Uncharacterized *Acetilactobacillus*-like genera represent the most abundant microorganisms in honeys produced by native stingless bee

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Abstract

Meliponiculture is deeply rooted in Mexico, especially in the Yucatán Peninsula and Veracruz, where it serves as both an economic activity and a cultural tradition. While *Lactobacillus* species are known to be abundant in the microbiomes of honeybees and their honey, most studies have focused on *Apis mellifera*, leaving stingless bee honey largely unexplored. Here, we report the first shotgun metagenomic analysis of honey from two extensively managed stingless bee species: *Melipona beecheii* and *Scaptotrigona mexicana*. From these samples, we reconstructed 25 metagenome-assembled genomes (MAG), 15 of which showed no match any known species, with average nucleotide identity (ANI) around 79 against its closest relative *Acetilactobacillus jinshanensis*, suggesting they represent novel species. Clustering revealed four distinct groups (intragroup ANI $>$ 99; intergroup ANI $<$ 80), possibly representing four new species across two new genera. Metapangenomic analysis further confirmed that reads did not align to existing *Acetilactobacillus* references, indicating that *Acetilactobacillus*-like microbes are novel and abundant. Given that *Acetilactobacillus* is underrepresented genomically and exhibits a long evolutionary branch within *Lactobacillaceae*, our findings highlight the potential importance of these microbes. Stingless bee honey has been attributed to antioxidant and antibacterial properties, which may be influenced by its microbial content. *Acetilactobacillus*-like bacteria may play key roles in shaping these properties in acidic food microbiomes. Our study provides foundational insights into the unique microbial diversity of stingless bee honey and its potential contributions to honey's chemical and medicinal characteristics.

Key words: Stingless bees, honey microbiome, *Acetilactobacillus jinshanensis*, *Scaptotrigona mexicana*, *Melipona beecheii*

Introduction

Stingless bees' honeys are recognized for their antimicrobial properties [12, 68], therapeutic potential [28, 57], and traditional use in treating ailments [56, 57], including wound healing, respiratory and digestive disorders, and eye conditions. For centuries, indigenous groups have managed stingless bees to produce pot honey [22, 53]. Due to a growing global interest in stingless bees' products, such as honey [12, 55, 68], and propolis [59], beekeeping holds strong potential for promoting family [24] and community economic development [20, 38]. Additionally, stingless bees contribute significantly to ecosystem services, providing pollination of native plants and crops [5, 62] in

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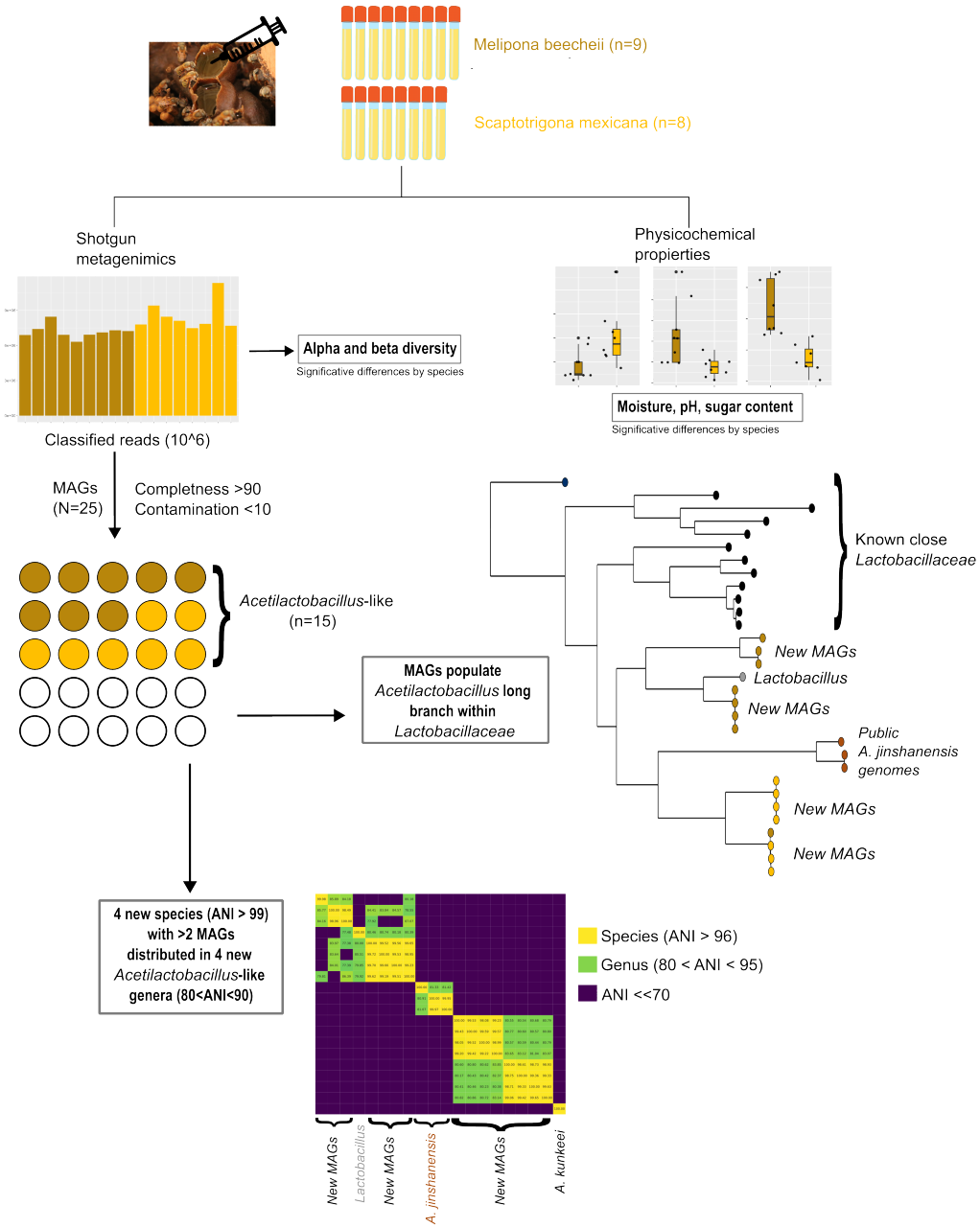

Fig. 0. Physicochemical and microbiological properties separate seventeen honey samples. Fifteen MAGs, reconstructed from shotgun metagenomics, have *Acetilactobacillus jinshanensis* as their closest relative, but phylogenetic reconstruction, metapangenome, and ANI, suggest these MAGs belong to new species and new genera of the Lactobacillaceae family.

tropical regions. Meliponiculture supports local livelihood and safeguards biocultural heritage by integrating traditional ecological knowledge, biodiversity conservation, and cultural identity.

Metagenomics and metabarcoding have transformed bacterial ecology by enabling the characterization of microbial communities and the discovery of uncultivated species [14, 31]. Shotgun metagenomics, when applied to food-associated microbiomes, facilitates the reconstruction of microbial genomes, providing insights into their community composition [13, 75], and metabolic potential [23]. Among food microbiomes, the honey's microbiome recently began to attract attention. However, to date, shotgun metagenomic research has been limited to *Apis mellifera* [9, 10, 18, 58]. In contrast, the microbiota of stingless bees and their honey, also called pot honey, have only been explored by 16S sequencing of isolates [3, 67] and metabarcoding [15]. Despite the advantages of shotgun metagenomics to asses genomic variability [25], and even trace honey origin and authenticity [58], no such studies have been conducted on pot honey, leaving a gap in our understanding of its microbial diversity.

The therapeutic effects of honey [44], and in particular of stingless bee honey [28], have been attributed to the presence of diverse bioactive compounds [69]. However, pot honey microbial communities also exhibit antimicrobial properties [37, 52, 64], making their identification particularly relevant. Honey bee associated microbiomes influence bee health [27, 29, 67]. Bees are known to contain *Lactobacillus* in their gut and surface [46], including Meliponini stingless bees [34]. New species of Lactic Acid *Lactobacillus* were discovered in the honey crop of bees by sequencing 16S on isolates [67]. In honey, *Lactobacillus*, including acid-resistant strains, has been consistently identified as a colonizer in both meliponini honey [6] and honey produced by *A. mellifera* [35, 9, 10, 58].

Here we focus on honey from *Melipona beecheii* and *Scaptotrigona mexicana*, two native species cultivated throughout southeastern Mexico. Although limited, studies in Melipona honey have also identified *Lactobacillaceae* as dominant members of its microbiome through metabarcoding [15]. Among these, one *Lactobacillaceae* reported in *Melipona* honey is *Acetilactobacillus* [15], an underrepresented *Lactobacillus* genus with only one known species [80]. *Acetilactobacillus jinshanensis* was first isolated and consistently found in solid vinegar [17, 75, 79], having a recognized role in fermentation [42]. The isolation of *Lactobacillus sp.* SY-1 from Malaysian stingless bee honey—closely related to *Acetilactobacillus* [65]—and the identification of a metagenome-assembled genome (MAG) corresponding to *A. jinshanensis* brasiliensis stingless bee larval food [60], suggest that the presence of *Acetilactobacillus* might be ubiquitous to stingless bee-associated environments. However, the microbial diversity of stingless bee honey remains largely unexplored.

To address this knowledge gap, we applied shotgun metagenomic sequencing to characterize the bacterial diversity in honey produced by two of the most culturally and economically significant stingless bee species in Mexico: *Melipona beecheii* and *Scaptotrigona mexicana* (Fig. 1). Our analyses revealed a high abundance of *Lactobacillus* in this pot honey. Our reconstructed MAGs share a maximum of 81% average nucleotide identity (ANI) with *Acetilactobacillus* public genomes, indicating a previously uncharacterized diversity of *Acetilactobacillus*-like species in these honeys. These bacteria may contribute to the bioactive properties of pot honey, including its reported antioxidant and antimicrobial activities.

Data availability

Metagenomes are available in the SRA BioProject PRJNA1235305. MAGs were deposited on Zenodo: `https://zenodo.org/records/15265413`. Phylogenetic analysis is available in `https://microreact.org/project/nkkrHqmRqRqsaZCqxazUT5-honey`. The code is available at: `https://github.com/HaydeePeruyero/honey_diversity`. The authors confirm that all supporting data, code, and protocols have been provided in the article or through supplementary data files.

Methods

During the 2022 harvest, we collected 17 honey samples: 8 from *M. beecheii* in the Yucatan Peninsula (Santa Gertrudis, Jose Maria Morelos and Mani) and 9 from *S. mexicana* in Papantla, Veracurz (Aguateno, El Colorado, and La Isla) (Fig. 1a, Table 1). Beekiping methods varies: *S. mexicana*, honey was harvested using modern boxes, blended honey, and traditional pitchers (Fig. 1b); *M. beecheii* using boxes, blend, and log hives "jobones" (Fig. 1c). All samples were physicochemically and microbially analyzed.

Sampling and Analysis Specifications

For each meliponary, the production unit, one to three colonies were sampled. Hives, boxes, or pitchers were opened to collect honey directly from the pot using a sterile syringe, while ensuring proper hygiene and biosafety measures, including the use of a cap, face mask, gloves, and gown. Manual honey extraction (320mL) was performed from one to three hives and distributed into eight sterile, pyrogen-free 50mL tubes. Following collection, samples were land-transported on ice in coolers, maintaining a temperature between 10° and 15°C. Upon arrival at the Centro de Innovación Apícola Sustentable at the Universidad Intercultural Maya de Quintana Roo, samples were stored at −40°C. Honey contains low quantities of environmental DNA per volume, and waxes that can inhibit the polymerase chain reaction (PCR) [7]. In this study, we standardized DNA extraction by condensing the maximum amount of DNA molecules. After the extraction, samples were sequenced (WGS), and metagenomes were subjected to quality control, taxonomic assignment, and binning. Every metagenome contained more than 6×10^7 raw reads (considering both forward and reverse). GC content was, on average, higher in *M. beecheii* ($40.84 \pm 1.08\%$) than in *S. mexicana* ($35.05 \pm 2.46\%$) samples, Table 1.

Physicochemical Analysis of Sampled Honey

Physicochemical parameters analyzed in this study include moisture, color (Pfund scale), hydroxymethylfurfural (HMF), total sugars, electrical conductivity, pH, and ash content. Parameters were measured as described by Xolalpa in [78].

Genomic DNA Extraction and Purification

DNA was extracted and concentrated from 50g of honey into a 1.5mL tube. The pre-treatment step, prior to applying any extraction method, followed the protocols described by [63] and [70]. The Qiagen DNeasy Blood & Tissue Kit protocol was followed after determining the optimal dilution for each honey sample. The extraction used a TissueLyser II Qiagen at 30Hz for four minutes, with 3mm tungsten carbide beads. The procedure includes adding 180μL of buffer ATL and 20μL of Proteinase K (included in the kit), 200μL of ethanol, 650μL of the mixture in a column DNeasy mini spin (included in the kit) and added to the tube collector of 2mL (included in the kit). The honey was centrifuged at 6000g for one minute in a centrifuge 5425 R Eppendorf™, 25μL of buffer AE was added, the mixture was incubated for one minute, and centrifuged at 6000g for one minute two times. DNA was obtained using silica columns, ensuring yields above 20ng/μL, with total extractions exceeding 500ng/μL. DNA purity was assessed using a QIAxpert System Spectrophotometer (Qiagen), with 260/280 absorbance ratios above 1.7, indicating high-quality DNA. Finally, microorganisms were sampled following Syed [65] isolation method.

Library Preparation and Shotgun Metagenomic Sequencing

The seventeen DNA samples extracted from honey (Table 1) were dried using an Eppendorf® Vacuum Concentrator Plus

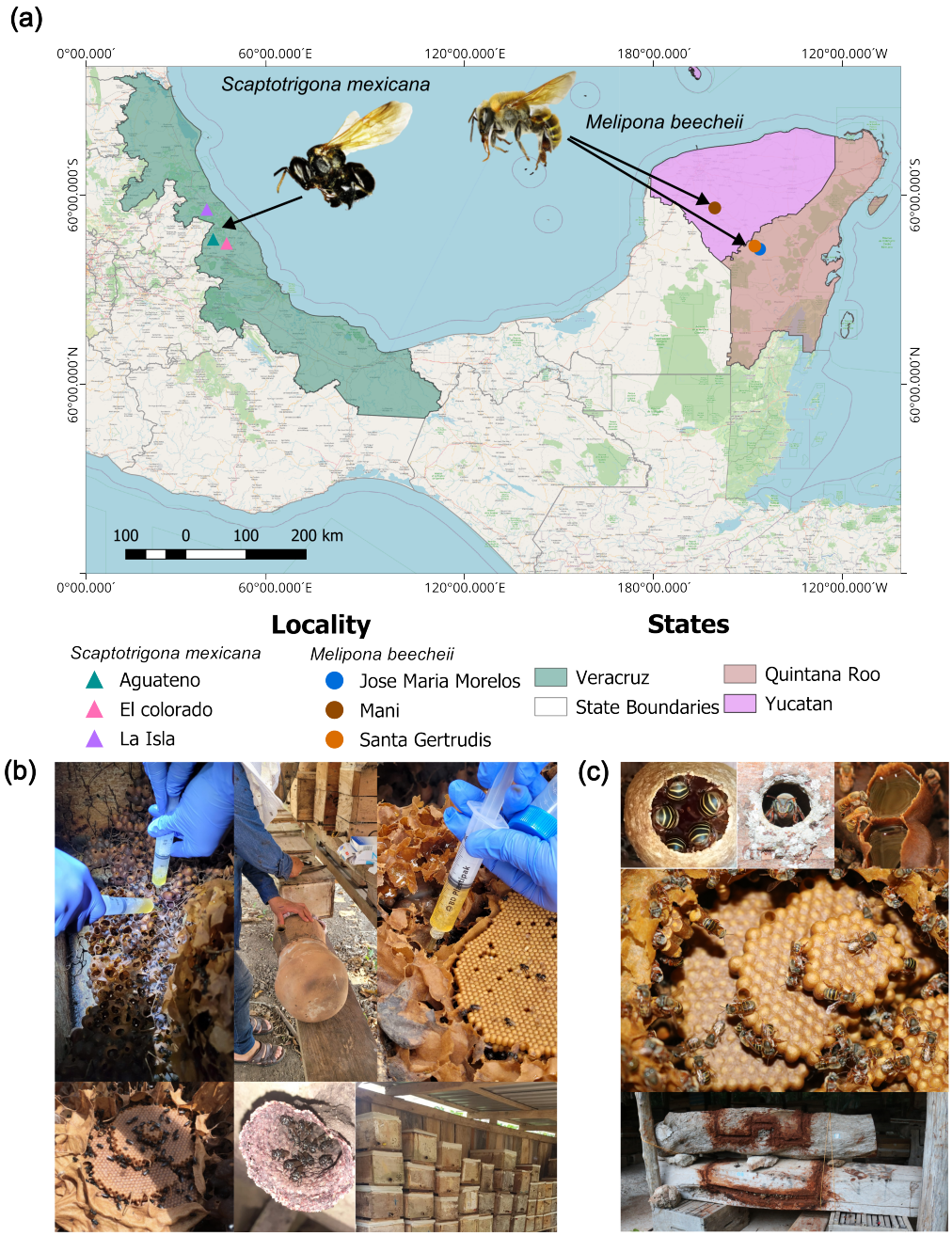

Fig. 1. Honey from *S. mexicana* and *M. beecheii* were collected in Mexico. (a) Samples come from three states and six different localities. (b) Sampling *S. mexicana* honey from different zootechnical managements, including boxes and pitcher hives. (c) *M. beecheii* bees honey samples come from blended honey, beehive boxes, and traditional "jobones".

for one hour to remove ultrapure water through mechanical vacuum centrifugation with heat application. The resulting DNA concentrations ranged from 500 to 1000 ng. Samples were sequenced using shotgun metagenomics in Macrogen, Inc. (South Korea). Library construction used the TruSeq Nano DNA Library Prep Kit Reference Guide 15041110 D (Illumina), with insert sizes of 350 bp. Sequencing was performed on the NovaSeq 6000 platform (Illumina), generating reads with an average size of 150 bp.

Metagenome analysis

From quality control to diversity analysis, we followed "The Carpentries Metagenomics Pipeline", with the only modification being the assembly tool [81]. Reads were processed to filter low-quality nucleotides, followed by taxonomic assignment, binning, gene prediction, and functional annotation. The quality of raw reads was evaluated using FastQC v0.11.9 [4]. To ensure that values remained within a Phred score of 30, low-quality nucleotides and adapters were removed using Trimmomatic v0.39 [8](`SLIDINGWINDOW:25:28`,

Table 1. Origin of honey samples, species, state, and locality, zootechnical management used in native communities in Mexico. Total read content considers both forward and reverse raw reads.

Species Bee	Sample ID	Zootechnical Management	State	Locality	GPS_X	GPS_Y	Read Count (10^7)	GC %
M. beecheii (n=9)								
M. beecheii	Melli_21	Blended honey	Quintana Roo	Santa Gertrudis	19.80	-88.77	6.632	42.0
M. beecheii	Melli_22	Jobon	Quintana Roo	Santa Gertrudis	19.80	-88.77	7.726	41.1
M. beecheii	Melli_39	Beehive box	Quintana Roo	Santa Gertrudis	19.80	-88.77	7.758	42.4
M. beecheii	Melli_40	Beehive box	Quintana Roo	Jose Maria Morelos	19.75	-88.70	7.372	39.8
M. beecheii	Melli_41	Blended honey	Quintana Roo	Jose Maria Morelos	19.75	-88.70	6.655	38.9
M. beecheii	Melli_42	Jobon	Quintana Roo	Jose Maria Morelos	19.75	-88.70	7.043	40.5
M. beecheii	Melli_45	Beehive box	Yucatán	Mani	20.39	-89.39	7.138	40.8
M. beecheii	Melli_46	Jobon	Yucatán	Mani	20.39	-89.39	6.806	41.6
M. beecheii	Melli_47	Blended honey	Yucatán	Mani	20.39	-89.39	6.961	40.5
S. mexicana (n=8)								
S. mexicana	Scapto_43	Traditional Pitcher	Veracruz	Aguateno	19.90	-97.14	7.538	33.3
S. mexicana	Scapto_23	Blended honey	Veracruz	Aguateno	19.90	-97.14	7.009	33.2
S. mexicana	Scapto_51	Beehive box	Veracruz	Aguateno	19.90	-97.14	7.729	34.1
S. mexicana	Scapto_89	Traditional Pitcher	Veracruz	La Isla	20.36	-97.24	7.149	33.9
S. mexicana	Scapto_91	Beehive box	Veracruz	La Isla	20.36	-97.24	7.503	33.9
S. mexicana	Scapto_90	Blended honey	Veracruz	La Isla	20.36	-97.24	7.381	34.4
S. mexicana	Scapto_92	Beehive box	Veracruz	El Colorado	19.84	-96.94	7.739	40.2
S. mexicana	Scapto_93	Blended honey	Veracruz	El Colorado	19.84	-96.94	6.628	37.4

`MINLEN:35`, `ILLUMINACLIP: TruSeq_LT_CD:2:40:15`). **Assembly:** Trimmed reads were assembled with MEGAHIT v1.2.9 [39]. **Taxonomic assignment:** For taxonomy classification, we used Kraken v2.1.2 [74] with the database PlusPFP [73, 36]. We use kraken-biom v.1.2.0 [19] to construct a biom file and an abundance matrix with the Kraken outputs. Bacterial and fungus abundances were integrated into separate abundance tables. **Diversity:** We assessed the honey microbiome using classical metagenomic diversity indices. Beta diversity (Bray-Curtis distance) was visualized via NMDS, revealing between-sample variation. Alpha diversity, reflecting within-sample richness, was evaluated with Observed OTUs, Shannon, and Chao1 indices. Shannon accounts for abundance distribution, while Chao1 estimates unseen diversity from rare OTUs. Diversity and abundance plots were generated in R v4.4.2 using `phyloseq` v1.50.0 [47].

Metagenome-Assembled Genomes

Binning and quality control: We obtained MAGs with MaxBin v2.2 [76] and assess their quality in terms of completeness and contamination with CheckM v1.2.1 [54]. **MAGs taxonomic assignment:** For each MAG we find with the closest genome available in the Genome Taxonomy Database (GTDB) with GTDB-Tk v2.1.1 [16] **Phylogenetic reconstruction:** A pangenome was calculated using Anvi'o v7.60 [26] for our 15 MAGs and 16 publicly available close *Lactobacillus* and *Acetilactobacillus* genomes. Then, 59 core single-copy protein families (`anvi-get-sequences-for-gene-clusters` setting to one the minimum and maximum number of genes per genome, and then concatenating the resulting sequences). Phylogenetic tree was reconstructed using `anvi-gen-phylogenomic-tree`. The best evolutionary model was automatically selected (`-m MFP`) in IQ-TREE v2.1.4-beta[48] and ultrafast Bootstrap support was applied with 1000 replicates (`-bb 1000`). Finally, an approximate likelihood ratio test (`-alrt 1000`) was conducted.

Metapangenome

A metapangenome maps reads from metagenomes to a pangenome[66, 21]. All *Acetilactobacillus*-like MAGs (n=15, 8 from *M. beecheii* and 7 *S. mexicana*), one *A. kunkeei*, *Lactobacillus* sp. SY-1, and all available *A. jinshanensis* genomes (n=3) were included in this analysis. Initially, a genome database was created using anvi'o v.8 [26]. Open reading frames (ORFs) were identified via Prodigal v2.6.3[32]. Gene families were calculated from the genome database using `anvi-pan-genome -ncbi-blast --mcl-inflation=10`. Honey metagenomes were mapped to MAGs and genomes with bowtie2 [36] with default parameters (`--sensitive`). **ANI:** For each pair of MAGs or genomes considered in the metapangenome we computed ANI with the FastANI v1.34 [33]. Results were visualized in a heatmap using Python v3.9.19 packages `Seaborn` v0.13.2 and `Matplotlib` v3.9.0.

Results

Honey samples from *M. beecheii* and *S. mexicana* differed in their physicochemical properties and their microbial communities.

Samples were grouped based on their bee species of origin, enabling a comparative analysis of physicochemical and microbiological properties to determine whether species-specific characteristics influence honey bacterial communities. We found significant differences only in three of the six features tested: pH, moisture content and total sugar concentration. The pH of stingless bee honey has been reported to range between 3.5 and 5.5 [7]. In this study, we found average pH value of 4.65 ± 0.76 for *M. beecheii* and 3.88 ± 0.23 for *S. mexicana* (Table S1). A Kruskal-Wallis (K-W) test revealed a significant difference between the two species (p-value = 0.01). Honey produced by stingless bees generally has lower total sugar concentrations and higher moisture content than honey from *A. mellifera* [50]. In our study, *M. beecheii* honey

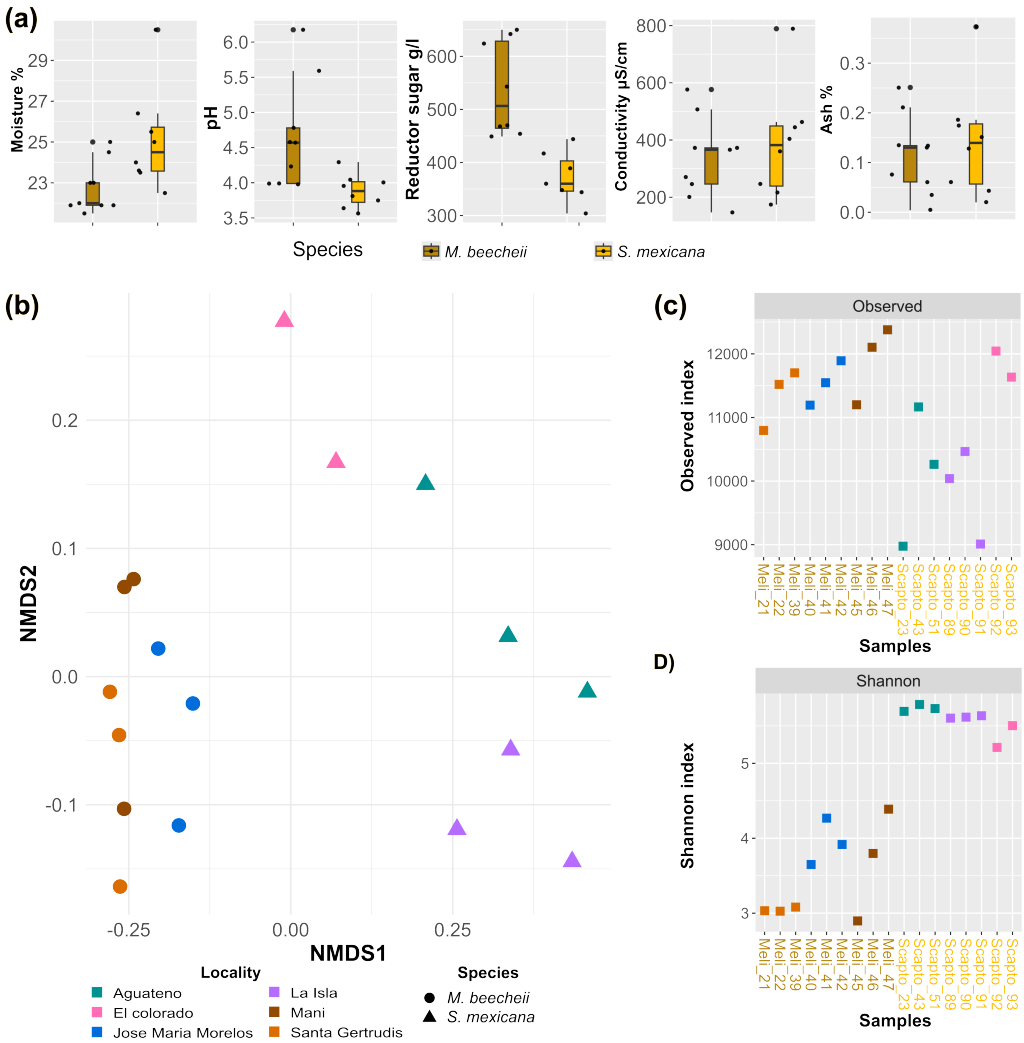

Fig. 2. The diversity of honey's microbial communities is grouped by bee origin. (a) Honey moisture, pH, sugars, conductivity, and ashes are shown in box plots according to species. (b) Species distribution and abundance are linearly separated in beta distance according to bee species. (c) *M. beecheii* has more alpha diversity than *S. mexicana*, measured by the observed index. (d) Contrary to taxonomic units, *S. mexicana* has more alpha diversity when measured by the Shannon Index.

contained an average of 537.58 ± 8.86 g/L of reducing sugars (fructose and glucose), while *S. mexicana* honey had an average of 372.28±47.65 g/L (Fig. 2a, Table S1,S2). A Tukey's multiple comparison test confirmed a statistically significant difference between the species (p-value = 0.0007).

No significant differences were found in electrical conductivity, ash content, color, or hydroxymethylfurfural levels. Organic acids make up approximately 0.5% of honey, with gluconic acid being the primary by-product of glucose enzymatic breakdown [51]. The electrical conductivity of *A. mellifera* honey from Thailand has been estimated at 0.26 ± 0.04 mS/cm [72]. In our study, *M. beecheii* honey had an average electrical conductivity of 339.87 ± 140.03 μS/cm, while *S. mexicana* honey averaged 387.28 ± 194.83 μS/cm (Table S1, S2). An ANOVA test revealed no significant differences between the two species (p-value = 0.56). High electrical conductivity can complicate DNA extraction, likely due to trace minerals in honey (0.17%), with potassium being the most abundant [7, 51]. The mean ash content was 0.11 ± 0.08% for *M. beecheii* and 0.14 ± 0.11% for *S.mexicana* (ANOVA, p-value = 0.56). In terms of colorimetry, the average values were 48.05±8.9 for *M. beecheii* and 46.12 ± 16.35 for *S. mexicana* (K-W, p-value = 0.77). Lastly, HMF content averaged 2.02±1.24 for *M. beecheii* and 1.87 ± 2.20 for *S. mexicana* (K-W, p-value = 0.47).

When evaluating beta diversity, microbial communities formed two distinct clusters based on the bee species producing the honey (Fig. 2b). The first cluster comprised samples from *M. beecheii* honey, whereas the second, which exhibited greater dispersion, included microbial communities from *S. mexicana* honey. Differences are statistically significant between the microbial communities of honey produced by the two bee species (PERMANOVA pseudo-F = 42.362, p-value = 0.0009, 1000 permutations)suggesting that bee species play a key role in shaping honey-associated microbiomes (Fig. 2b). In contrast, the zootechnical management does not seem relevant for clustering when samples are compared by beta diversity (Fig. S1a). Nevertheless, bee species are correlated with geographical distribution. Samples of *M. beecheii* honey were collected in Quintana Roo and Yucatán, whereas those of *S. mexicana* originated from the State of Veracruz. With these

data, it is impossible to determine if the differences are due to the samples' geographical location or the bee species. Due to the significant differences in pH, moisture, and sugar content, it may be some of these variables which influence microbiome differences.

The observed diversity was higher in *M. beecheii* honey samples (Fig. 1c), whereas the Shannon index was higher in *S. mexicana*, (Fig. 2d). These results implied that OTU abundances are more evenly distributed among *S. mexicana* samples. Alpha diversity measured with the Chao1 index is higher in the metagenomes from honey produced by *M. beecheii*, (Fig. S1b). Alpha diversity was found to vary significantly across all three indices: observed (Welch t-test, p-value=0.025), Shannon (Kruskal-Wallis, p-value=0.005), and Chao1 (ANOVA followed by a post hoc Tukey test, p-value=0.0015). We observed significant differences between honey from both species only in terms of three physicochemical variables: moisture content, pH, and reducing sugar content. In contrast, microbiologically, all the tests conducted on Alpha and Beta diversity indicated significant differences between the species.

Potentially new species of *Acetilactobacillus* are consistently found in the honey microbiome

After quality control, the reads were taxonomically assigned using Kraken2 with a database that includes plants, fungi, viruses, and bacteria. On average, most reads remained unclassified (68.80%), although we managed to classify $> 10^6$ reads in most samples, (Fig. S2). Classified reads were distributed as follows: 76.51%, to Bacteria; 20.52%, to Eukaryota; 2.82%, to Viruses; and 0.14%, to Archaea (Fig. 3a). Bacillota is the most abundant bacterial phylum followed by Pseudomonadota (Fig. S3a). The fungal reads were distributed across four phyla, with *Ascomycota* being the most abundant (83.46% in *M. beecheii* and 67.41% in *S. mexicana*), followed by *Mucoromycota* (9.39% and 16.18% respectively). In contrast, the remaining reads belonged to *Basidiomycota* (6.21% and 13.43%) and *Microsporidia* (0.92% and 2.96%), (Fig. S3b). The 17 most abundant genera are shown in (Fig. 3b). Genera from phylum *Ascomycota* are represented in shades of blue, those from *Basidiomycota* in green, *Microsporidia* in brown, and *Mucoromycota* in purple (Fig. 3b). Most Eukaryota reads belonged to plants, whereas fungal reads represented, on average, 0.46% of the data (Fig. S4).

Overall, the results showed that bacteria predominated in the microbial community of both types of honey, especially the *Lactobacillaceae* family. Therefore, we focused on bacterial communities (Fig. 3c). Genus with an abundance $> 5\%$ included *Acetilactobacillus*, *Apilactobacillus*, *Fructobacillus*, *Lactobacillus*, and *Nicoliella* within *Lactobacillaceae* family from the Bacillota Phylum, and *Pantoea* and *Tatumella* from Pseudomonadota. In *M. beecheii* honey, most bacterial reads were mainly classified as *Acetilactobacillus* (52.72% of the total reads), whereas in *S. mexicana* honey, reads were more frequently classified as *Apilactobacillus* (9.77%), followed by *Lactobacillus* (4.75%). Microorganisms from *Apilactobacillus* genus have been previously identified in flowers, honey bees, and hives [49].

Bacterial MAGs were assembled from reads after binning the samples. MAGs were filtered, retaining only those with completeness higher than 90% and contamination lower than 15%. Twenty-five MAGs from samples of both bees survived this filtering procedure. Among these, only seven were high-quality MAGs with completeness $> 95\%$ and contamination $< 5\%$. Thus, we searched for the closest known genome of each MAG by contrasting them against the Genome Taxonomy Database (GTDB) (Table 2). Results included *Rosenbergiella*, *Fructobacillus*, *Bombella*, *Pantoea*, *Acetobacter*, and *Acetilactobacillus* (Fig. 3d).

Interestingly, the fifteen MAGs having *A. jinshanensis* HSLZ-75 [79] as their closest GTDB match had a maximum ANI of 79.07%. In 2019, the *Lactobacillaceae* family was reclassified [80], showing species ANI rounding thresholds above 95%, whereas most genera range between $70 - 90\%$ of average intra-genus ANI similarity. Given these facts, an ANI of 79.07% suggests a new species or genus *Acetilactobacillus*-like is consistently present in both honeys microbial communities.

The genus *Acetilactobacillus* currently contains only one recognized species, *A. jinshanensis*, which is phylogenetically distinct from other Lactobacillaceae genera. To explore the phylogenetic relationships of our MAGs we searched the ten closest relatives of *A. jinshanensis*, as identified in the Lactobacillaceae reclassification study, and included *L. senioris* as an outgroup [80]. To identify publicly available genomes related to *Acetilactobacillus*, we searched NCBI and found three genomes: HSLZ-75 the reference genome of *A. jinshanensis*, originally isolated from solid-state vinegar [79]; *A. jinshanensis* subsp. *aerogenes*, also from vinegar [71]; and *A. jinshanensis* brasiliensis, a MAG recovered from bee larval food [60]. Additionally, a *Lactobacillus* sp. Sy-1 strain isolated from fresh Malaysian honey from *Heterotrigona itama* was identified as phylogenetically related to *Acetilactobacillus*, although its closest relative is *Apilactobacillus micheneri* with an ANI of only 69.33% [65]. Notably, both *A. jinshanensis* brasiliensis and *Lactobacillus* sp. Sy-1 are associated with bees or honey. Using our *Acetilactobacillus*-like MAGs, the 11 closest *Lactobacillus* relatives (Table S3), *Lactobacillus* sp. Sy-1, and the available *A. jinshanensis* genome sequences, we constructed a multilocus phylogeny, (Fig. 4). The tree clearly separated *Acetilactobacillus*-related sequences—including our MAGs—from other *Lactobacillaceae* members. Interestingly, *Lactobacillus* sp. Sy-1 is located in a clade separated from *A. jinshanensis*, and clustered with three of our *Acetilactobacillus*-like MAGs. Since the tree shows variation in our MAGs we refined population studies in the following section by constructing a metapangenome and by calculating FastANI distances between *Acetilactobacillus*-like MAGs and publicly available genomes related to *Acetilactobacillus*.

To determine whether the obtained *Acetilactobacillus*-like MAGs were represented in the metagenomic reads and to understand their population structure, we calculated a metapangenome [66, 21]. This approach assesses whether metagenomic reads map to a set of genomes while simultaneously comparing gene families across those genomes. In the initial phase of the pangenome analysis, gene families were identified and their distribution across genomes was systematically examined to assess shared genetic diversity. Our analysis included the fifteen *Acetilactobacillus*-like MAGs from *S. mexicana* and *M. beecheii* honey samples, all genomes related to *Acetilactobacillus* as described in the previous section, and one *A. kunkeei* genome as outgroups (Fig. 5). *Acetilactobacillus*-like MAGs were detected in twelve out of the seventeen metagenomic samples, with samples 45_Melli, 89_Scapto, and 92_Scapto each harboring two MAGs. In the pangenome visualization, each circle represents a MAG or genome, while each radius corresponds to a gene family. The bottom-right quadrant highlights the core genome, comprising

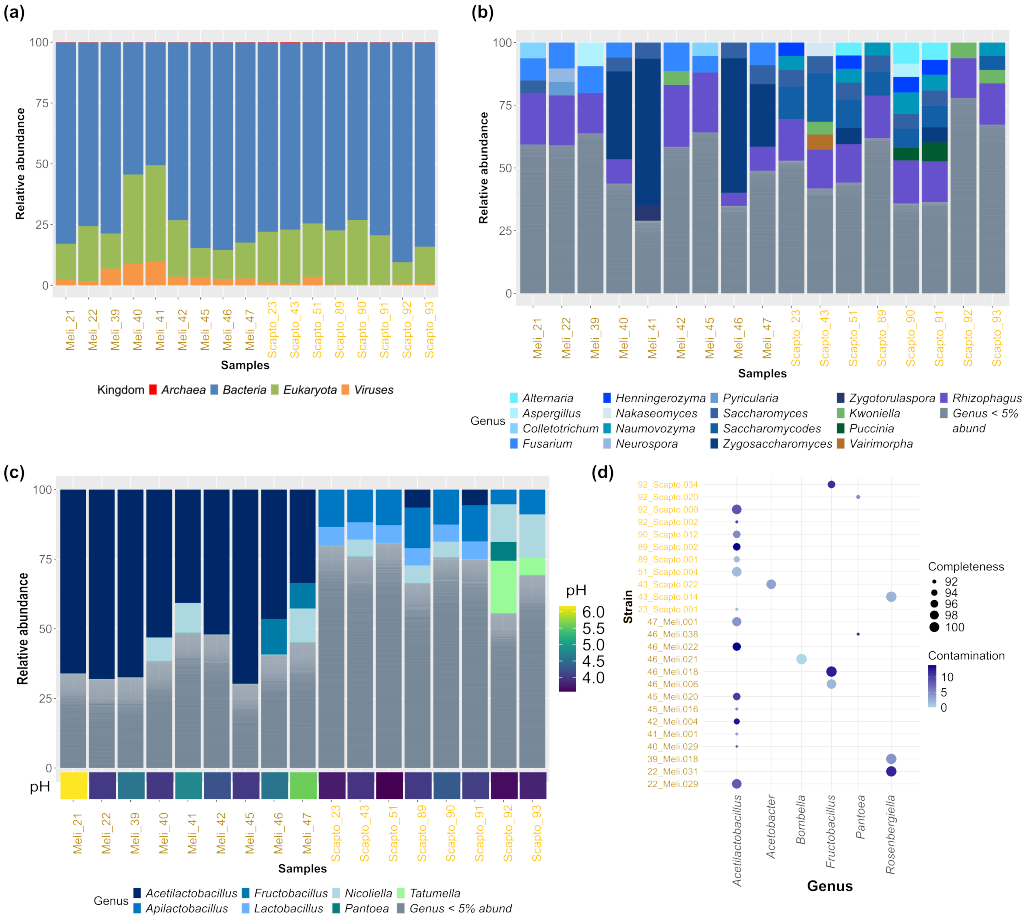

Fig. 3. *Acetillactobacillus*-like lineage is consistently found in honey microbial communities. (a) Most classified reads are from the kingdoms of bacteria, followed by Eukaryota, and viruses. (b) Distribution of fungal genera, whereby those above 5% belonging to phyla Ascomycota are shown in blue, those belonging to Basidiomycota in green, those belonging to Microsporidia in brown, and those corresponding to Mucoromycota in purple. (c) Bacterial reads above 5% comprise the genera *Acetilactobacillus*, *Apilactobacillus*, *Fructobacillus*, *Lactobacillus*, and *Nicoliella*, from the family *Lactobacillaceae* within the Bacillota phylum (blue), and genera *Tatumella* and *Pantoea* within the Pseudomonadota phylum (green). (d) Twenty-five MAGs were recovered with more than 90% of completeness and less than 10% of contamination. Its closest genus, according to the GTDB, is on the x-axis, *Acetilactobacillus*

conserved gene families shared across all genomes, whereas the left half of the figure displays variable gene families. Based on gene family conservation, our nineteen MAGs/genomes clustered into six groups: four of them were composed of our MAGs, another group contained the publicly available *A. jinshanensis* genomes, whereas *A. kunkeei* remained as a singleton in the sixth clade. The square in the right upper quadrant of the circle results from mapping all reads in the metagenomes to each genome in the pangenome. For each metagenomic sample and each genome, there is a bar representing the proportion of the genome covered by the reads in that metagenome. We observed reads from *M. beecheii* metagenomes honey map to the first and a lesser extent, to the second group, whereas they barely mapped to the fourth group and only mapped to one MAG in the third group from the 42_Melli sample. Conversely, *S. mexicana* honey was mapped to the fourth group and, in lesser amounts, to the third group. *A. kunkeei* showed very little proportion of its genome covered by the reads, possibly due to the gene families shared with the other genomes or to a very low presence of *A. kunkeei* in the samples. Among the genomes classified as *A. jinshanensis*, only the *A. jinshanensis* brasiliensis [60] is covered by reads from *M. beecheii* honey. In contrast, the other *A. jinshanensis* were covered by *S. mexicana* reads and had a very low coverage from *M. beecheii* samples.

The difference in coverage in the genomes from *A. jinshanensis* brasiliensis and the other two *A. jinshanensis* genomes raised the question of whether they belong to the same species. Thus, we calculated ANI values for each pair of genomes in the pangenome, (Fig. 6). Values above 98% are shown in yellow in (Fig. 6), and suggest that the pair of genomes under comparison belong to the same species. *A. jinshanensis* brasiliensis only showed a yellow square with itself in the figure, suggesting that it is the only representative of its species. The closests relatives to *A. jinshanensis* brasiliensis were *A. jinshanensis* HSLZ75 (81.07% ANI) and *A. jinshanensis* subsp. *aerogenes* (80.91% ANI), whereas HSLZ75 and aerogenes had an average ANI of 99.96% between them, reinforcing that *A. jinshanensis* brasiliensis should be reclassified. *Lactobacillus* sp Sy-1 also only showed a yellow square with itself, indicating that it is the only representative of its species. Finally, the green squares shared with group 1 and group 2 suggest that the isolate sp Sy-1 could belong to the same genus of MAGs in group 1 and/or group 2.

Table 2. MAGs classification, contamination and completeness.

Genome ID	Completeness	Contamination	Size (Mbp)	ANI to Closest GTDB Genome
22_Melli.029	98.28	8.23	1.9	77.49 (*A. jinshanensis HSLZ-75*)
45_Melli.020	94.83	10.34	1.9	75.98 (*A. jinshanensis HSLZ-75*)
47_Melli.001*	97.41	4.31	2.0	77.02 (*A. jinshanensis HSLZ-75*)
41_Melli.001	91.38	3.45	1.4	79.07 (*A. jinshanensis HSLZ-75*)
45_Melli.016	91.38	5.33	1.7	77.10 (*A. jinshanensis HSLZ-75*)
40_Melli.029	91.35	8.62	1,7	75.81 (*A. jinshanensis HSLZ-75*)
46_Melli.022	95.98	13.89	3.9	76.82 (*A. jinshanensis HSLZ-75*)
89_Scapto.002	94.83	13.79	1.3	77.82 (*A. jinshanensis HSLZ-75*)
51_Scapto.004*	98.28	1.72	1.3	76.79 (*A. jinshanensis HSLZ-75*)
92_Scapto.009	98.28	8.62	2.3	77.50 (*A. jinshanensis HSLZ-75*)
90_Scapto.012	94.83	5.17	2.7	76.76 (*A. jinshanensis HSLZ-75*)
23_Scapto.001	91.38	1.72	1.0	76.53 (*A. jinshanensis HSLZ-75*)
89_Scapto.001	93.26	1.72	1.1	76.06 (*A. jinshanensis HSLZ-75*)
92_Scapto.002	91.38	10.16	1.1	76.68 (*A. jinshanensis HSLZ-75*)
42_Melli.004	93.10	12.93	1.7	78.34 (*A. jinshanensis HSLZ-75*)
22_Melli.031	99.69	11.99	3.6	— (*Rosenbergiella*)
39_Melli.018*	99.69	4.36	3.2	— (*Rosenbergiella*)
43_Scapto.014*	99.84	2.30	3.5	96.67 (*Rosenbergiella epipactidis*)
46_Melli.006*	98.28	2.04	1.6	92.55 (*Fructobacillus sp001038455*)
46_Melli.018	100.00	12.38	3.2	— (*Fructobacillus*)
92_Scapto.034	94.83	11.29	1.4	— (*Fructobacillus*)
46_Melli.021*	100.00	0	2.2	— (*Bombella*)
46_Melli.038	91.38	11.76	5.6	98.07 (*Pantoea stewartii*)
43_Scapto.022*	98.28	3.45	1.8	— (*Acetobacter*)
92_Scapto.020	91.68	4.89	5.0	99.18 (*Pantoea ananatis*)

* Indicates a high quality MAGs with < 5% of contamination and > 95% of completeness.

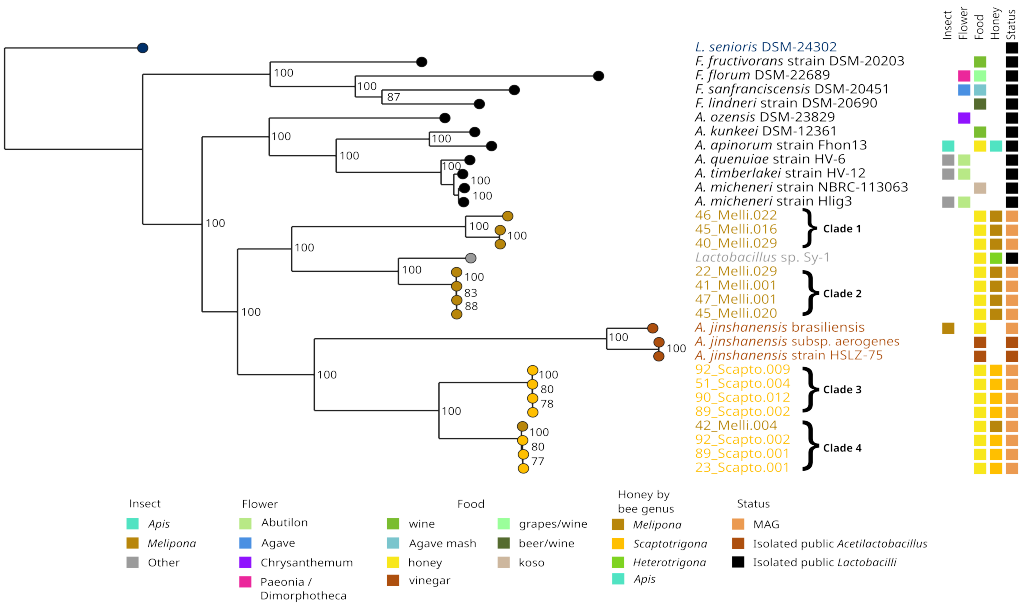

Fig. 4. *Acetilactobacillus*-like MAGs populate the long branch of *A. jinshanensis* found during the genus revision. Multilocus tree with XX concatenated proteins. Metadata shows the isolation source, including flowers and bees, while our MAGs group is enriched with honey isolates.

All *Acetilactobacillus*-like MAGs recovered from honey samples had ANI values below 80% to *A. jinshanensis* genomes, which suggests that they belong to a different genus. In fact, the clustering pattern of our 15 MAGs were the same four groups, regardless of the clustering method being used: gene families' presence/absence or ANI values. These results indicate that regardless of whether the comparison is based on the variable or conserved gene families, these four groups are consistently formed. The first group gathered four MAGs from *M. beecheii*-honey samples with an average ANI of 990.09%. The second group integrated three genomes obtained from *M. beecheii*-honey with an average ANI of 937.2%. The third group

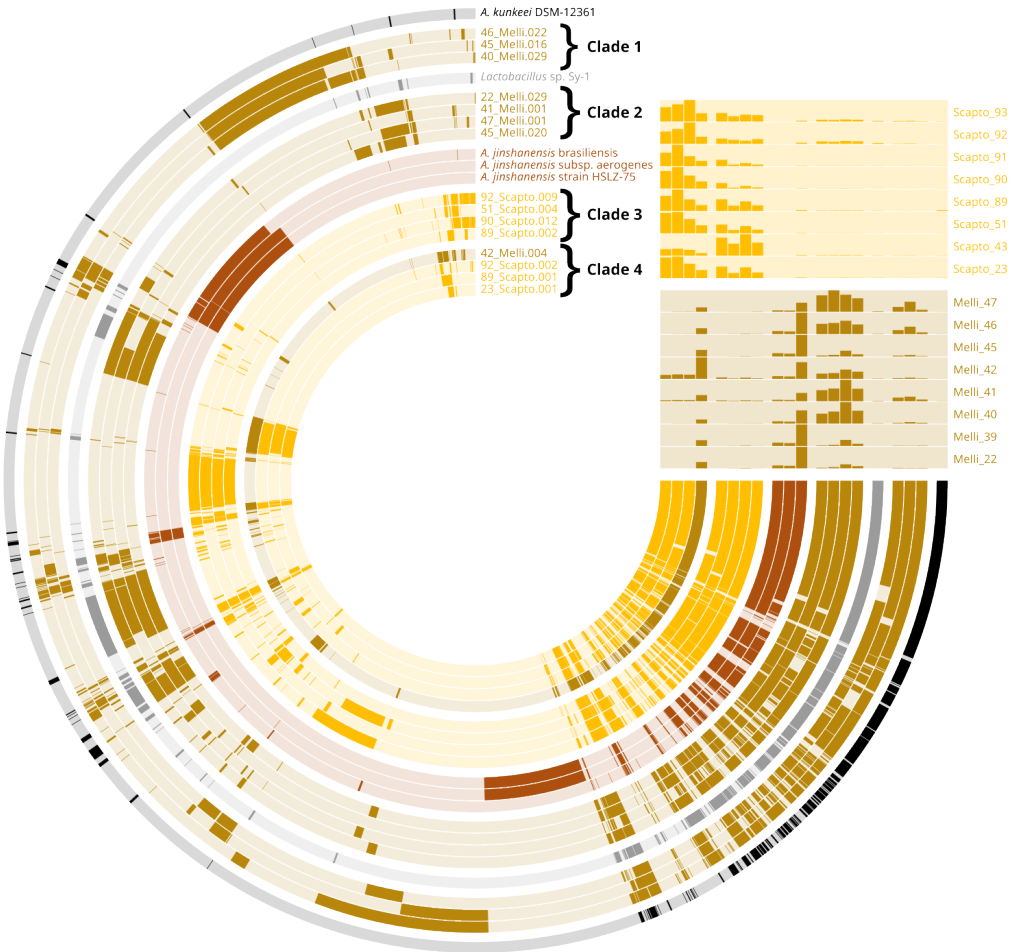

Fig. 5. MAGs close to *Acetilactobacillus* are clustered in four groups that could constitute a new genus. The circles in the pangenome are the fifteen MAGs close to *Acetilactobacillus*, accompanied by *A. jinshanensis* and *A. kunkeei* as outgroups. The radius on circles represents gene families, with dark colors indicating the presence of the gene in the genome and pale colors indicating gene absence. The presence/absence patterns clustered our MAGs in four groups do not include *A. jinshanensis*. The heatmap in the right upper quarter of the metapangenome has rows of the metagenomic samples and the MAGs and genomes as columns. The bars show each genome's coverage by the metagenome reads.

comprised four MAGs from *S. mexicana*-honey with an average ANI of 990.57%, and the fourth group, from *M. beecheii*-honey, had an average ANI of 990.48% (Table S4). These ANI values suggest that groups 1, 3, and 4 only contain genomes from the same species, whereas only two are from the same species in group 2. Moreover, ANI values are below 80% between groups, and group1 and group 2 together are separated from group 3 and group 4 for more than 70% ANI (Table S5). Taken together, these results suggest that each group contained at least two genomes of the same species and the potential existence of two to four new genera close to *Acetilactobacillus*.

Discussion

The bacterial microbiome in stingless bee honey is dominated by *Lactobacillus*

Indeed, the physicochemical parameters of honey varied between species (*M. beecheii* and *S. mexicana*). High moisture content can cause the growth of yeasts and filamentous fungi, leading to fermentation [1], whereas lower levels are directly associated with extended shelf life [61]. In *A. mellifera* honey, the average moisture content has been reported at 18.3% [11] while in stingless bees honey, moisture levels range from 23.4 to 36.4% [2, 45, 78, 50]. In our samples, the average moisture content was 22.75 ± 1.25% for *M. beecheii* vs 25.12 ± 2.5% for *S. mexicana* (Fig. 2a). A Kruskal-Wallis (K-W) confirmed that this difference was statistically significant between the two species ($p = 0.01$, $\alpha = 0.05$).

The bee microbiome has been extensively studied, followed by research on the honey microbiome of *A. mellifera*. In contrast, the microbiome of stingless bee honey has received significantly less attention and has primarily been characterized through 16S amplicon-based studies. Our approach contributes 17 additional shotgun metagenomes, further supporting previous findings that *Lactobacillus* dominates the bacterial microbiome, whereas the fungal community is more variable. Our results suggest that honey microbiomes can be distinguished by the bee species producing them. However, since our samples came from different locations, this variation could also be influenced by differences in the plants they forage on rather than the bee species. Zootechnical management does

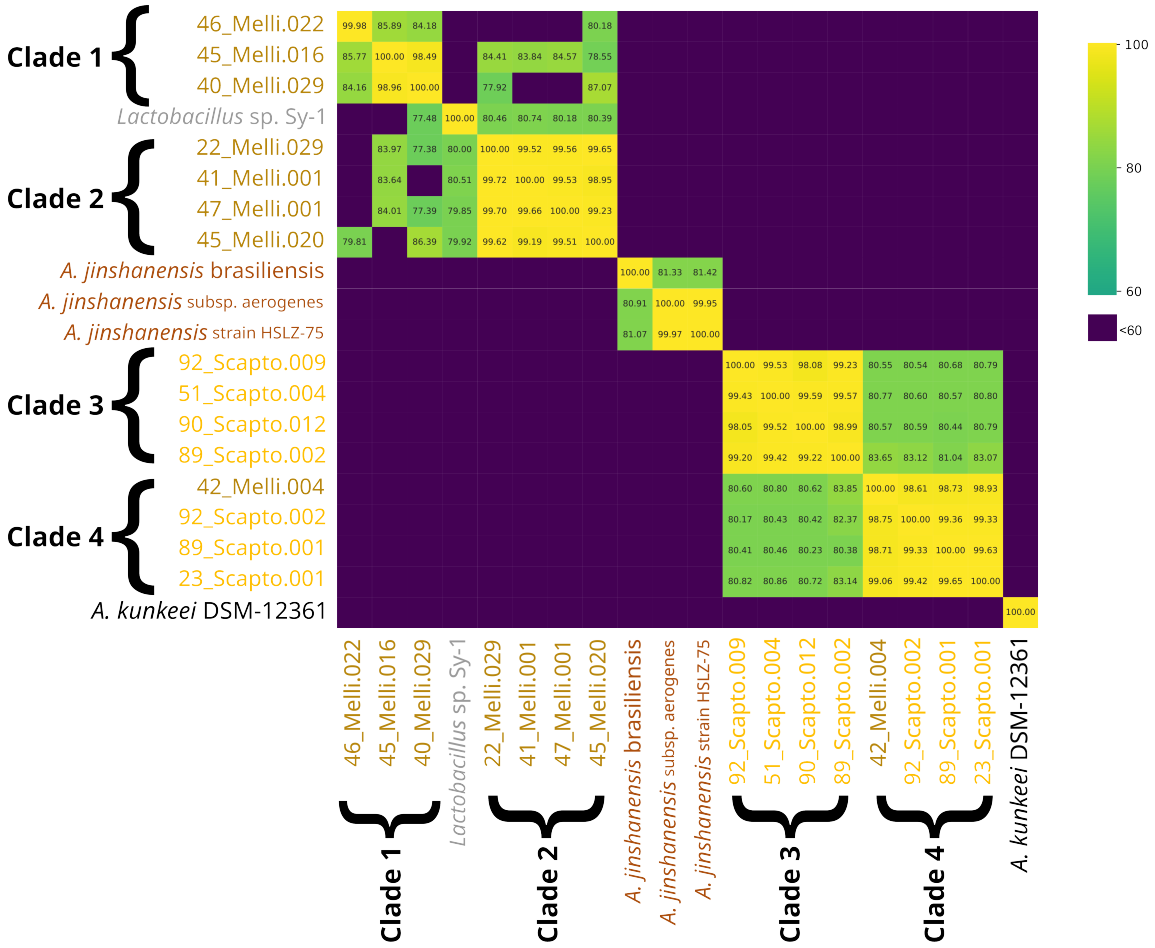

Fig. 6. ANI groups genomes and MAGs according to their conserved sequences. MAGs with ANI above 98% are shown in yellow, while pairs with ANI close to 80 are shown in green.

not appear to be the main factor shaping the honey microbiome. Among the *Lactobacillus* species, the taxonomic assignment of reads using Kraken2 suggested that *Acetilactobacillus* is the most abundant genus. However, these reads did not map to *Acetilactobacillus* genomes in the metapangenome analysis. Thus, we found no evidence that they belonged to *Acetilactobacillus*, but to new *Acetilactobacillus*-like genera instead.

Acetilactobacillus is the Closest Genus to Most of the Recovered MAGs

Our shotgun metagenomic approach to native bee honey enabled the reconstruction of high-quality MAGs. Filtering for completeness above 90% and contamination below 10%, we recovered 25 MAGs, with 15 having *A. jinshanensis* as their closest known genome in GTDB, showing an ANI of at most 79.09%. Applying stricter quality thresholds, with completeness above 95% and contamination below 5%, we retained seven of these 25 MAGs, spanning the phyla Proteobacteria (*Bombella* 46_Melli.021 and *Acetobacter* 43_Scapto.022), Pseudomonadota (*Rosenbergiella* 39_Melli.018 and *Rosenbergiella* 43_Scapto.014), and Bacillota (*Fructobacillus* 46_Melli.006, *A. jinshanensis* 47_Melli.001, and *A. jinshanensis* 51_Scapto.004). PPrevious studies have identified *Lactobacillales* [77] in particular *Lactobacillus* as a dominant lineage in honey microbiomes [10, 58, 15]. The genera *Fructobacillus* and *Acetilactobacillus* belong to the Lactobacillaceae family, highlighting the importance of this group in honey-associated microbial communities. Our findings suggest that *Acetilactobacillus* is the closest genus to most recovered MAGs, reinforcing its potential ecological significance in this environment, and its hypothetical role in honey properties, granting further research in this area.

New *Acetilactobacillus*-like related genera occur in honey

Given the high genetic diversity within the *Lactobacillus* lineage, a taxonomic revision was proposed to reclassify several species into 23 new genera [80]. According to this proposal, the *Acetilactobacillus* genus includes only a single species, *A. jinshanensis*, which has a long evolutionary branch and very few representatives. Our findings indicate that the *A. jinshanensis*-like MAGs that were recovered clustered into four distinct groups, with ANI values below 80 compared to known *A. jinshanensis* and *A. kunkeei* genomes. This finding strongly suggests that these MAGs represent new species, expanding one of the underrepresented clades that was previously reported [80].

Table 3. Reported Acetilactobacillus genus in the literature.

Taxon	Accession	Sample origin	Ref	Abundance	Collection date	Annotation date	Num samples	Technology	Size (Mb)	pH
A. jinshanensis HSLZ-75	CP034726	Zhenjiang aromatic vinegar	[79]	-	2015	2018	1	Illumina isolate strain	1.6	4.0
Lactobacillus	PRJNA379892	Baijiu alcohol fermentation stage	[43]	>95%	2020	2017	5 Baiyunbian baijiu fermentation batches	16S rDNA amplicons	-	-
Lactobacillaceae	-	Spoiled Sichuan bran vinegar	[71]	>90%	2020	2020	10 samples	16S rDNA amplicons	-	-
A. jinshanensis subsp. *aerogenes* Z-1	CP051512-13	Spoiled Sichuan bran vinegar	[71]	-	2020	2020	36 isolates	Illumina MiSeq and PacBio isolate strain	1.6 +1 plasmid	3.6
A. jinshanensis BJ01	PRJNA886850	Baijiu fermented grains	[41]	-	2019	2022	9 lactic and/or acetic acid	RNA Illumina HiSeq 2500	3.5	-
A. jinshanensis brasiliensis	CP171343.1	Larval food of stingless bees	Santos 2024 (submitted)	-	2019	2024	1 MAG	BGISEQ	1.1	-
Two clades of *Acetilactobacillus*	PRJNA1111860	Mellipona bees and honey samples	Silva Cerqueira, 2024	More in bees than honey	2017	2024	18 samples	16S rRNA Illumina	-	-
A. jinshanensis L214	PRJNA886850	Baijiu fermented grains	[17]	-	-	-	CRA015377 CRA015377 and CRA013941	Illumina	-	-
A. jinshanensis		Sichuan Baoning vinegar	[75]	23.02%	2022	2024	297 samples	Illumina HiSeq X platform in the paired-end 150 bp mode	-	-
A. jinshanensis		Sichuan Baoning vinegar	[42]						-	-

Currently, only three publicly available genomes of *A. jinshanensis* exist: two isolated from vinegar and one MAG from stingless bee larval food. *A. jinshanensis* and *A. kunkeei* have been classified as closely related species. In our study, *Acetilactobacillus*-like MAGs were predominant in honey samples. The ANI values below 80% indicate that these MAGs do not belong to *A. jinshanensis* but rather to a related species.

Among the three available *A. jinshanensis* reference genomes, the brasiliensis strain is the most phylogenetically distant (average ANI: 80.99%), suggesting that it may require taxonomic reclassification. Additionally, reads from *M. beecheii* honey mapped exclusively to the brasiliensis genome and not to the other *A. jinshanensis* strains, reinforcing the hypothesis that honey and bees harbor distinct *Acetilactobacillus*-like genera. Many studies classify sequencing reads as *Acetilactobacillus* merely because it is the closest available reference genome. However, our findings suggest that such classifications may require revision to account for newly identified genera within this lineage.

Acetilactobacillus Dominates Acidic Environments Over Time

The species *A. jinshanensis* was first described in 2020 [79] and remains the only recognized member of the *Acetilactobacillus* genus. It thrives in acidic environments, with an optimal pH range of 3.0 – 5.0, and was initially isolated from solid-state vinegar (*A. jinshanensis* strain HSLZ-75). In 2023, a second genome was described: *A. jinshanensis* subsp. *aerogenes* strain Z-1, isolated from Sichuan bran vinegar [71]. Metabarcoding studies have identified *A. jinshanensis* as the most abundant bacterium in vinegar. Additionally, it has been reported as the dominant bacterium in Baijiu, a traditional Chinese fermented beverage. Its presence was confirmed through 16S sequencing, and ANI analysis between strain BJ01 and *A. jinshanensis* HSLZ-75 (1.6 Mb) showed a 99.62% similarity. Transcriptomic studies [41] further confirmed its acid tolerance, with specific genes identified in strain Z-1 [40].

Metagenomic studies of Baijiu [17] have identified over 30 MAGs of varying quality, though they have yet to be publicly released. Both metagenomic and metatranscriptomic analyses suggest that *A. jinshanensis* thrives during acetic acid fermentation as acidity increases over time. It appears to contribute to the flavor of vinegar, but it may also play a role in its spoilage [30]. The mechanisms that allow *A. jinshanensis* to survive in acidic environments have been characterized and may be shared by these newly proposed species. Hence, further studies are needed to identify the bioactive molecules that are potentially produced by the dominant bacterial species in honey from native bees.

In summary, our metagenomic approaches suggest the potential presence of novel species which closely related to *A. jinshanensis*, that would populate its solitary branch and eventually could serve as future markers for characterizing native honey. Further studies are needed to isolate and confirm these proposed new genera and determine whether they contribute to the antibiotic properties of honey.

Conclusion

This study reveals unexpected *Acetilactobacillus*-like microbial diversity in stingless bee honeys from different locations and bees, identifying previously uncharacterized taxa. Shotgun metagenomics enabled the reconstruction of 25 high-quality MAGs, 15 of which shared 80% ANI with *A. jinshanensis*, indicating they likely represent novel species. Clustering analysis grouped these MAGs into four distinct clades, expanding an underrepresented lineage within *Lactobacillaceae*. Although previous studies reported *Acetilactobacillus* as abundant in honey [15], our metapangenomic reads did not map to reference genomes, suggesting the presence of novel genera. The closest genome, *A. jinshanensis* brasiliensis (from stingless bee larval food), shares only 81% ANI with our MAGs and vinegar-associated strains, suggesting it too may warrant reclassification. Given that *Acetilactobacillus* contributes to acidic food fermentations, these novel taxa may underlie the antioxidant and antibacterial properties of stingless bee honeys. Our results advance microbial taxonomy and deepen understanding of culturally significant honeys, with implications for authenticity, functional properties, and probiotic development.

Competing interests

No competing interest is declared.

Author contributions statement

Conceptualization, conception, and design of study, writing, and submission of manuscript A.X.A. and E.J.D.S.; methodology. A.X.A., E.J.D.S., N.S.M and B.E.R.C.; analysis of DNA extraction and preparation for sequencing A.X.A, D.I.H.M, W.I.M.C; C.L.C. and L.R.O. isolate miscroorganisms; Statistical analysis, A.X.A. and H.C.P.; analysis of metagenomic data H.C.P., N.S.M and A.X.A; metapangenome analysis J.A.L.F; physicochemical analyses. A.X.A. and H.C.P.; writing and preparation of the original draft, A.X.A, N.S.M, H.C.P, and E.J.D.S.; writing and review and editing A.X.A, N.S.M, H.C.P, and E.J.D.S.; Writing of the article with contributions from D.I.H.M., W.I.M.C., B.E.R.C, J.F.R.C., F.B.G., B.E.O.V., H.C.P.; Management and acquisition of financial funds for the project, A.X.A, and E.J.D.S. All authors have read and accepted the published version of the manuscript.

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