohydrate metabolism, energy production, cofactor biosynthesis, and nutrient acquisition were enriched across multiple bee genera, whereas other functional categories showed more restricted enrichment patterns. Overall, the predicted functional profiles suggest that, despite taxonomic differences among bacterial communities, core metabolic functions are broadly conserved across stingless bee genera, while specific metabolic pathways vary in their relative enrichment among hosts.

4. Discussion

Gut microbial communities in stingless bees studied here exhibit a taxonomically rich but numerically uneven structure, characterized by a stable core alongside a diverse fraction of low-abundance taxa. While higher taxonomic ranks are well-resolved, current reference databases fail to classify a major portion of the microbiota at finer scales, particularly at the genus and species level. This low representation of assigned sequences across both variant diversity and total abundance demonstrates that uncharacterized bacteria are not merely rare or transient members but constitute a dominant component of the stingless bee gut. This taxonomic limitation highlights a critical gap in global genomic

repositories (such as SILVA), which remain historically biased toward honey bees and bumblebees (Haag et al., 2026; Kwong et al., 2017; Sarton-Lohéac et al., 2023). Because Neotropical Meliponini harbor ancient, host-specific bacterial lineages that co-evolved with their hosts (Kueneman et al., 2023; Sarton-Lohéac et al., 2023), relying on reference databases built primarily for temperate managed pollinators fails to capture the true scope of tropical symbiont diversity.

Stingless bee gut bacteria represent ancient sister lineages to core symbionts found in honey bees and bumblebees with conserved functional traits, confirming their common evolutionary origin (Haag et al., 2026; Kwong et al., 2017; Sarton-Lohéac et al., 2023). This phylogenetic relatedness provides an evolutionary context for the recurrent occurrence of bacterial genera across different groups of social bees. In our dataset, several of the most consistently detected genera, including *Lactobacillus*, *Bifidobacterium*, *Bombilactobacillus* and *Snodgrasella*, were also among the bacterial lineages commonly reported from honey bees and bumblebees (Haag et al., 2026; Mazel et al., 2025). Rather than an evolutionary trajectory governed strictly by host-symbiont codiversification, the topological incongruence between host and bacterial phylogenies suggests that social bee microbiota evolution was driven by host-switching events alongside independent symbiont acquisitions and losses (Cerqueira et al., 2021; Ramírez-Ahuja et al., 2025; Santini et al., 2026; Sarton-Lohéac et al., 2023). Supporting this dynamic, our findings reveal marked variations in bacterial genus profiles, not only distinguishing honey bees and bumblebees from stingless bees, but also segregating *Melipona* species from non-*Melipona* ones. These compositional divergence patterns align closely with former investigations on corbiculate bee microbiota assembly, suggesting adaptation to different host species (Haag et al., 2023; Kueneman et al., 2023; Sarton-Lohéac et al., 2023) .

A particularly informative feature of stingless bee microbiome evolution is the recurrent loss or reduction of *Snodgrassella* and *Gilliamella* across several Meliponini. These bacteria are prominent members of the social bee microbiota, yet their distribution across stingless bees is highly heterogeneous, with reduced prevalence or apparent absence reported in *Melipona* and in several Afrotropical, Indomalayan, Australasian, and Neotropical genera (Cerqueira et al., 2021;

Figueroa et al., 2021; Kueneman et al., 2023; Ramírez-Ahuja et al., 2025; Tola et al., 2021). Our results further support this heterogeneous distribution. Among the ten *Melipona* examined, *Snodgrassella* was detected in only three species, and it was likewise detected in only three of the non-*Melipona* analyzed. In contrast, *Gilliamella* was not detected at the genus level in any of the 29 species examined here.

Sequences assigned to the family Orbaceae were detected in 17 species, encompassing both *Melipona* and non-*Melipona*. Because our V4–V5 16S rRNA dataset did not resolve these Orbaceae sequences to genus or species level, their taxonomic identity remains uncertain. These sequences could include *Gilliamella*-related lineages, including potentially divergent strains associated with stingless bees, although this possibility cannot be established from the present data alone. Conversely, both *Snodgrassella* and *Gilliamella* are retained in other stingless bee lineages, including *Tetragonisca*, *Trigona*, *Partamona*, *Frieseomelitta*, and *Tetragonula*, while some genera show an apparent decoupling of their distributions, with one symbiont occurring at high abundance and the other being rare or undetected (Botina et al., 2019; Figueroa et al., 2021; Kueneman et al., 2023; Ramírez-Ahuja et al., 2025; Sarton-Lohéac et al., 2023). Together, these patterns indicate that the distribution of these bacterial lineages is not uniform across Meliponini and that associations with the ancestral social-bee microbiota have been repeatedly modified during stingless bee diversification.

The absence or reduction of *Snodgrassella* and *Gilliamella* in *Melipona* also raises the possibility that other bacterial lineages contribute to functions otherwise associated with these core symbionts (Cerqueira et al., 2024). *Floricoccus* is particularly relevant in this context, as it was detected in all ten *Melipon*a and was also present in six non-*Melipona*. This broad occurrence among Brazilian *Melipona* is consistent with previous reports proposing *Floricoccus*-related bacteria as putative symbionts associated with this host genus, potentially acquired from floral environments and subsequently maintained through social transmission (Cerqueira et al., 2024; Santini et al., 2026; Vidigal et al., 2026). However, this association does not appear to be geographically universal. A recent survey of stingless bees from Mexico, including

Melipona beecheii, *Melipona solani*, and *Melipona fasciata*, did not detect *Floricoccus* in these species (Ramírez-Ahuja et al., 2025). This contrasting distribution suggests that the association of *Floricoccus* with *Melipona* may be context dependent and could reflect geographic variation, host population structure, environmental exposure, or differences in microbial acquisition and transmission. Thus, rather than representing a fixed *Melipona*-associated symbiont, *Floricoccus* may constitute a recurrent but geographically and ecologically variable component of the stingless bee microbiota.

The *Melipona* ileum lacks *Snodgrassella* and *Gilliamella* but contains a putative *Floricoccus*-related symbiont together with *Bifidobacterium*, *Lactobacillus*, and *Bombella* (Santini et al., 2026). Together with the distribution observed in our dataset, these findings raise the possibility that *Floricoccus* may contribute to ecological or metabolic functions associated with the gut community in *Melipona*. However, the detection of *Floricoccus* does not demonstrate functional replacement of *Snodgrassella* or *Gilliamella*, nor does it establish that *Floricoccus* performs equivalent functions. In honey bees and bumble bees, *Snodgrassella* and *Gilliamella* form a spatially organized ileal biofilm and participate in metabolic interactions, including cross-feeding (Hammer et al., 2021; Li et al., 2022). Determining whether *Floricoccus* occupies a comparable niche and contributes to analogous functions in *Melipona* and non-*Melipona* species will therefore require spatially resolved and functional approaches beyond amplicon-based community profiling.

Bacterial alpha diversity varied among bee species, but this variation was unevenly distributed between the two major host groups considered here. Shannon diversity did not differ significantly among the ten *Melipona* examined, whereas significant differences were detected among non-*Melipona*. Several *Melipona* species and *P. lucii* displayed relatively high Shannon diversity, whereas lower values were observed in *C. capitata*, *T. hyalinata*, and *S. bipunctata*. The absence of significant differences in Shannon diversity among *Melipona* species suggests a relatively conserved level of bacterial alpha diversity within this genus. A similar pattern has been reported among closely related *Melipona*, as no significant differences in Shannon diversity were detected among *M. bicolor*, *M. mondury*, and *M. quadrifasciata* (Cerqueira et al., 2024).

Likewise, no significant differences in alpha diversity were detected across 16 Mexican species, including *M. beecheii*, *M. solani*, and *M. fasciata*, despite variation in Shannon values among genera (Ramírez-Ahuja et al., 2025). In contrast, differences in alpha diversity were detected between *M. mondury* and *M. quadrifasciata* when the full-length 16S rRNA gene was analyzed (Haag et al., 2026). Together, these observations suggest that relatively similar levels of bacterial alpha diversity can occur among closely related stingless bees, although differences may emerge depending on the host comparison and methodological resolution.

In contrast, alpha diversity varied significantly among the non-*Melipona* species studied. This greater heterogeneity is consistent with evidence that host species can contribute substantially to variation in bacterial diversity among Neotropical stingless bees, with significant differences in ASV richness reported among species (Figueroa et al., 2021). Differences in bacterial diversity have also been reported between *A. australis* and *T. carbonaria*, as well as between wild and managed *T. carbonaria* colonies, indicating that bacterial diversity can vary with both host identity and ecological context (Liu et al., 2023).

Beta-diversity analyses indicated substantial differences in gut bacterial community composition among bee species. Host identity consistently explained more variation in community structure than geographic factors, suggesting that bacterial community assembly is more strongly associated with species than with broad spatial variation. This pattern is consistent with previous studies showing that host species is an important determinant of gut microbiota composition in stingless bees and other social bees (Figueroa et al., 2021; Kwong et al., 2017; Ramírez-Ahuja et al., 2025; Sarton-Lohéac et al., 2023; Vidigal et al., 2026). Although some bacterial taxa were shared among hosts, microbial communities remained distinctive at the host-species level, indicating that the recurrence of common bacterial lineages does not necessarily result in equivalent community structures across species (Sarton-Lohéac et al., 2023). In our dataset, geographic variables contributed less to community differentiation than host identity, and geographic distance was not significantly associated with microbiota dissimilarity. Nevertheless, the sampled species occupied contrasting environmental settings, including the warm and humid Amazonian biome and the

Atlantic Forest, which encompasses cooler and often montane environments in southeastern Brazil (Engel et al., 2023). Together, these patterns suggest that host-associated factors play an important role in structuring stingless bee gut microbiota, while environmental conditions and microbial dispersal may contribute to additional variation among communities.

The significant association between host phylogeny and gut microbiota composition observed in our study suggests that host evolutionary history contributes to the structuring of bacterial communities in stingless bees. This pattern is consistent with phylosymbiosis, a phenomenon in which the similarity of host-associated microbial communities reflects the evolutionary relationships among their hosts (Lim and Bordenstein, 2020). Similar associations between host phylogeny and gut microbiota composition have been reported in social bees, with closely related hosts tending to harbor more similar microbial communities (Kwong et al., 2017). Comparable phylogenetic patterns have also been observed in ants, where differences in gut microbiota among species were associated with host phylogeny despite overall community stability (Sanders et al., 2014). However, the moderate strength of the association observed in our study and the deviations from phylogenetic correspondence among some lineages indicate that host evolutionary history does not fully determine microbiota composition. Instead, the observed pattern is consistent with a dynamic microbiota in which host evolutionary history provides an important framework for community structure, while ecological and microbial processes may contribute to variation among hosts (Kwong et al., 2017; Lim and Bordenstein, 2020; Vidigal et al., 2026).

The phylogenetic reconstruction of dominant bacterial genera revealed substantial intra-genus ASV diversification across Meliponini. Within *Lactobacillus* and *Bifidobacterium*, ASVs associated with *Melipona* were polyphyletic, branching across distinct clades rather than forming a single host-monophyletic group. This pattern aligns with evidence that core symbionts of corbiculate bees diversify into complex strain clusters maintained via host switching and niche partitioning (Kwong et al., 2017; Mazel et al., 2025). Moreover, the observation that specific ASVs reach high prevalence in a primary host while occurring sporadically in sympatric species supports host-preferred

lineage persistence with occasional cross-host spillover (Haag et al., 2026; Sinotte et al., 2020).

In contrast to lactic acid bacteria, *Snodgrassella* exhibited low ASV diversity, being mostly restricted to non-*Melipona* such as *F. varia* and *T. angustula*, mirroring reported lineage losses in specific stingless bee clades (Cerqueira et al., 2021). Conversely, *Floricoccus* ASVs were strongly enriched in *Melipona* while remaining distinct from environmental isolates, supporting its status as a host-adapted lineage acquired from floral niches (Cerqueira et al., 2024, 2021; Santini et al., 2026). Meanwhile, the high ASV richness in Orbaceae highlights a vast reservoir of gammaproteobacterial diversity, likely encompassing uncharacterized *Gilliamella* strains (Kwong et al., 2017; Praet et al., 2017).

Collectively, the heterogeneous distribution of bacterial ASVs provides key insights into microbiota assembly in stingless bees. While community-level similarity often reflects host phylogeny (phylosymbiosis) (Lim and Bordenstein, 2020; Mallott and Amato, 2021), finer ASV-level incongruence refutes a model of rigid host-symbiont co-speciation. Instead, the Meliponini gut microbiota has been dynamically reshaped by host transfers, lineage acquisitions, and symbiont losses (Prasad et al., 2025; Sarton-Lohéac et al., 2023). Ultimately, social interactions, floral environments, and host physiology interact to drive strain-level adaptation across Neotropical stingless bees (Cerqueira et al., 2024; Leonhardt and Kaltenpoth, 2014).

The significant association between host phylogeny and gut microbiota composition observed in our study suggests that host evolutionary history contributes to the structuring of bacterial communities in stingless bees. Primary pathways (including amino acid biosynthesis, nucleoside/nucleotide production, cofactor and vitamin synthesis, carbohydrate degradation, and energy metabolism) constituted the dominant functional categories. This overlap suggests high functional redundancy, where taxonomically distinct bacterial communities converge on shared metabolic capabilities essential for host nutrition and gut homeostasis (Engel et al., 2012; Sarton-Lohéac et al., 2023). Similar functional conservation is well-documented across social corbiculate

bees, where core phylotypes (such as *Lactobacillus* Firm-5, *Bifidobacterium*, and *Gilliamella*) share conserved machinery for sugar fermentation and pollen-derived glycan cleavage, while aerobes like *Snodgrassella* supply respiratory energy metabolism (Kwong et al., 2014; Quinn et al., 2024).

The differential enrichment of specific metabolic pathways among bee genera demonstrates that taxonomic turnover introduces host-specific functional variation. This pattern aligns with recent comparative metagenomic evidence in *A. mellifera* and Neotropical Meliponini, showing that while core metabolic capabilities are maintained across hosts, specific lineage-level adaptations [(such as specialized glycoside hydrolase repertoires (CAZymes) or strain-specific biosynthetic auxotrophies)] drive fine-scale functional differentiation (Prasad et al., 2025; Wardman et al., 2022). Furthermore, in social bees, functional capacity is not static; it scales with host identity, developmental stage, and caste, reflecting a balance between environmental resilience in foraging workers and fermentative specialization in queens and larvae (Vidigal et al., 2026).

Because these metabolic pathways were inferred from 16S rRNA marker-gene profiles using PICRUSt2, they represent predicted metabolic potential rather than directly measured functional activity or expressed phenotypes. Nonetheless, these findings indicate that stingless bee microbiotas reconcile a highly conserved core functional architecture with host-associated metabolic tailoring, optimizing symbiont contributions across diverse Neotropical pollinator niches.

Future studies should expand phylogenetic and biogeographic sampling across additional Brazilian biomes and include multiple colonies per species, together with individual-level and colony-level traits such as body size, colony size, nesting biology, social organization, and resource use. Longitudinal sampling could further clarify the temporal stability and transmission dynamics of gut microbiota. Combining amplicon sequencing with culture-dependent and metagenomic approaches would also improve the taxonomic and functional resolution of bacterial lineages that remain poorly differentiated by 16S rRNA sequencing, including members of the Orbaceae. Such integrative approaches

will help clarify how host traits, evolutionary history, and environmental acquisition interact to shape microbiota assembly in Neotropical stingless bees.

5. Conclusion

Gut microbiota assembly in stingless bees is structured primarily by host identity, with evolutionary history also contributing to community composition, whereas geographic distance explained a comparatively smaller proportion of the observed variation. This host-associated structuring may reflect the combined influence of host physiology, species-specific ecological traits, and microbial transmission processes within and among colonies. The observed association between host evolutionary history and microbiota composition further suggests that related bee species may retain partially similar microbial communities, although this pattern was not consistent across all lineages. These findings highlight the importance of considering host identity and evolutionary history when investigating microbiota diversity in stingless bees. Given the ecological and conservation importance of several stingless bee species, including threatened taxa such as *M. capixaba*, understanding species-specific microbial associations may provide additional context for biodiversity conservation and host-microbiome interactions.

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Declarations

The authors have no financial or any other kind of competing interest related to this work.

Credit for authorship contribution statement

LLB: Conceptualization, Methodology, Investigation, Validation, Writing – original draft, Writing – review & editing. **MLV:** Investigation, Validation, Formal analysis (Bioinformatic analysis), Writing – review & editing. **JRG:** Validation, Supervision,

Writing – review & editing. **GFM:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Data availability

The raw DNA sequencing reads generated during this study are available in the NCBI SRA repository under BioProject PRJNA1374264. The computational code, scripts, and processed data tables are archived in Zenodo (https://doi.org/10.5281/zenodo.22864094) and are also available via GitHub at: https://github.com/MateusLordelo/Ecological-drivers-of-gut-microbiota-diversity-and-evolution-in-brazilian-stingless-bees. All other supporting data, including ASV tables, taxonomic assignments, and PICRUSt2 functional output, are provided as Supplementary Materials accompanying this article.

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Figures

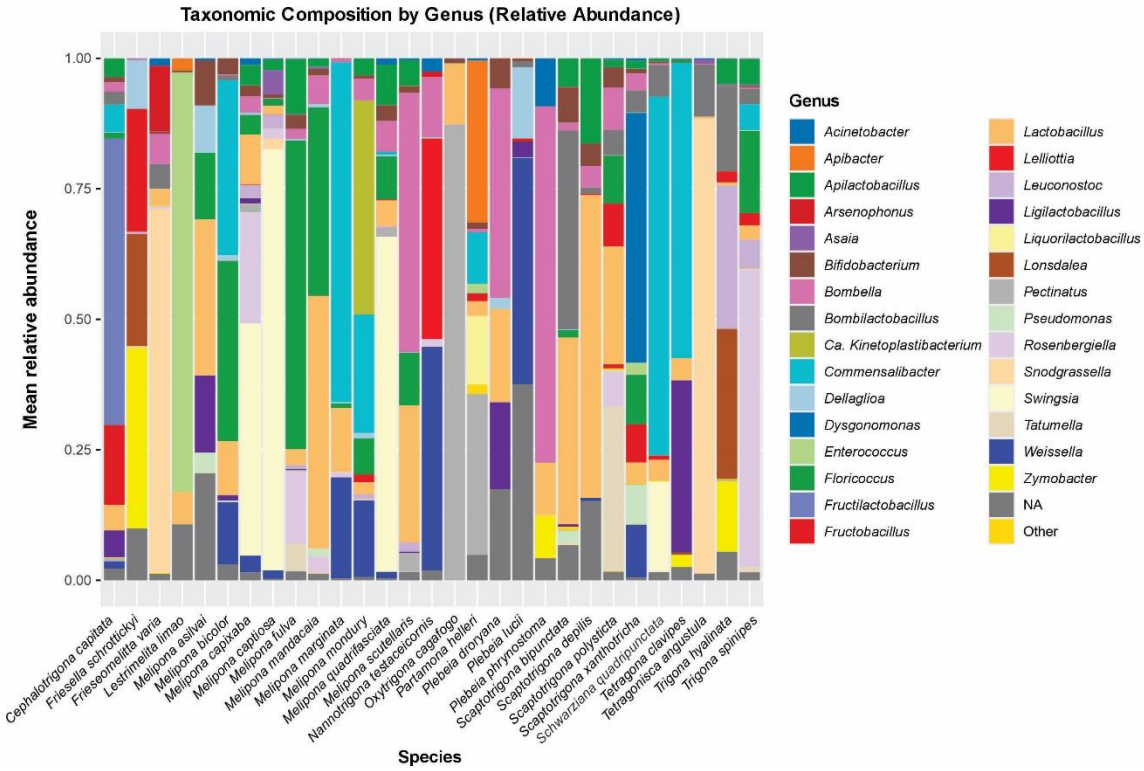


Figure 1. Bacterial composition of the gut microbiota in 29 species of Brazilian stingless bees at the genus level. Stacked bar plots show the mean relative abundance of bacterial genera identified in each bee species' gut microbiota, based on 16S rRNA gene amplicon sequencing. Colors represent different bacterial genera, and the length of each colored segment indicates the relative abundance of that genus within the respective species. Genera whose mean relative abundance was < 0.01 are grouped under “Other”. Only the most abundant genera, covering up to 90% of total relative abundance, are shown. Bee species names are listed on the x-axis, and the corresponding bacterial genera are indicated in the color legend on the right side of the figure.

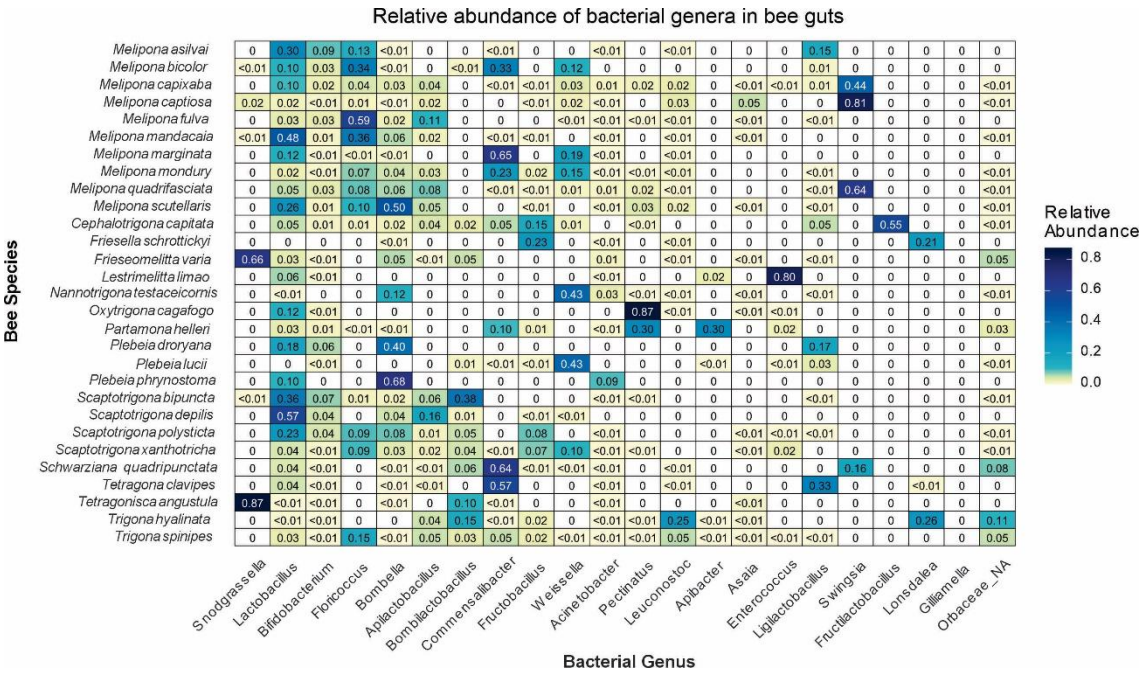


Figure 2. Heatmap showing the relative abundance of the most abundant bacterial genera detected in the gut microbiota of 29 stingless bee species. Taxonomic identification was performed using the SILVA database, and abundance values are based on the relative frequency of 16S rRNA gene sequences (amplicon sequence variants; ASVs) attributed to each genus. Each row represents a bee species, and each column corresponds to a bacterial genus. Color intensity reflects relative abundance: dark blue indicates high relative abundance (up to 0.87), while lighter shades toward yellow represent low (<0.05) or undetectable abundance. Cells marked with "<0.01" indicate genera detected at extremely low relative abundance.

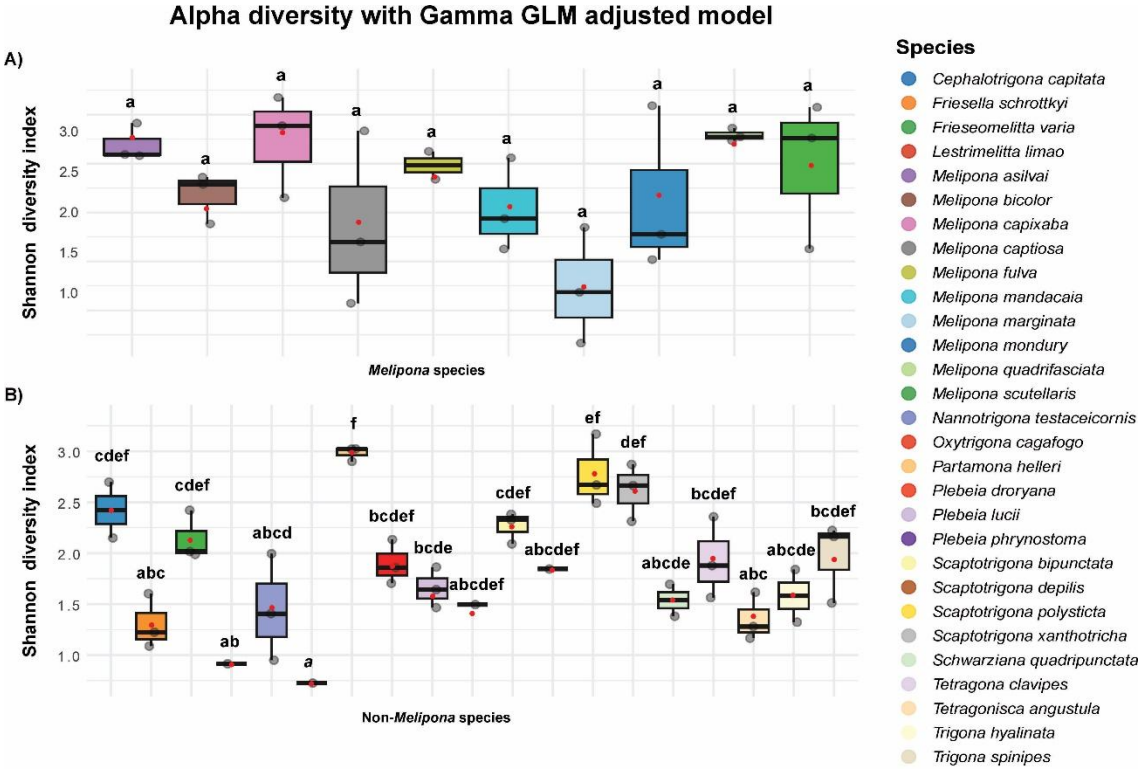


Figure 3. Alpha diversity of the gut microbiota across Brazilian stingless bee species at the bacterial genus level. **A)** *Melipona* species, highlighting intra-genus homogeneity in gut microbiota diversity. **B)** Corresponding diversity patterns for non-*Melipona* stingless bee species. Shannon diversity index values were calculated to assess alpha diversity, incorporating both richness and evenness of bacterial genera. Boxplots denote the median (center line) and interquartile range (IQR; box bounds), with whiskers extending to 1.5 x IQR and semi-transparent jitters representing individual sample distributions. Red dots indicate Estimated Marginal Means (EMMs) derived from a Gamma Generalized Linear Model (GLM) adjusted for log-transformed library size. Overall species effects were evaluated using Type III Wald Chi-Square tests (alpha = 0.05), with pairwise comparisons adjusted using Tukey's method. Distinct lowercase letters indicate statistically significant pairwise differences ($p < 0.05$). Colors correspond to host species as indicated in the legend.

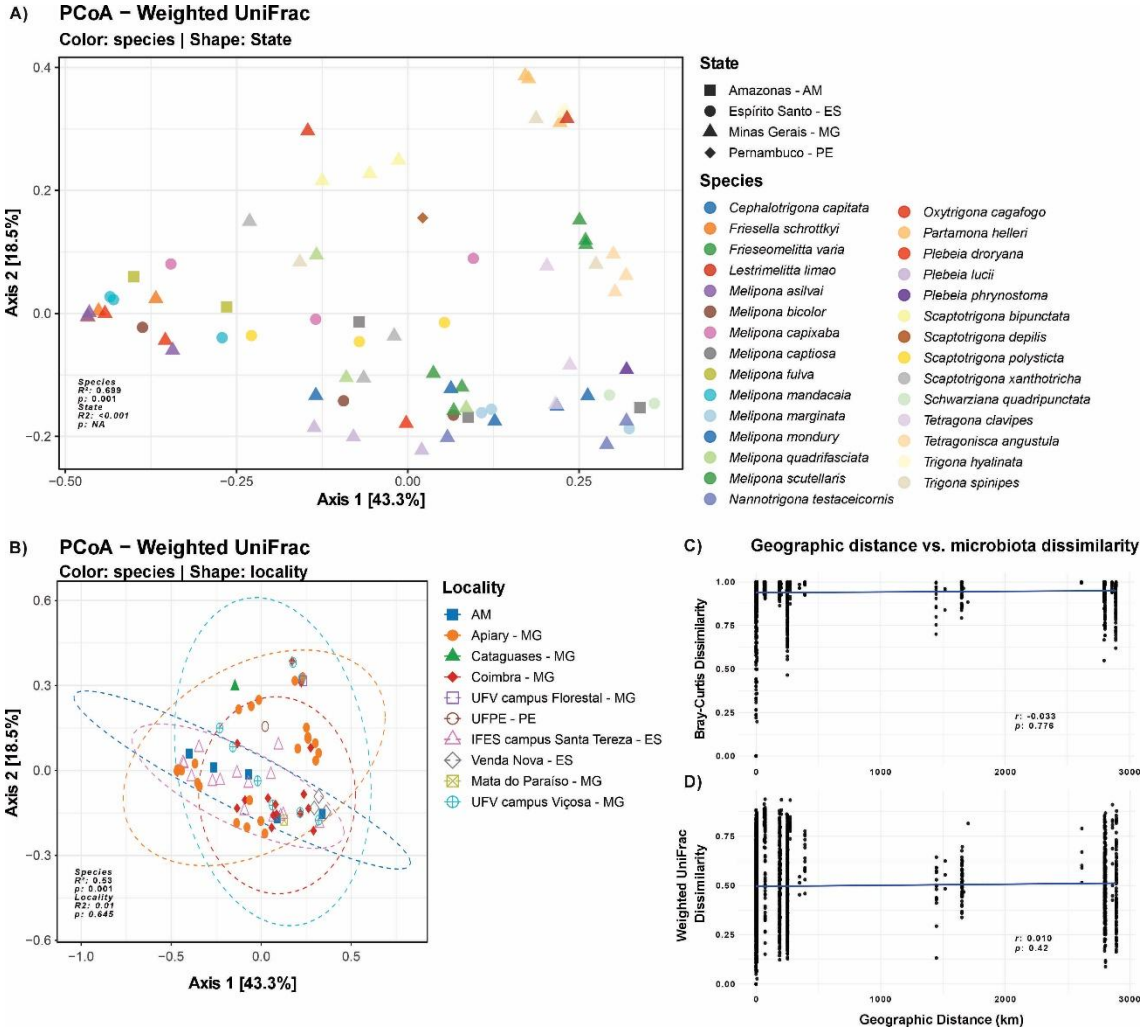


Figure 4. Beta diversity analyses of gut microbiota in stingless bees. **A)** Principal coordinates analysis (PCoA) based on Weighted UniFrac distances, showing differences in gut bacterial community composition among 29 Brazilian stingless bee species. Each point represents a pooled gut sample from a single colony. Symbols indicate the state of origin (AM, ES, MG and PE), and colors represent bee species. **B)** Principal coordinates analysis (PCoA) based on Weighted UniFrac distances, showing the clustering of samples according to sampling location (apiary, UFV campus, Coimbra, Mata do Paraíso, and states AM, ES, and MG). Ellipses represent group confidence intervals. **C)** Relationship between geographic distance (km) and gut microbiome dissimilarity based on Bray-Curtis distances. **D)** Relationship between geographic distance (km) and gut microbiome dissimilarity based on Weighted UniFrac distances. Differences in community composition were significant (PERMANOVA, $p < 0.001$).

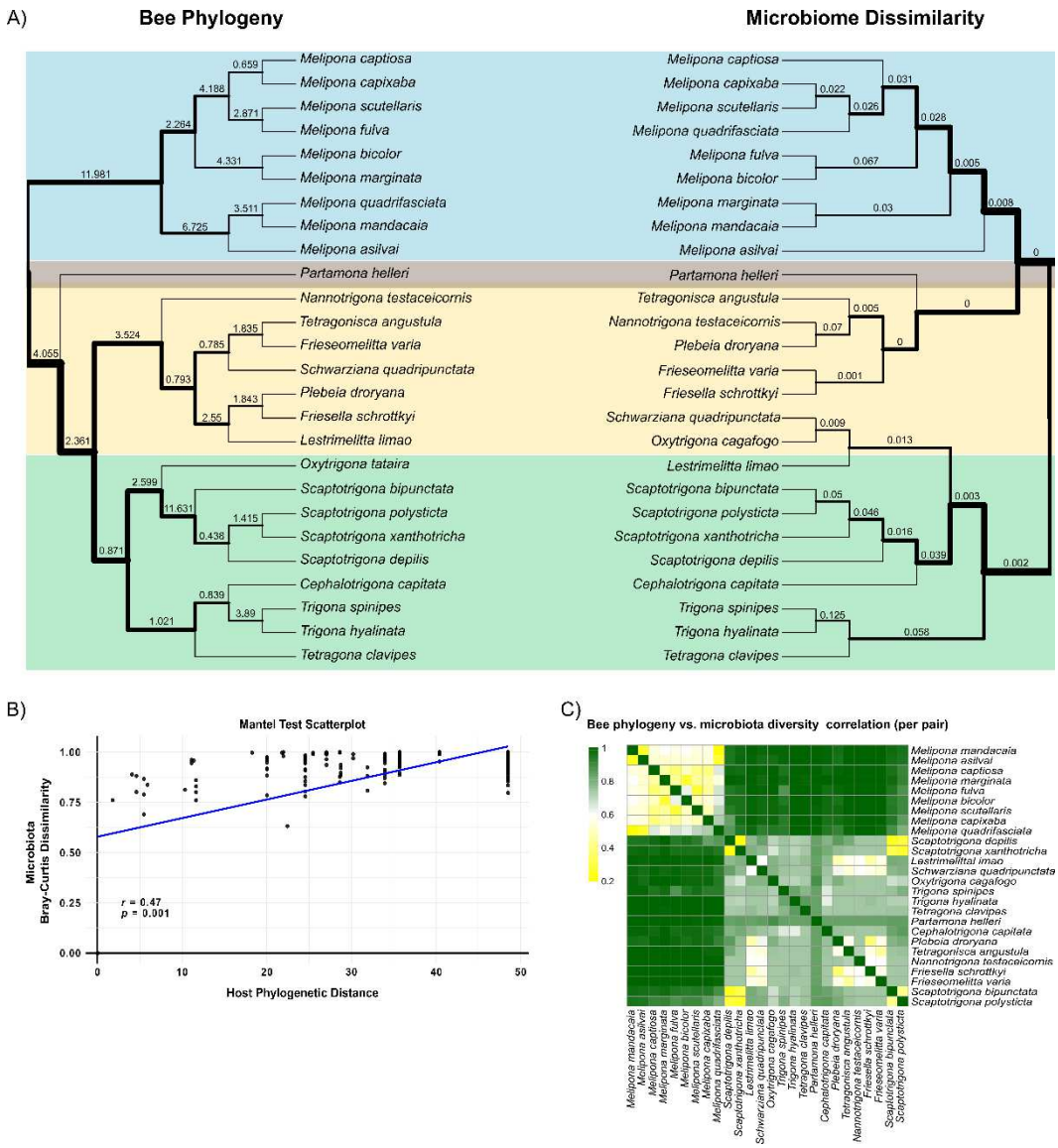


Figure 5. Relationship between bee phylogeny and gut microbiota composition. **A)** Tanglegram comparing the host phylogenetic tree of 29 Brazilian stingless bee species (left) with the dendrogram of gut microbiota dissimilarities (right). The host phylogeny was derived from a time-calibrated chronogram using published molecular data available from the Bee Tree platform. Numeric labels in branches represent branch divergence time in millions of years. Colored backgrounds indicate the major host groups represented in the phylogeny: blue, *Melipona* species; gray, *Partamona helleri*; yellow, other non-*Melipona* species including *Nannotrigona*, *Tetragonisca*, *Frieseomelitta*, *Plebeia*, *Schwarziana*, *Lestrimelitta*, and *Oxytrigona*; and green, species belonging mainly to *Scaptotrigona* and *Trigona*, together with *Cephalotrigona*. The colors are used only to facilitate

visual identification of host groups and do not represent statistical clusters of microbiota composition. The microbiota dissimilarity tree was constructed using hierarchical clustering based on average pairwise Bray-Curtis dissimilarities. Numeric labels on the microbiota branches indicate divergence from a hypothetical ancestor, where values approaching 1.0 indicate total community turnover. To facilitate visual comparison, both dendrograms were untangled (stepwise rotation) and plotted with uniform branch lengths. **B)** Correlation between the host phylogenetic distance matrix (millions of years) and the bacterial Bray-Curtis dissimilarity matrix. Statistical significance was assessed via a Mantel test (999 permutations) using Pearson's product-moment correlation. The regression line represents the linear relationship between host and microbial divergence, with the Mantel statistic and significance level (p = 0.001). **C)** Heatmap illustrating the normalized correspondence between host evolutionary distance and gut microbiota dissimilarity for each species pair. Values represent a similarity index calculated as 1 − |dhost − dmicro|, where d represents the min-max scaled distance for each matrix. Dark green cells (1.00) indicate high phylosymbiotic congruence, in which phylogenetic distances between host species directly correspond to dissimilarity in their gut microbiota. Conversely, yellow cells indicate lower congruence, where host evolutionary distances do not correspond to the degree of microbiota dissimilarity.

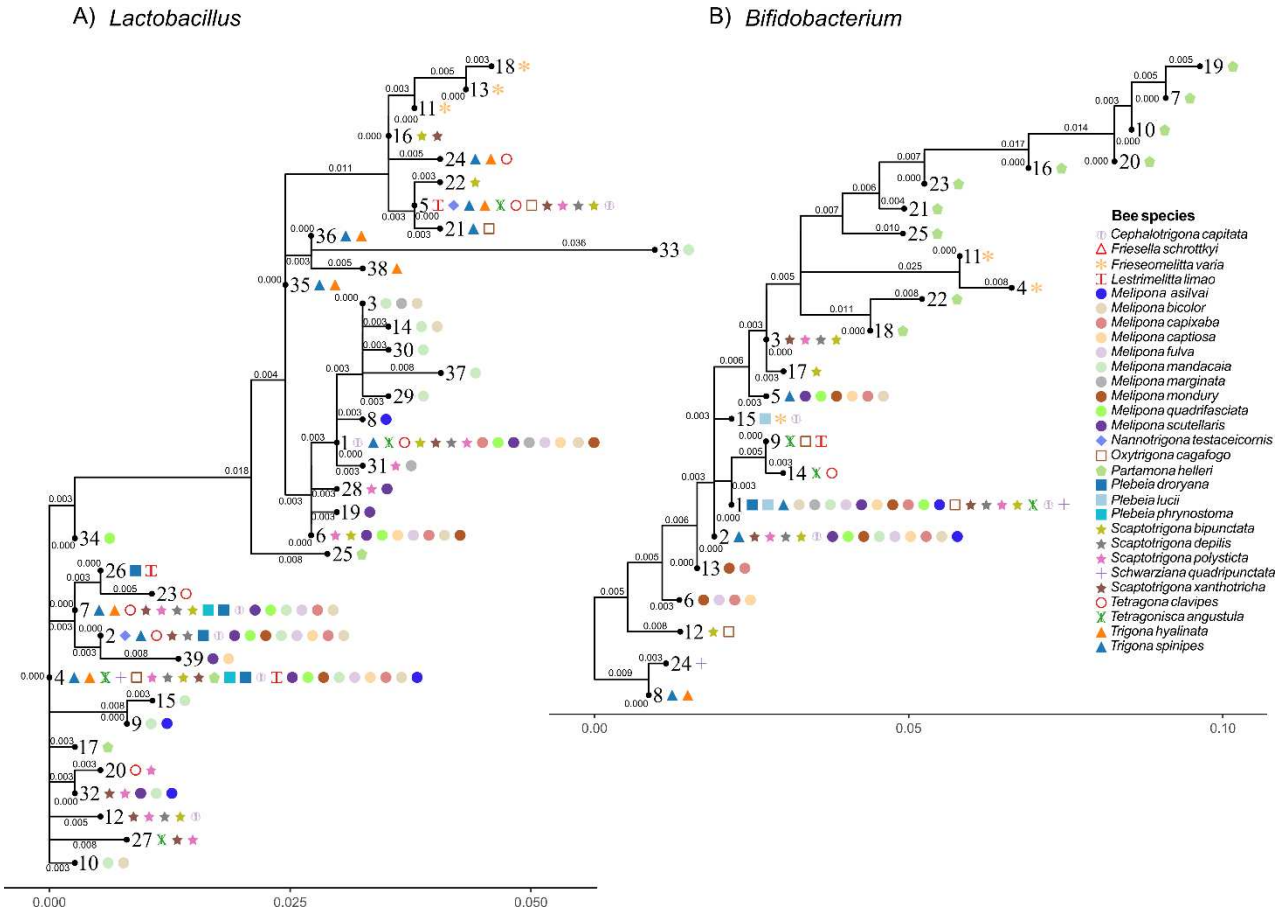


Figure 6. Phylogenetic relationships among amplicon sequence variants (ASVs) obtained through 16S rRNA gene amplicon sequencing and assigned to *Lactobacillus* **(A)** and *Bifidobacterium* **(B)** recovered from the gut microbiota of 29 stingless bee species. Each tip represents a unique ASV, numbered according to its position in the phylogenetic tree. Colored symbols indicate the bee species in which each ASV was detected, while symbol shapes represent bee genera. Branch lengths correspond to nucleotide substitutions per site

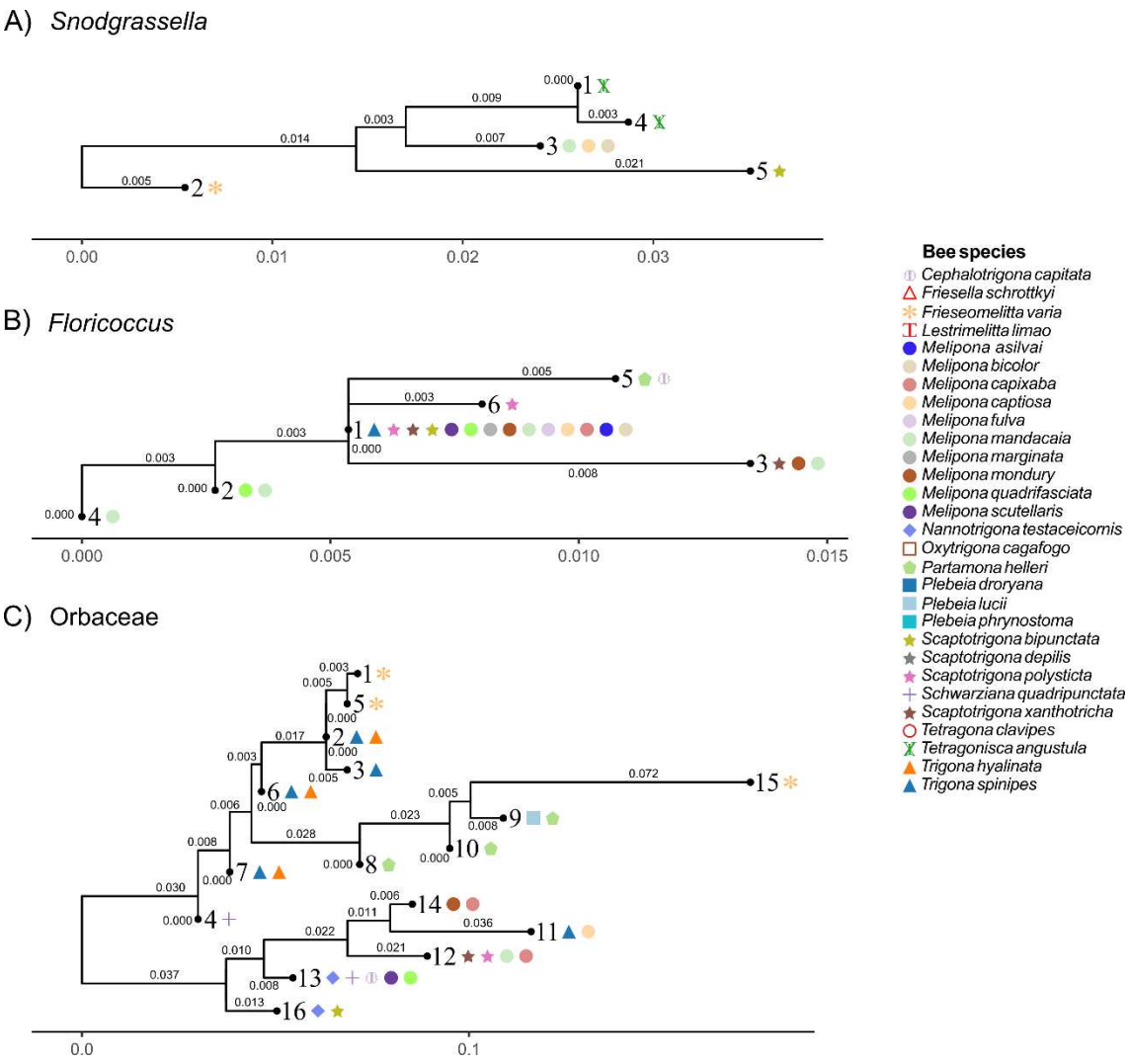


Figure 7. Phylogenetic relationships of *Snodgrassella* **(A)**, *Floricoccus* (**B**), and Orbaceae **(C)** amplicon sequence variants (ASVs) detected in Brazilian stingless bees. Phylogenetic trees were generated from the phylogenetic tree contained in the *phyloseq* object after subsetting ASVs assigned to each bacterial genus. Each terminal node represents a unique ASV and is labeled with a numeric identifier corresponding to its position in the phylogenetic tree. Colored symbols indicate the stingless bee species in which each ASV was detected. Branch lengths represent nucleotide substitutions per site. Trees were generated in R using the packages phyloseq, ggtree, and ggplot2. ASVs assigned to Orbaceae correspond to sequences that remained classified at the family level after taxonomic assignment.

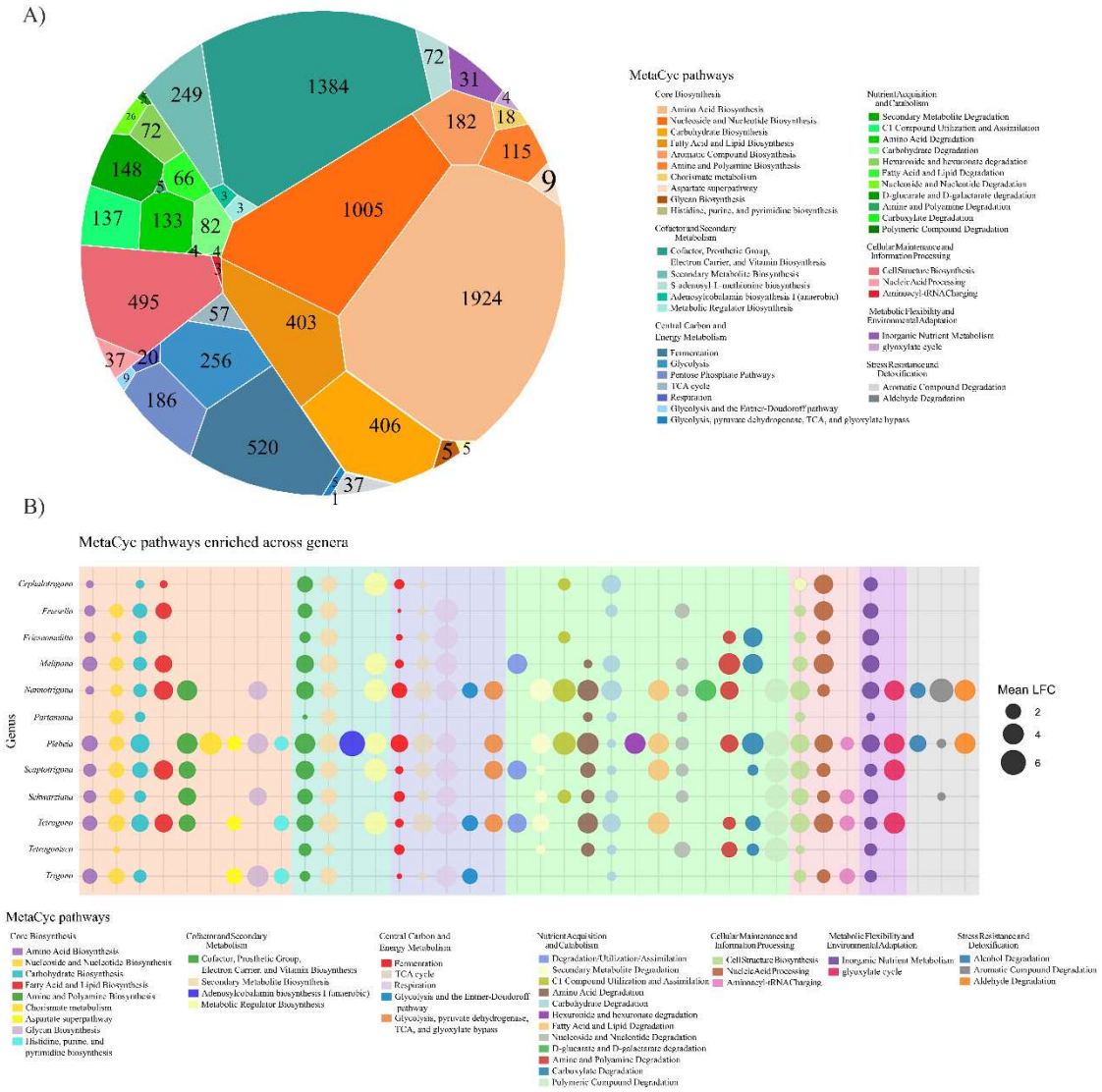


Figure 9. Predicted functional potential of the gut microbiota of Brazilian stingless bees based on MetaCyc pathway inference. **A)** Distribution of predicted MetaCyc pathways grouped into major functional categories inferred using PICRUSt2. Numbers within each segment indicate the number of pathways assigned to each functional category. **B)** Differential enrichment of predicted MetaCyc pathways among stingless bee genera. Bubble size represents the mean log-fold change (LFC) of enriched pathways, while colors correspond to individual MetaCyc pathway categories.