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Honey characterization and identification of fructophilic lactic acid bacteria of fresh samples from *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia llorentei*, and *Plebeia jatiformis* hives

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Stingless bees are essential to preserve tropical ecosystems. They pollinate native flora, producing honey with properties for traditional health uses. Lactic acid bacteria spontaneously ferment honey in stingless bee honey (SBH). This study aims to determine the main physicochemical characteristics of *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia jatiformis* and *Plebeia llorentei* honey and to isolate and identify FLAB present in SBH samples. The physicochemical properties of SBH, such as color, pH, acidity, sugars, protein, total soluble solids, water activity, total polyphenols, and antioxidant activity, were determined since these parameters can be related to the presence of some bacteria groups, and with health benefits for humans and the hive ecosystems. FLAB harvested from honey, taken directly from storing pots of the hives, were identified by 16S ribosomal RNA sequencing and preserved for future biotechnological use due to their resistance to non-ionic osmotic stress. The results showed significant differences in the physicochemical characteristics of SBH samples. Seven FLAB from four stingless bee species were identified as *Fructobacillus pseudoficulneus* and *F. tropaeoli*. In addition, three other strains of *Fructilactobacillus* spp. were identified only at the genus level. All species showed the ability to grow under different carbon sources, resulting in negative hemolysis and sensitivity to cefuroxime, erythromycin, and chloramphenicol. To the best of our knowledge, this is the first time that the physicochemical and FLAB characterization of SBH from *P. jatiformis* and *P. llorentei* has been reported. Therefore, the future following research should be focused on the environmental, health and food biotechnological applications implications of FLAB from SBH.

KEYWORDS

honey characterization, fructophilic lactic acid bacteria, stingless bees, antioxidant properties, 16S ribosomal RNA, stingless honey

1. Introduction

Stingless bees, meliponines, or indigenous bees comprise a broad group of social bees (Nordin et al., 2018). They can be found in tropical or subtropical regions, such as Australia, Africa, Southeast Asia, and tropical America, mainly in warm and humid forests. Most of these species are native to Central and South America, and many are still wild. They form a honeycomb with a similar structure (with pots, breeding panels, wax and propolis), as shown in Figure 1. Some species are farmed due to the medicinal and nutritional properties of their honey, royal jelly, and pollen (Leonhardt, 2017). More than 46 species of stingless bees have been reported in Mexico, most of them in the country's southwest (Quezada-Euán, 2018).

Honey is a natural viscous sweetener made by several bees. The honey produced by the *Apis mellifera* bee is the most studied worldwide. Generally, it contains 80–85% carbohydrates, 15–17% water, 0.3% proteins, 0.2% ashes and minor quantities of amino acids, phenols, pigments and vitamins (Alvarez-Suarez et al., 2018; Khan et al., 2018). However, over 600 species of Meliponini (stingless bees) produce honey classified as SBH. Significant differences have been found between the honey produced by *Apis mellifera* honey and the different species of SBH, which difficult their quality regulation and characterization (Nordin et al., 2018). SBH generally has more moisture, a peculiar flavor, a varied color range, and a more pronounced aroma (Rozman et al., 2022).

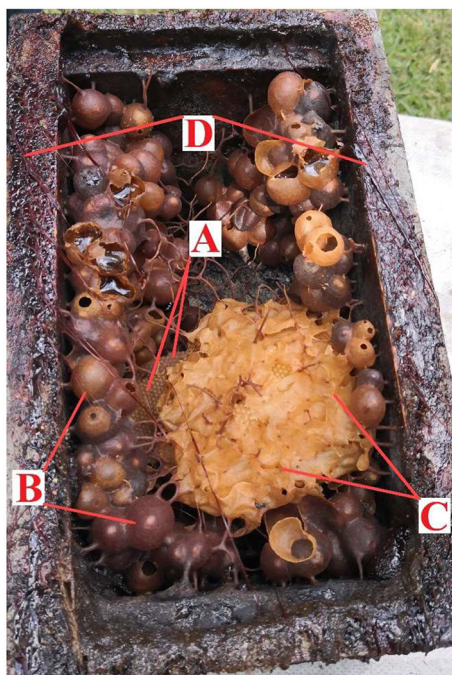


FIGURE 1
Stingless bee honeycomb structure of *Plebeia jorenteii*: brood nest (A), storing pots of honey and pollen (B), protective wax (C), and propolis to seal (D).

The SBH has more moisture than *Apis* spp. honey, so some microorganisms, such as Lactic Acid Bacteria (LAB), consume part of the sugar and transform it into lactic acid through anaerobic fermentation (Souza et al., 2021). Because of the nutritional compounds of honey, the growth of bacteria is allowed without spoiling the honey. These bacteria modulate the properties of honey through fermentation. Thus, an interaction between the food and the living microorganism is generated (Mărgăoan et al., 2020). Some microorganisms contribute to honey conservation and nutrient digestion (Belina-Aldemita et al., 2020; Souza et al., 2021).

Fructophilic lactic acid bacteria (FLAB) are a recently discovered group of previously considered LAB with some unique biochemical characteristics. They prefer fructose as substrate, and their growth in glucose is poor. FLAB use fructose as a substrate and electron acceptor because they usually live in fructose-rich habitats (Filannino et al., 2019). So, they can be found in flowers, fruits, fermented food derived from fruits, and honey (Endo et al., 2018). FLAB are heterofermentative since they generate other products (carbon dioxide, acetate, ethanol and mannitol) in addition to lactate. Moreover, they metabolize different carbohydrates, and some strains can metabolize *p*-coumaric acid (the main phenolic acid in pollen) to phloretic acid or *p*-vinylphenol (Endo et al., 2018) and have shown high tolerance to non-ionic osmotic stress (Ali et al., 2017).

The richness of bioactive metabolites in SBH could be responsible for traditional medical and therapeutic uses. For example, honey is rich in antioxidant compounds like flavonoids, phenolic compounds, and bioactive peptides, which can prevent health issues (Nordin et al., 2018). Among the SBH beneficial properties, the antimicrobial activity, the antioxidant and hypolipidemic effect, the protection from injuries caused by dyslipidemia, the anti-inflammatory activity against chronic subclinical systemic inflammation, and the prevention of *Staphylococcus aureus* infection have been reported (Pimentel et al., 2022). In addition, SBH has been used to prevent and treat diverse diseases in Mexico (Sánchez Cano, 2019), Kenya (Sabella et al., 2022) and Guatemala (de la Roca, 2018), like:

- Respiratory diseases (cough, pneumonia, sinusitis, laryngitis, and asthma);
- Stomach upsets (diarrhea, ulcers, and indigestion);
- Skin problems (wounds, blemishes, measles, allergy, itching, burns, and hemorrhoids);
- Eye conditions (cataracts, cernosities, conjunctivitis);
- Prevent anemia or recover energy during pregnancy or after childbearing.

However, despite its potential importance for health and food, there are few studies about the characterization of fresh SBH to differentiate it from an old SBH. Most of the research has been carried out on the genera *Melipona* and *Scaptotrigona* (Jimenez et al., 2016; Ávila et al., 2018), and for the genus *Plebeia*, there are only three reports to date (Duarte et al., 2012, 2018; Echeverrigaray et al., 2021).

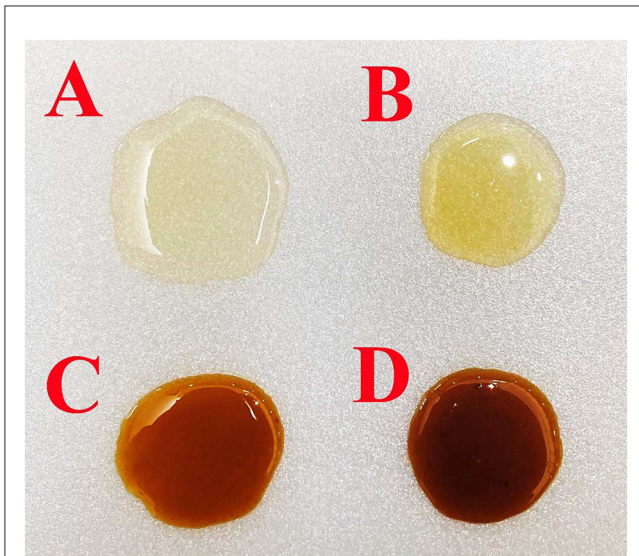


FIGURE 2
Melipona beecheii (A), *Scaptotrigona pectoralis* (B), *Plebeia jatiformis* (C), and *Plebeia llorentei* (D) honey samples taken from the same honeybee farm.

LAB have been studied from the gastrointestinal tract of *Apis* spp. and some stingless bees, but not in honey samples. In *A. mellifera* were identified *Lactobacillus johnsonii* and *Enterococcus faecium* (Carina Audisio et al., 2011), and in *A. dosarta* honey, *Weissella* spp. were found. In addition, Torres-Moreno et al. (2021) recently isolated LAB from the intestine of *M. beecheii*, *S. pectoralis*, and *P. jatiformis* (stingless bees), finding strains of *Apilactobacillus* spp., *Lactiplantibacillus plantarum*, *W. paramesenteroides* and *Leuconostoc citreum*. All these unique characteristics of FLAB have made them a novel candidate for environmental, food and health biotechnology (Agagüdüz et al., 2022). Therefore, this research aimed to determine the main physicochemical characteristics of honey from *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia jatiformis* and *Plebeia llorentei* (Figure 2) and to isolate and identify FLAB in the honey samples.

2. Materials and methods

2.1. Samples

Mr. Juan Pale kindly donated the SBH samples. The samples were collected in September 2020 from honeycombs belonging to “El Rinconcito”, a honeybee farm in Teocelo, Veracruz, Mexico (19.393640787641715, -96.97934590967033). Each sample (30 mL) of SBH was taken with sterile material directly from the honey-storing pots (Figures 1, 3) of four stingless bee specimens and kept in tubes under refrigeration. The bees were previously identified as *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia llorentei*, and *Plebeia jatiformis* (Torres-Moreno et al., 2021).

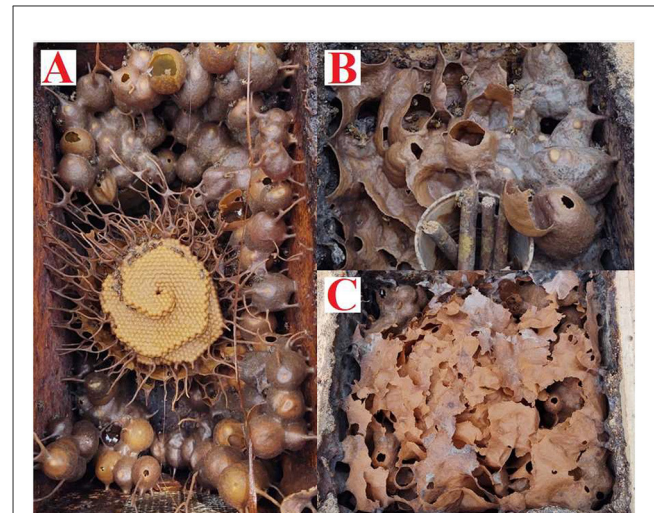


FIGURE 3
 Honeycombs of *Plebeia llorentei* (A), *Melipona beecheii* (B), and *Scaptotrigona pectoralis* (C).

2.2. Analytical methods to determine physicochemical parameters in honey

2.2.1. Color intensity: ABS_{635nm}

The color intensity of SBH was measured according to Smetanska et al. (2021) with slight modifications. First, one gram of each honey sample was diluted 1:1 with distilled water. Then, the homogenized solution was centrifuged at $14,000 \times g$ for 5 min, and the absorbance was measured at 635 nm (spectrophotometer VE-5100UV, Científica Velaquin, Mexico). Finally, the color intensity was determined by converting the absorbance value to the Pfund scale with equation (1).

$$Pfund (mm) = -38.70 + 371.39*(Abs) \quad (1)$$

2.2.2. pH, free acidity, lactonic acidity, and total acidity

The pH was determined with a potentiometer. The acidity (free, lactone, and total) was measured according to the AOAC methods (Lee et al., 2005). The free acidity was calculated according to equation (2), the lactonic acidity with equation (3), and the total acidity with equation (4).

$$FA = \frac{(mL \text{ of } 0.05 \text{ M NaOH of the sample}) - (mL \text{ of } 0.05 \text{ M NaOH of the control})}{g \text{ of the sample}} \quad (2)$$

$$LA = \frac{(10 - mL \text{ of } 0.05 \text{ M HCL}) * 50}{g \text{ of the sample}} \quad (3)$$

$$TA = FA + LA \quad (4)$$

Where FA means free acidity, LA means lactonic, and TA is total acidity.

2.2.3. Sugar and soluble protein content

Reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNS) method. First, the sample was diluted to 0.001% (100 mg of honey in 100 mL of distilled water), and 200 μ L of the diluted sample were taken and mixed with 200 μ L of the DNS reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The mixture was heated for 15 min at 100°C and then diluted with 8 ml of distilled water in an ice bath. Then, the percentage of reducing sugar was determined by measuring the absorbance of the sample and making a standard curve of 100 mg of glucose in 100 mL of distilled water, which would be 100% glucose, and their respective dilutions (80, 60, 40, 20, and 0%) at 540 nm in a multiplate reader (Multiskan go, ThermoFisher, USA) (Susilowati and Azkia, 2022).

The total sugar content was determined by the phenol-sulfuric acid method, described by Trinh et al. (2022), with some modifications. First, the honey (1 g) was dissolved in 20 mL of water and then made up to 100 mL with distilled water. The previous solution was mixed, and 1 mL was taken to bring it to a volume of 100 mL with distilled water. Later, 5 mL of concentrated H₂SO₄ was added to the second dilution of honey (1 mL) in a test tube, mixed carefully and allowed to react for 10 min and placed in an ice bath. When it reached room temperature, phenol 5% (3 mL) was added and mixed. The solution was allowed to stand for 30 min and then, absorbance measured spectrophotometrically at 490 nm. A calibration curve was made with different concentrations of glucose. This assay was repeated three times.

The Bradford method was used to determine the soluble protein content in the SBH samples (Naree et al., 2021). The assay was carried out in a multiplate reader (Multiskan go, ThermoFisher, USA) at 595 nm. Honey was first diluted 1:1 with distilled water. Then, for the quantification, 10 μ L of the diluted sample was placed in the wells, and 190 μ L of Bradford reagent was added, allowing the mixture to react for 5 min. The calibration curve used a serial dilution of 0.2 to 1.4 mg of ovalbumin per 1 mL of distilled water.

2.2.5. Total soluble solids (TSS), moisture, and water activity (a_w)

The TSS, refractive index, moisture and a_w are related parameters that were determined according to Anguebes et al. (2016). The TSS was measured by °Brix of the honey with a refractometer (ATAGO, Pocket Refractometer model PAL-1) at 20°C. Moisture was calculated by subtracting the amount of TSS from the total content of the product to determine the water content in honey (Bogdanov et al., 2002). The water activity (a_w) was determined by the dew point method, according to Balzan et al. (2020); the AquaLab 4TE-Decagon equipment (Decagon Devices, Pullman, WA, USA) was used at an ambient temperature of 25°C, calibrating the equipment with activated carbon.

2.2.6. Phenolic compounds and antioxidant activity

The total phenolic content of honey samples was determined using the Folin-Ciocalteu method, according to Alvarez-Suarez et al. (2018) with slight modifications. So, 1 g of honey was diluted

to 10 mL with distilled water and filtered through a 0.45 μ m pore filter. Total phenolic content results were expressed as mg of gallic acid equivalents (GAE) per 100 g of honey (mg GAE 100 g⁻¹ of honey) with a gallic acid standard curve.

To determine the antioxidant content or antioxidant activity, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and the potassium ferricyanide reduction (FRAP) methods were used (Badrulhisham et al., 2020). The dilution and filtration of the sample were carried out in the same way as in the total polyphenol content method. Ten μ L of diluted honey and 190 μ L of previously prepared ABTS solution (0.700 \pm 0.05 nm) were mixed and incubated in the dark at 30°C for 6 minutes. After the incubation, the absorbance of the mixture was measured at 734 nm. Ten μ L of 80% methanol were used as a blank. For DPPH, 10 μ L of diluted honey and 290 μ L of a previously prepared DPPH solution (0.04 mg mL⁻¹) were mixed and incubated in the dark at room temperature (22°C) for 30 min. As a blank, 80% methanol was used, and the absorbances were measured at 517 nm. For the potassium ferricyanide reduction method, the FRAP reagent was prepared by mixing 1% of K₃Fe(CN)₆ solution (w/v, in Milli-Q water) with 1 M HCl (v/v, in Milli-Q water), 1% sodium dodecyl sulfate solution (w/v, in Milli-Q water), and 0.2% ferric (III) chloride solution (w/v, in Milli-Q water) at a ratio of 3:3:1:1. Then, 50 μ L of the diluted honey sample were mixed with 200 μ L of the prepared FRAP, and incubated for 20 min at 50°C. The absorbances were read at 750 nm, using the FRAP reagent as blank. All absorbance measurements were performed in triplicate on a microplate spectrophotometer (Multiskan go, ThermoFisher, USA).

A Trolox standard curve (0, 5, 15, 25, 40, 55, 70, 90, and 120 μ g Trolox mL⁻¹) was performed for ABTS, DPPH and FRAP. The reducing antioxidant power of SBH samples was expressed in milligrams of Trolox equivalents of antioxidant capacity per kilogram of honey (mg TEAC kg⁻¹ of honey).

2.2.7. Enumeration of LAB

The enumeration of LAB present in SBH was carried out according to Mathialagan et al. (2018), with some modifications. Plate count was used to enumerate LAB and FLAB populations under aerobic and static conditions. Plates were first prepared with the Man, Rogosa and Sharpe (MRS) agar supplemented with 2% fructose and 0.8% calcium carbonate (CaCO₃). Then, serial dilutions were made with 1 ml of the honey sample up to 1 \times 10⁻⁴ dilution, with the drop plate technique (Naghili et al., 2013), three micro drops of 10 μ L were taken from each dilution to place them on the plates. The plates were incubated at 30°C for 3 days. The bacterial colonies were enumerated and expressed as Colony Forming Units per mL (CFU mL⁻¹).

2.5. Isolation of lactic acid bacteria from honey samples

LAB isolation was performed according to Aween et al. (2012), with some modifications. A diluted honey solution was made using

1 mL of fresh honey diluted 1:10 with peptone water (0.1% w/v). To induce bacteria growth, 1 mL of the diluted honey solution was added to 9 mL of MRS broth supplemented with 2% fructose (MRS-2F) and 0.8% of CaCO₃. The MRS-2F broth supplemented with CaCO₃ and honey was incubated for 2 days under microaerophilic conditions at 30°C. This culture was used to streak in a Petri dish with MRS-2F agar added with 0.8% CaCO₃. In addition, striatal inoculation was performed with the diluted honey solution directly to the Petri dishes (MRS-2F agar added with 0.8% CaCO₃) without previously inducing growth in broth. All the plates were incubated for three days under aerobic and static conditions at 30°C. Colonies that grew were replated (three to four times) in new Petri dishes (in the same conditions) to ensure isolation. Well-isolated colonies were picked and transferred to MRS-2F broth, adding the same volume of glycerol 1:1 with distilled water. For further studies, the strains were stored at -80°C and maintained on glycerol stocks (Arencibia et al., 2008).

2.6. Identification of isolated colonies

Phenotypic identification of seven isolated bacteria was made to verify that the strains were catalase-negative, Gram-positive and rod-shaped. The size was measured by image analysis using ImageJ 1.53t (public domain, USA). The strains that did not meet the characteristics of LAB were discarded. A carbohydrate fermentation profile with API 50 CHL was carried out for the presumptive LAB under anaerobic and static conditions (Syed Yaacob et al., 2018). A molecular identification was carried out according to Torres-Moreno et al. (2021), with slight modifications. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Aboul-Maaty and Oraby, 2019), using the 16S rRNA gene to identify LAB. The PCR amplification programs consisted of (1) 7 min at 95°C, (2) 35 cycles of 1 min at 94°C+1 min at 63°C+1 min at 72°C and (4) and a final extension of 10 min at 72°C, using the primer UniBac-Forward (GAT CCT GGC TCA GGA TGA AC) and UniBac-Reverse (GGA CTA CCA GGG TAT CTA ATC) in T100 thermal cycling (BIO-RAD, USA). PCR products were sequenced using the Genetic Analyzer 3130xl sequencer (Applied Biosystems, USA) to obtain approximately 790 bp sequences.

2.7. Phylogenetic analysis of gene 16S rRNA

The obtained sequences were compared to published sequences of 16S rRNA genes from different species of the Order *Lactobacillales*. Sequences were aligned using the MUSCLE algorithm in UGENE v33.0 (Okonechnikov et al., 2012) and edited with PhyDE-1v0.9971 (<http://www.phyde.de/download.html>). The phylogenetic analysis was conducted for Bayesian Inference using MrBayes 3.2.5 software (Ronquist et al., 2012) using the model for molecular evolution TVMef +I+G selected according to the best fit using jModelTest v0.1.1 (Posada, 2008). The phylogenetic analysis was run for 10 million generations, sampling every 1000 generations. Bayesian posterior probability values were calculated, considering nodes significantly supported if posterior probabilities

were ≥ 0.95 . Trees were visualized in FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

2.8. Safety of the strains

Hemolysis and antibiotic resistance assays were determined (Mohammad et al., 2020) by activating the strains with MRS-2F broth until reaching 1×10^8 CFU mL⁻¹. Subsequently, each strain was seeded on the entire surface of blood agar for the hemolysis test and MRS-2F agar for the antibiotic susceptibility test, placing on the MRS-2F agar the usual antibiotic disks used for Gram-positive bacteria immediately after the strains were spread. All plates were incubated for 48 h at 37°C under aerobic conditions. *Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* ATCC7644 were positive controls for α -hemolysis and β -hemolysis. For antibiotic resistance, the diameter (mm) of growth inhibition of each antibiotic was measured.

2.9. Statistical analysis

All measurements were performed ($n = 3$) to obtain the mean, standard deviation, and one-way analysis of variance (ANOVA). For honey, the independent variable was the origin (species of bee) since the samples were taken simultaneously and in the same place. Comparisons between species were made in pairs using the Tukey test with a confidence of $\alpha = 0.05$. All statistical analyzes were performed using SigmaPlot 14.0 (Systat Software Inc., London, UK).

3. Results and discussion

3.1. Physicochemical characterization of stingless bee honey

The physicochemical data of the honey produced by *M. beecheii*, *S. pectoralis*, *P. llorentei*, and *P. jatiformis* is shown in Table 1. Most of the parameters are within the range of the results reported by other authors. Different factors influence the composition of honey, like the bee species, the environment and the extraction method. In this study, to reduce these parameters, the procedure was standardized to reduce variability, since all the samples were taken freshly from one pot from each hive belonging to each bee specie (Mohammed, 2020). The physicochemical parameters of the SBH were essential to predict the presence of different LAB and FLAB in the hives and their stress-related characteristics (Braghini et al., 2021).

The Pfund scale determined the color of the samples. All SBH samples were statistically different ($p \leq 0.001$). There are no food regulations regarding the color of SBH (Tan et al., 2021). In this research, the *P. llorentei* and *P. jatiformis* honey was dark, and lighter in the samples from *S. pectoralis* and *M. beecheii*. Nordin et al. (2018) and Ávila et al. (2019) analyzed the color of 24 honey samples from the *Melipona* spp. with a color ranking from clear to dark (0.3 to 152 mm Pfund units) and eight *S. bipunctata* samples (5.87 to 118.03 mm). These results indicated that the color of the

TABLE 1 Physicochemical characterization of the honey produced by stingless honeybees: *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia llorentei*, and *Plebeia jatifformis*.

Honey stingless bee	<i>M. beecheii</i>	<i>S. pectoralis</i>	<i>P. llorentei</i>	<i>P. jatifformis</i>	Comparison. mean (range)
Pfund colorimeter (mm)	White ^{B,C,D} (21.59 mm ± 0.73)	Extra white ^{A,C,D} (14.16 mm ± 0.80)	Dark amber ^{A,B,D} (171.94 mm ± 1.49)	Dark amber ^{A,B,C} (133.07 mm ± 1.42)	92.9 (16–150) (Nordin et al., 2018)
pH	3.59 ± 0.04 ^{B,D}	3.36 ± 0.02 ^{A,B,D}	3.5 ± 0.01 ^{B,D}	3.23 ± 0.06 ^{A,B,D}	3.93 (2.93–6.64) (Souza et al., 2021)
Free acidity (mEq kg ⁻¹)	8.83 ± 0.62 ^B	49.25 ± 2.25 ^A	30.58 ± 0.88	31.5 ± 1.25	28.8 (13.5–46.8) (Cardona et al., 2019)
Lactonic acidit (mEq kg ⁻¹)	8.58 ± 2.01 ^B	22.67 ± 0.87 ^A	12.5 ± 0.75	18.17 ± 0.14	9.0 (0.62–38.0) (Cardona et al., 2019)
Total acidity (mEq kg ⁻¹)	17.41 ± 2.63 ^B	71.91 ± 1.38 ^A	43.01 ± 0.14	49.67 ± 1.13	37.9 (23.6–62.9) (Cardona et al., 2019)
Reducing sugars (g 100 g ⁻¹) by DNS	77.49 ± 0.55 ^{B,C,D}	62.68 ± 1.51 ^{A,C,D}	57.87 ± 1.23 ^{A,B,D}	71.61 ± 1.69 ^{A,B,C}	58.87 (7.4–80.9) (Souza et al., 2021)
Total sugar content (g 100 g ⁻¹) by the Dubois method	78.25 ± 2.45 ^{B,C}	65.28 ± 3.28 ^{A,C,D}	59.10 ± 2.40 ^{A,B,D}	76.71 ± 3.25 ^{B,C}	78.3 (57.4–91.9) (Echeverrigaray et al., 2021)
Protein content (mg g ⁻¹)	0.68 ± 0.09 ^{B,C,D}	1.03 ± 0.08 ^A	0.95 ± 0.11 ^A	1.06 ± 0.06 ^A	1.12 (0.1–5.74) (Souza et al., 2021)
Total soluble solids (% of °Brix)	76.47 ± 0.23 ^{C,D}	77 ± 0.2 ^{C,D}	69.26 ± 0.31 ^{A,B,D}	74.73 ± 0.12 ^{A,B,C}	72.9 (64.7–83.3) (Souza et al., 2021)
Moisture (g 100 g ⁻¹)	23.53 ± 0.23 ^{C,D}	23 ± 0.2 ^{C,D}	30.73 ± 0.31 ^{A,B,D}	25.27 ± 0.12 ^{A,B,C}	27.3 (13.3–43.0) (Souza et al., 2021)
Water activity (a _w)	0.631 ± 8.5 × 10 ⁻⁴ ^{B,C,D}	0.641 ± 4.9 × 10 ⁻⁴ ^{A,C,D}	0.754 ± 2.8 × 10 ⁻⁴ ^{A,B,D}	0.710 ± 7.8 × 10 ⁻⁴ ^{A,B,C}	0.70 (0.6–0.86) (Echeverrigaray et al., 2021)
Total polyphenols (mg of gallic acid kg ⁻¹)	237.83 ± 12.01 ^(B,C,D)	464.47 ± 22.54 ^{A,C,D}	659.34 ± 21.76 ^{A,B,D}	547.41 ± 14.37 ^{A,B,C}	791.6 (6–8,546.2) (Souza et al., 2021)
ABTS (mg of TEAC kg ⁻¹)	1,281.82 ± 85.54 ^(B,C,D)	227.57 ± 63.41 ^{A,C,D}	498.41 ± 40.75 ^{A,B,D}	380.87 ± 70.42 ^{A,B,C}	776.3 (106–2,006.8) (Badrulhisham et al., 2020)
DPPH (mg of TEAC kg ⁻¹)	76.72 ± 44.77 ^C	103.53 ± 40.45	268.46 ± 112.43 ^A	162.48 ± 74.32	364.3 (41.5–781.7) (Badrulhisham et al., 2020)
FRAP (mg of TEAC kg ⁻¹)	72.63 ± 2.42	21.98 ± 3.09 ^{C,D}	170.86 ± 35.97 ^B	183.19 ± 61.72 ^B	2,316.2 (181.4–6,253.4) (Badrulhisham et al., 2020)
Enumeration of LAB by CFU of LAB mL ⁻¹ in MRS broth (SD)	1 × 10 ^{3D} (0.632 × 10 ³)	2.83 × 10 ³ (0.408 × 10 ³)	3.17 × 10 ³ (0.752 × 10 ³)	11.16 × 10 ^{3A} (1.941 × 10 ³)	1 × 10 ⁴ (1 × 10 ³ –1 × 10 ⁵) (Vásquez et al., 2012)

Results are expressed as the mean (n = 3) ± SD. Superscripts indicate that there was a significant difference with *M. beecheii* (A), *S. pectoralis* (B), *P. llorentei* (C) and *P. jatifformis* (D) by Tukey test with confidence of $\alpha = 0$.

honey is independent of the bee species and that it is normal to find light-colored honey like the one found in this work (*S. pectoralis* honey with 14.5 mm Pfund units). Finally, the color of *Plebeia molesta* has been described between light and dark amber without a Pfund measurement, so it is important to perform the color assay (Geisa et al., 2021).

3.1.1. Free acidity, lactonic acidity, total acidity, and pH

The pH of *S. pectoralis* and *P. jatifformis* honey was statistically different ($p = 0.012$), while the pH of *P. llorentei* and *M. beecheii* was similar ($p = 0.07$). All the pH values were lower than the average value reported by Souza et al. (2021). Nectar, mandibular bee substances, plant species, and soil composition influence the pH. Low pH prevents undesirable microorganisms (Nascimento et al., 2015).

The lactonic and total acidity were statistically different in all honey produced by the tested bee species ($p \leq 0.014$). For free acidity, *M. beecheii* and *S. pectoralis* were statistically different

($p \leq 0.001$), but free acidity values between *P. jatifformis* and *P. llorentei* had no significant difference ($p = 0.851$). The free acidity is an indicator of freshness, and the maximum limit, according to International Honey Commission for *Apis mellifera*, is 50 mEq kg⁻¹. So, the samples could be considered fresh (Bogdanov et al., 2002). In addition, *M. beecheii* honey from Mexico had 5.9 mEq kg⁻¹, as previously found (Santiesteban-Hernández et al., 2003). Therefore, lactonic acid is another indicator of the freshness in honeybee. The reported values were also like those previously reported (Lage et al., 2012; Cardona et al., 2019; Scholz et al., 2020). Therefore, results indicated the freshness of the honeybee samples collected directly from the beehives.

3.1.2. Sugar and soluble protein content

Table 1 shows the differences of sugar and protein content in the samples of *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia llorentei*, and *Plebeia jatifformis* honey.

All the reducing sugar results were statistically different ($p < 0.001$). The average of reducing sugars previously found was

TABLE 2 Characteristics of bacteria isolated from stingless bee honey.

Honey	Bacteria	Code (strain)	GenBank accession number	Activation temperature	Gram stain	Catalase test	Metabolism	Bacillus size (μm)
<i>Melipona beecheii</i>	<i>Fructilactobacillus</i> spp.	H-Mb-0-1	OP941514	30°C	+	-	FA	2.3 \pm 0.42
<i>Plebeia jatifformis</i>	<i>F. pseudoficulneus</i>	H-Pj-0-1	OP941513	30°C	+	-	FA	1.3 \pm 0.32
<i>Plebeia jatifformis</i>	<i>F. pseudoficulneus</i>	H-Pj-0-2	OP941512	30°C	+	-	FA	1.4 \pm 0.21
<i>Scaptotrigona pectoralis</i>	<i>F. pseudoficulneus</i>	H-Sp-0-1	OP941511	30°C	+	-	FA	1.2 \pm 0.17
<i>Scaptotrigona pectoralis</i>	<i>Fructilactobacillus</i> spp.	H-Sp-0-2	OP941515	30°C	+	-	FA	1.9 \pm 0.44
<i>Scaptotrigona pectoralis</i>	<i>Fructilactobacillus</i> spp.	H-Sp-0-3	OP941516	30°C	+	-	FA	2.1 \pm 0.36
<i>Scaptotrigona pectoralis</i>	<i>F. tropaeoli</i>	H-Sp-0-4	OP941510	30°C	+	-	FA	1.2 \pm 0.14

FA means facultative anaerobe. Results of bacillus size are expressed as the mean ($n = 3$) \pm SD.

between 7.45 to 97.10 g 100 g⁻¹ (Ávila et al., 2018; Nordin et al., 2018; Souza et al., 2021). The reduced sugar concentration was like that previously reported by Trinh et al. (2022), who found a range between 74.28 to 88.47 g 100 g⁻¹ in 13 honey samples. Moreover, as expected, the total sugar content was higher than the reducing sugars because of the presence of non-reducing sugars such as sucrose, trehalose and raffinose, carbohydrates that can be metabolized by FLAB (Mahmood and Abbas, 2020). For total sugars, all the honey samples were statistically different ($p \leq 0.007$), except for *M. beecheii* and *P. jatifformis*, which were statistically similar ($p = 0.789$). Honey is a carbohydrate source but not a food rich in protein. However, the protein present in the honey is vital for the quality of life in the hives to feed the bees (Camilli et al., 2020). Stingless bees are more selective in collecting pollen because it is their honey's primary protein source. However, enzymes involved in sugar metabolism (for example, glucose oxidase, amylases, invertases and glucosidases) produced by the hypopharyngeal glands of stingless bees also count in the protein concentration of honey (Ahmad et al., 2021). Generally, the samples analyzed here had higher soluble protein values than previous reports of SBH (Villacrés-Granda et al., 2021) and like those found in Taiwan and Thailand (Chen et al., 2019).

Furthermore, the difference in the protein content of SBH produced by *S. pectoralis*, *P. jatifformis* and *P. llorentei* was not statistically significant ($p \leq 0.001$). In contrast, the reduced protein content in *M. beecheii* honey was significantly different ($p \geq 0.194$) compared to the honey of the other bee species. The samples were taken in the same geographical region, and the environment where the bees look for the nectar is the same, but even so, the honey turned out to have differences as Ávila et al. (2018), demonstrating that there are variations in the chemical composition of SBH of the same species. The protein from pollen in honey is important because it modulates and enhances the development and growth of LAB, such as *Fructobacillus* spp. and *Lactobacillus* spp. (di Cagno et al., 2019).

3.1.3. Total soluble solids, moisture, and water activity

The results of total soluble solids (TSS) (%), moisture (%), and water activity (a_w) in the samples of *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia llorentei*, and *Plebeia jatifformis* honey are shown in Table 1.

All the samples were statistically different ($p \leq 0.001$) in TSS, moisture and a_w except for *M. beecheii* and *S. pectoralis* ($p \geq 0.074$) where TSS and moisture were different ($p \geq 0.074$). The results in this research were similar to those previously reported by Nordin et al. (2018), Cardona et al. (2019), and Echeverrigaray et al. (2021). In general, *Plebeia* spp. had a higher a_w and *P. jatifformis* had a higher bacteria count. Furthermore, the a_w of SBH was higher than that of *A. mellifera* (Shamsudin et al., 2019). The results obtained showed that when moisture increases or the number of soluble solids decreases, a_w increases. Therefore, high levels of moisture and a_w , coupled with favorable external conditions, could cause fermentation and alter the acidity, pH, bacterial load, and amount of carbohydrates and protein in the honey (Makhloufi et al., 2021).

3.2. Phenolic compounds and antioxidant activity

The total polyphenol compounds are shown in Table 1. All the results were statistically different ($p < 0.001$). Biluca et al. (2017) reported a higher content of polyphenols in the honey of *S. bicunctata* (≈ 500 mg of gallic acid kg⁻¹) compared to *M. bicolor*, *M. quadrifasciata*, *M. mondury* and *M. scutellaris*, as well as in this work. However, the results of the polyphenol content of this research are lower than the polyphenol content reported by Souza et al. (2021). Nonetheless, it is normal because the polyphenol content depends on the geographical origin. It turns out that honey samples taken in highlands (mountain areas), such as those in this research, have lower total polyphenol content values because the

floral variety and availability are less accessible than in the lowlands (Yayinie et al., 2022).

The antioxidant activity with ABTS, DPPH and FRAP is shown in Table 1. Although all the results of ABTS were statistically different ($p \leq 0.03$), *M. beecheii* had the highest antioxidant activity by ABTS, followed by *P. llorentei* and *P. jatiformis*. For DPPH assay, the honey samples were not statistically different ($p \geq 0.085$), except for *P. llorentei* ($p \leq 0.023$), which was different from the others and with greater antioxidant activity. In the FRAP method, samples were similar. There was no significant difference between *P. jatiformis* and *P. llorentei* ($p = 0.932$), showing a high antioxidant activity, compared to *M. beecheii* and *S. pectoralis*, which also had no significant statistical difference between them ($p = 0.099$).

Previous reports have shown a higher ABTS activity on honey from *S. bipuncata* and *S. mexicana* compared to the results from this research (Jimenez et al., 2016; Ávila et al., 2019). Furthermore, as in this report, *A. mellifera* honey usually has higher ABTS activity than DPPH (Attanzio et al., 2016). Alvarez-Suarez et al. (2018) found greater antioxidant activity in *M. beecheii* with 440.05 ± 27.10 mg of TEAC kg^{-1} by FRAP and similar antioxidant activity by DPPH with 105.69 ± 4.15 mg of TEAC kg^{-1} .

3.3. Enumeration of LAB from honey and its isolation

Each colony was counted to measure the bacterial load of LAB, then it was corroborated under an optical microscope since some yeasts can grow even in the presence of CaCO_3 and be confused with LAB colonies. Table 1 shows the total LAB count of the different samples.

There was no significant difference in the CFU of bacterial load between *M. beecheii*, *S. pectoralis* and *P. llorentei* honey samples ($p > 0.05$), but there was a significant difference for *P. jatiformis* honey ($p < 0.001$). It was not possible to appreciate any trend or relationship between the bacterial load and the physicochemical properties of honey since *P. jatiformis* honey had the highest bacterial count with 11.16×10^3 CFU mL^{-1} , almost four times higher than *P. llorentei* honey, even though these were the most similar honey sharing some similar properties (Table 1). As shown in Table 2, four strains from *S. pectoralis* were isolated, two from *P. jatiformis* and one from *M. beecheii*. Although *P. jatiformis* honey had a higher bacterial load, more bacteria could be isolated from *S. pectoralis* honey with greater bacterial diversity since it showed more LAB species such as *F. tropaeoli* H-Sp-04, *F. pseudoficulneus* H-Sp-01 and two *Fructilactobacillus* spp. (H-Sp-02 and H-Sp-03). A greater bacterial diversity in *S. pectoralis* honey could be due to the physicochemical conditions favorable to the development of LAB. Therefore, this honey presents the highest lactic acid (22.67 mEq kg^{-1}) and total (71.91 mEq kg^{-1}) acidity, which are related to the freshness, maturation and fermentation of honey.

Unfortunately, LAB cannot be isolated from *P. llorentei* since, during the replanting process, the colonies stopped growing, probably due to a lack of nutrients or by its obligate anaerobe metabolism. Torres-Moreno et al. (2021) showed that LAB could not be isolated from the stomach of *P. llorentei* bees, suggesting that this honey contains LAB that cannot be cultivated in MRS medium,

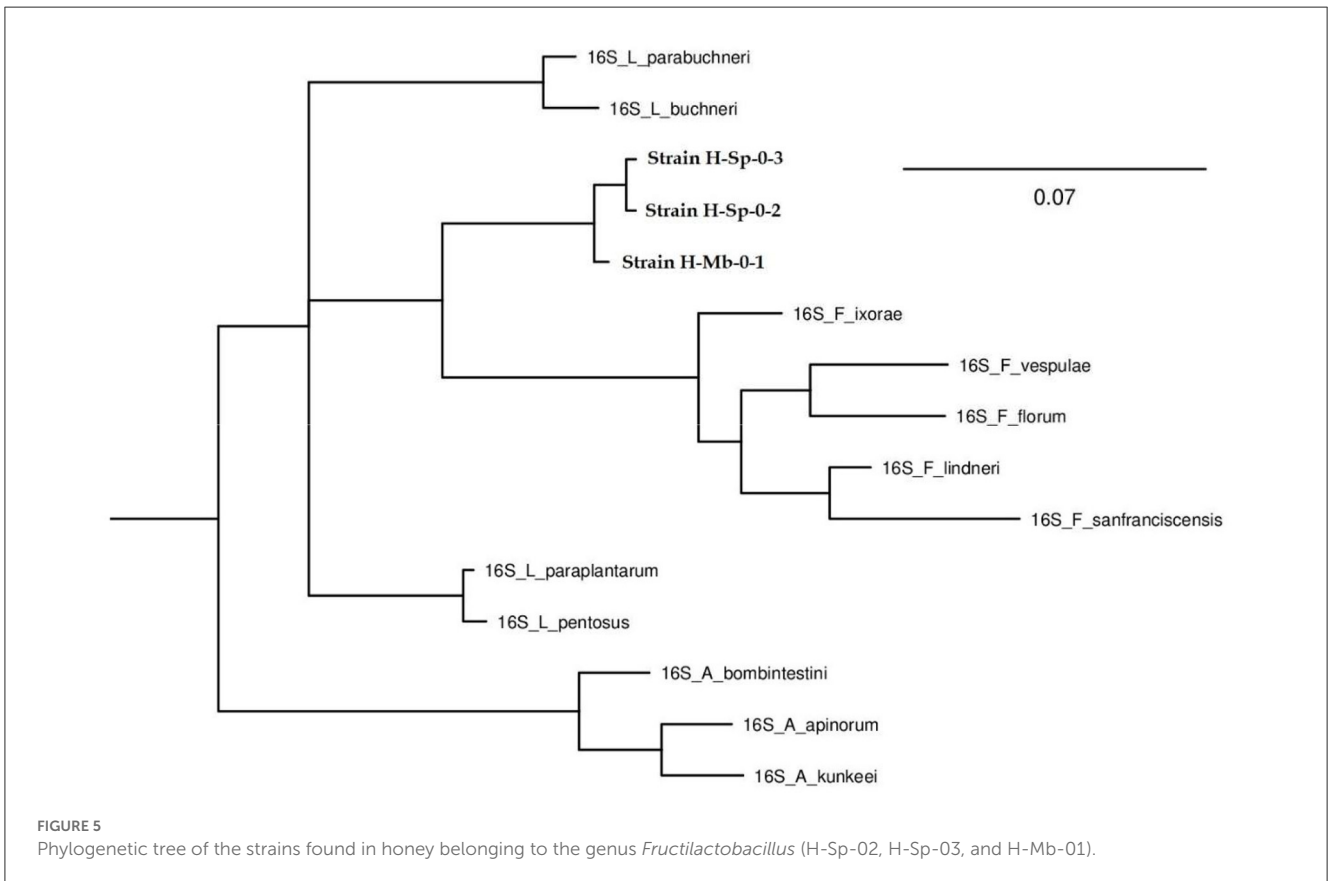
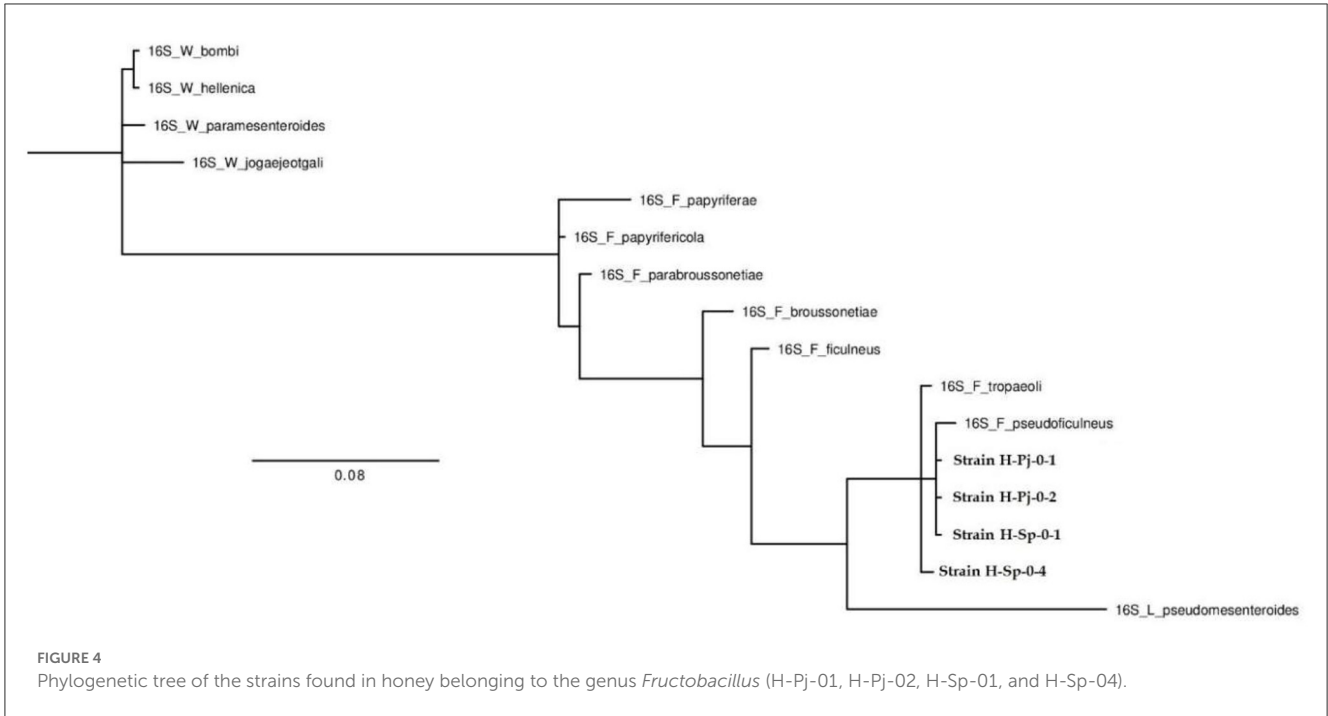
probably because some nutrient is missing or because this LAB is used only for honey's physicochemical properties and does not adapt to different conditions. The physicochemical properties of *P. llorentei* honey compared with *P. jatiformis* honey are statistically different since *P. llorentei* has the least amount of total and reducing sugars, the highest moisture and a_w , the lowest °Brix value, the highest content of polyphenols and antioxidant activity by DPPH. It may be that the difference between some or the combination of these properties has interfered in the null isolation of bacteria from *P. llorentei* honey. Obligate anaerobic bacteria could be present in SBH. Therefore, this characteristic could have prevented their cultivation in a conventional culturing attempt since they do not grow in atmospheric oxygen concentrations (Mattila et al., 2012). The need for an anaerobic environment may explain the lack of bacteria identified in *P. llorentei* honey.

3.4. Characterization of LAB isolated from honey samples

The interest in isolating LAB from SBH is because honey is a stressful environment for bacteria. Therefore, this food should have resistant bacteria, as demonstrated by Reale et al. (2020), who isolated LAB from sourdough, a food that shares some of the characteristics of honey. After all, it is an acid product with low moisture, high solute content, and contains fructose. Reale et al. (2020) found resistant bacteria, such as *F. sanfranciscensis*, that can be used in fermentation technology. In addition, it is known that honey has phenolic compounds with antioxidant activity. According to Cortés-Rodríguez et al. (2019) some LAB can grow and develop in the presence of phenolic compounds, while pathogenic bacteria cannot. Thus, there will be a biocontrol of pathogens due to resistant bacteria such as desirable, beneficial or probiotic LAB in food rich in polyphenols.

The genetic identification (Figures 4, 5) and optical microscope photos (Figures 6–8) of the strains from SBH indicated three of the isolates of *Fructilactobacillus* spp. could not be identified to the species level. Moreover, three *Fructobacillus pseudoficulneus* and one *Fructobacillus tropaeoli* species were identified. All the isolates were Gram-positive, catalase-negative, able to grow at 30°C, non-hemolytic and had a size around 2 μm for *Fructilactobacillus* spp. and 1 μm for *Fructobacillus* spp. (Table 2). The identified strains were added to the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) database with the following access numbers: OP941514, OP941513, OP941512, OP941511, OP941515, OP941516 and OP941510 as shown in Table 2.

Due to the taxonomic rearrangement, the genus *Fructilactobacillus* is derived from the genus *Lactobacillus*, and both belong to the *Lactobacillaceae* family (Zheng et al., 2020). On the other hand, the *Fructobacillus* genus forms monophyletic clades with the *Oenococcus* genus since they belong to the *Leuconostocaceae* family (Bello et al., 2022). The comparative genomic analyses found four new species of the genus *Fructobacillus* (*F. broussonetiae*, *F. parabroussonetiae*, *F. papyriferae* and *F. papyrifericola*). Therefore it is believed that new species of the genus *Fructilactobacillus* can also be found, and the fact that some bacteria could not be identified to the species level in



this research was because many species have not yet been reported (Lin et al., 2022). Torres-Moreno et al. (2021) isolated LAB (three strains of *Apilactobacillus* spp., two of *Lactiplantibacillus plantarum*, three of *Weissella paramesenteroides*, two *Leuconostoc*

citreum and two undetermined LAB) at the same time and place, from the same beehives of the same bee species, with the only difference that the honey sample was taken from the honey stomach of stingless bees and not from the honey of the beehive.

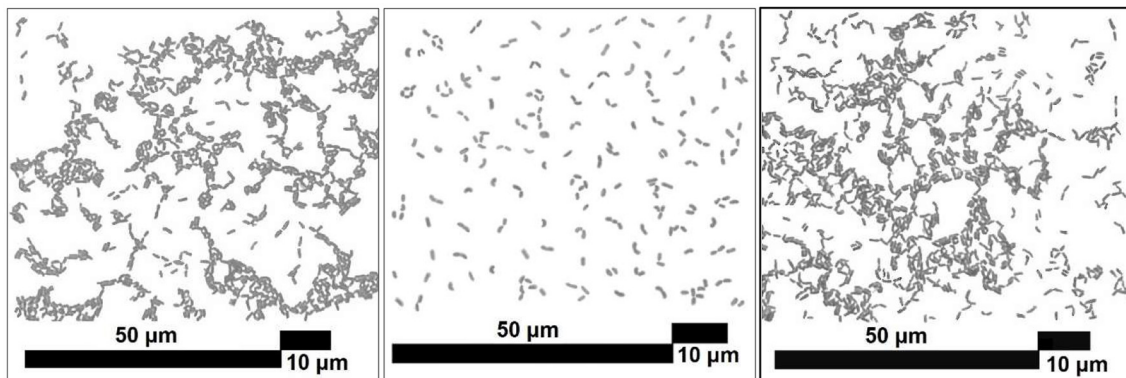


FIGURE 6

Optical microscope micrograph of the *Fructobacillus pseudoficulneus* H-Pj-0-2 (left), *Fructobacillus tropaeoli* H-Sp-04 (center) and *Fructobacillus pseudoficulneus* H-Pj-0-1 (right).

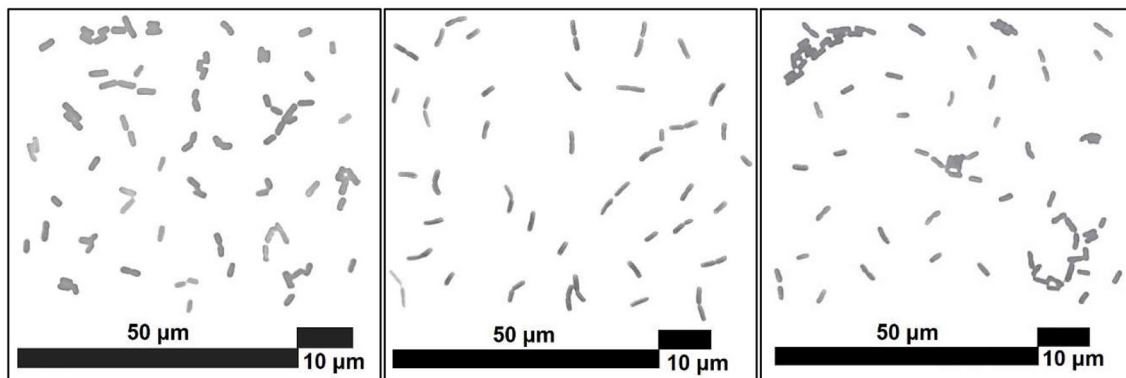


FIGURE 7

Optical microscope micrograph of the *Fructilactobacillus* spp. H-Sp-0-3 (left), *Fructilactobacillus* spp. H-Mb-01 (center) and *Fructilactobacillus* spp. H-Sp-0-2 (right).

Since no bacteria of the genus *Fructobacillus* or *Fructilactobacillus* were found in the research carried out by Torres-Moreno et al. (2021), it is believed that these LAB and FLAB proceed from the environment or the beehive and not from the gastrointestinal tract of honeybees because bacteria of these genera were found in all samples of honey from stingless bees taken from the hives.

3.4.1. Carbohydrate metabolism of LAB isolated from honey samples

F. tropaeoli H-Pj-04, *F. pseudoficulneus* H-Pj-0-1 and *F. pseudoficulneus* H-Sp-0- are FLAB (Table 3) because they grew faster with fructose (one day) than glucose (2 days). Moreover, all the *Fructobacillus* spp. grew in D-xylose (from 3 to 5 days) and with potassium 5-ketogluconate (from 2 to 3 days), and the bacteria of the genus *Fructilactobacillus* usually cannot, although they grow with squalene (in 1 day). Finally, some reports indicated that *Lactobacillaceae* could ferment potassium 5-ketogluconate (Buron-Moles et al., 2019). However, in general, *Fructobacillus* spp. are unable to ferment this carbohydrate (Endo et al., 2011; Ma'unatin et al., 2020). *F. tropaeoli* H-Pj-04 could ferment D-trehalose but not D-raffinose as *F. pseudoficulneus* H-Pj-0-1, H-Pj-0-2 and H-Sp-01.

F. pseudoficulneus H-Sp-0-1 differs from *F. pseudoficulneus* H-Pj-0-1 and H-Pj-0-2 in that it does not ferment mannose or D-turanose. The strain *Fructilactobacillus* spp. H-Sp-0-2 is the only one that can ferment erythritol (within 5 days), and it is also the only one that cannot metabolize methyl- α D-glucopyranoside. The strain *Fructilactobacillus* spp. H-Sp-0-3 differs from the other two strains of the same genus in that it can grow on D-maltose (within 3 days) but does not grow with D-raffinose.

3.4.2. Haemolytic activities and susceptibility to antibiotics of LAB isolated from honey

The ability of seven LAB and FLAB to induce hemolysis *in vitro* was tested. Non-hemolytic activity is considered a safety requirement for biotechnological or probiotic use in the food industry; positive hemolytic activity is considered a typical virulence factor in pathogenic microorganisms (Hazwani Hasali et al., 2018). The results indicated that all bacteria did not exhibit β -hemolytic activity, meaning negative hemolysis or that the bacteria exhibited γ -hemolysis.

The antibiogram (Table 4) reveals that all strains of the genus *Fructilactobacillus* were sensitive to amoxicillin and piperacillin

(both β -lactam antibiotics); azithromycin (macrolide antibiotic); and sulfamethoxazole-trimethoprim (sulfonamide), while all the strains of the genus *Fructobacillus* showed resistance to these antibiotics. Resistance to the antibiotics mentioned above that inhibit beta-lactamase, protein synthesis, and purine binding for DNA formation could be risky for *Fructobacillus* spp. Furthermore, it was found that *F. trophaeli* and *A. kunkaei* isolated from bee gut were resistant to kanamycin, streptomycin, erythromycin and clindamycin, drawing attention to the health industry (Simsek et al., 2022). Furthermore, all strains of the *Fructobacillus* genus of this research have similar results except *F. trophaeli* H-Sp-0-4 with an inhibition diameter of 2 mm for cefuroxime, while all strains of

F. pseudoficulneus were inhibited by 0.5 mm and because it was the only one which was inhibited by ciprofloxacin (0.5 mm). The strain *Fructilactobacillus* spp. H-Sp-0-3 was more sensitive than all the others because it has larger diameters of inhibition, especially with amoxicillin (7 mm), cefixime (5 mm), cefuroxime (5 mm), sulfamethoxazole-trimethoprim (7 mm), and penicillin (3 mm).

According to Hoerr et al. (2016), the sensitivity of bacteria to cephalosporins such as cefazolin and cefuroxime (β -lactam antibiotics more stable against β -lactamases) is due to a fragile cell wall since the mechanism of action of the antibiotic is to inhibit the synthesis and binding of peptidoglycan causing cell lysis. Also, all strains were sensitive to erythromycin, the most used macrolide, since it inhibits the protein synthesis by binding to the 50S subunit of the ribosome, causing bacteriostatic activity. However, azithromycin was only effective for strains of the genus *Fructilactobacillus* spp. This genus was also sensitive to amoxicillin and piperacillin, which are β -lactam derivatives of penicillin that differ from cephalosporin because 7-cephalosporanic acid is replaced with 6-aminopenicillanic acid and they are less stable against β -lactamase, making *Fructobacillus* spp. strains more resistant to antibiotics than strains of *Fructilactobacillus* spp.

Most of the LAB isolates of this research were resistant to ofloxacin and ciprofloxacin (both synthetic quinolone antibiotics of second generation), and penicillin (β -lactam antibiotic). Although first and second-generation quinolones are usually more effective against some Gram-positive bacteria than third and fourth-generation quinolones, these drugs are used mostly for Gram-negative bacteria (Pham et al., 2019). Unfortunately, there is an increasing resistance to penicillin by Gram-positive bacteria, even pathogenic bacteria, because of horizontal spreading of penicillinase plasmids or by horizontal gene transfer that synthesizes penicillin-binding protein (Jubeh et al., 2020). Then, more vigilance is required for these risk factors such as prohibiting the use of antibiotics in beekeeping, although there is a trend to allow the limited presence of antibiotics in honey and honey bee colonies in Belgium, France, United Kingdom, Switzerland, United States of America, Canada, India, and Argentina (Pachla et al., 2021).

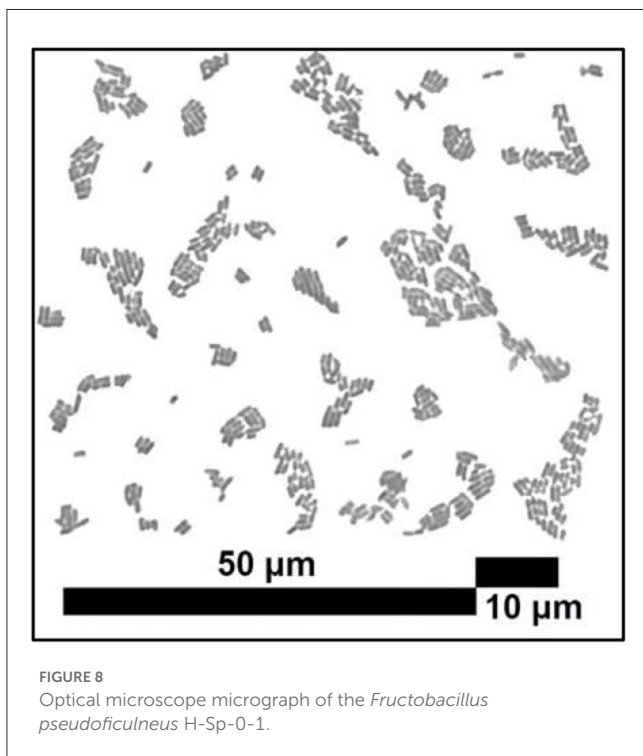


FIGURE 8
Optical microscope micrograph of the *Fructobacillus pseudoficulneus* H-Sp-0-1.

TABLE 3 Results of bacterial growth in anaerobiosis with different carbon sources with the API 50 CHL.

Bacteria	Erythritol (2)	D-xylose (6)	Glucose (11)	Fructose (12)	Mannose (13)	D-mannitol (18)	Methyl- α -D-glucopyranoside (21)	Squaline (25)	D-Maltose (28)	Sucrose (31)	D-Trehalose (32)	D-Raffinose (35)	D-Turanose (40)	Potassium gluconate (47)	Potassium 5-ketogluconate (49)
<i>F. trp</i> ; H-Pj-0-4	-	+5	+2	+1	-	+3	+2	-	+3	+1	+1	-	+3	+2	+2
<i>F. psf</i> ; H-Pj-0-1	-	+3	+2	+1	+5	+3	+5	-	+5	+1	-	+3	+4	+2	+3
<i>F. psf</i> ; H-Pj-0-2	-	+3	+1	+1	+5	+3	+3	-	+3	+1	-	+2	+4	+2	+3
<i>F. psf</i> ; H-Sp-0-1	-	+3	+2	+1	-	+3	+4	-	+5	+1	-	+2	-	+2	+3
<i>Frilb</i> spp.; H-Mb-0-1	-	-	+1	+1	-	+2	+3	+1	-	+1	+2	+2	+4	+2	-
<i>Frilb</i> spp.; H-Sp-0-2	+5	-	+1	+1	-	+2	-	+1	-	+1	+3	+2	+5	+2	-
<i>Frilb</i> spp.; H-Sp-0-3	-	-	+1	+1	-	+1	+2	+1	+3	+2	+1	-	+3	+1	-

(-) means negative growth, (+) means growth and the number means the days elapsed for the positive growth.

TABLE 4 Antibiogram results for bacteria isolated from stingless bee honey.

Antibiotic	<i>F. psf</i> H-Pj-0-1	<i>F. psf</i> H-Pj-0-2	<i>F. psf</i> H-Sp-0-1	<i>F. trp</i> H-Sp-0-4	<i>Ficb</i> spp H-Mb-0-1	<i>Ficb</i> spp H-Sp-0-2	<i>Ficb</i> spp H-Sp-0-3
Amoxicillin (AMX 10)	+	+	+	+	1	2	7
Amoxicillin/clavulanic acid (AMC 20/10)	0.5	+	0.5	+	1	1	7
Cefixime (CFM 30)	0.5	5	0.5	5	+	+	5
Cefazolin (CFZ 30)	1	3	1	3	3	5	4
Cefuroxime (XM 30)	0.5	0.5	0.5	2	1	3	5
Azithromycin (AZ 15)	+	+	+	+	3	2	3
Sulfamethoxazole-trimethoprim (SXT 25)	+	+	+	+	+	1	7
Piperacillin (Pi100)	+	+	+	+	4	1	5
Erythromycin (MS15)	1	1	1	1	4	7	5
Chloramphenicol (S30)	2	2	2	1	2	+	1
Tetracycline (TE 30)	+	0.5	0.5	0.5	2	3	2
Penicillin (Q10)	+	+	+	+	1	+	3
Ciprofloxacin (IC 5)	+	+	+	0.5	+	+	+
Ofloxacin (OF 5)	+	+	+	+	1	+	+
Hemolysis	-/γ	-/γ	-/γ	-/γ	-/γ	-/γ	-/γ

(+) means growth without inhibition, the number means the size of bacterial inhibition diameter and (-) means negative hemolysis or gamma hemolysis.

The safety of LAB concerning antibiotic resistance is due to the possible lateral transfer of antibiotic resistance genes such as tetracycline, erythromycin, and chloramphenicol; clinically relevant antibiotics to which FLAB isolates from this research were sensitive (Li et al., 2019). Nevertheless, it is always necessary to characterize the resistance to antibiotics of LAB for future biotechnological purposes or as probiotics in food to guarantee public health. Therefore, using bacteria that are sensitive to a greater number of antibiotics is recommended so that they are not infectious bacteria.

4. Conclusion

This work shows significant differences between the stingless bee species regarding the physicochemical characteristics of the freshly honey samples collected from the same place simultaneously. Since bees foraged in the same area, these differences can be attributed to the foraging preference of each bee genus and species. Since fresh honey was characterized, the results obtained can be used as quality indicators in Meliponini honey. Furthermore, the antioxidant activity depended on the stingless species because the foraged area was the same. Then, the Meliponini bees can produce honey with a different antioxidant activity.

Finally, a total of seven LAB were identified from the SBH, three of them, of the genus *Fructobacillus*, with fructophilic character (FLAB), three bacteria whose 16S rRNA genes did not match the previously reported species, leaving the possibility that they belong to new species of the genus *Fructilactobacillus*. SBH is a natural sweetener of interest for its healthy properties and beneficial microbiota. However, more studies are necessary to characterize the bacteria present in it, as well as to determine which compounds are the ones that generate its antioxidant activity.

Data availability statement

The original contributions presented in the study are publicly available. The identified strains were added to the GenBank (<https://www.ncbi.nlm.nih.gov/Genbank/>) database with the following access numbers OP941514, OP941513, OP941512, OP941511, OP941515, OP941516, and OP941510.

Author contributions

GM-L: conceptualization, supervision, formal analysis, writing the original draft, writing review, editing, and supervision. AA-V: formal analysis, research, and writing the original draft. HH-S: conceptualization, supervision, writing the original draft, writing review, editing, and supervision. LD-Á: conceptualization and supervision. BP-S and RT-M: research, methodology, and writing the original draft. DH-R: research, methodology, formal analysis, writing the original draft, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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