LWT - Food Science and Technology 140 (2021) 110737

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Honey quality parameters, chemical composition and antimicrobial activity in twelve Ecuadorian stingless bees (Apidae: Apinae: Meliponini) tested against multiresistant human pathogens

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ARTICLE INFO

Keywords:
Stingless bee (Meliponini) honey
Ecuadorian honey
Antioxidant activity
Antibacterial activity
Physicochemical analysis

ABSTRACT

Ecuadorian honey samples of eight genera and 12 stingless bee species from three geographic regions (Andes, Amazon and Pacific) were studied for their physicochemical parameters, chemical composition, mineral elements and antimicrobial activity. Meliponine honey is acidic and has a high water content, but our study revealed substantial chemical variation. *Oxytrigona mellaria* was highest in proteins, while *Melipona* sp. had the most free amino acid content. Five species of honey contained vitamin C, which was highest in *O. mellaria*. The most abundant minerals were the macronutrients, potassium and calcium. All honey inhibited microbial growth in gram-negative and gram-positive multiresistant human pathogens, with *O. mellaria* and *Trigona silvestriana* being most effective against both bacteria.

1. Introduction

Stingless bees are a pantropical group, which are most diversified among countries in the American tropics, with over 400 species in these areas (Vit, Pedro, & Roubik, 2018). They build nests of combined wax, gums, resin and other organic materials (Vit, Pedro, Maza, Ramírez, & Frisone, 2018). Honey or "pot honey" (Vit, Roubik, & Pedro, 2013) is stored in pot-like structures made of those organic constituents, usually in tree hollows or cavities where meliponines maintain colonies of 100 to 100,000 workers. From collected floral and organic ingredients and the glandular, fungal and microbial elements present in the nest and bees, honey acquires its characteristic color, acidity, taste, chemical composition and biological properties (Fletcher et al., 2020; Paludo et al., 2018). These attributes make it different from the honey produced by the introduced Western honeybee, *Apis mellifera* (Apidae: Apinae: Apini), which only recently began to inhabit Neotropical habitats (Avila, Beux, Ribani, & Zambiazi, 2018; Vit et al., 2018).

Although pot honey's characteristics differ from those of *A. mellifera* honey, both have been used in a similar way in food and medicine (Scepankova, Saraiva, & Estevinho, 2017). Honey has been recognized as an important functional food with relevant medical effects. Its functional properties have been attributed mainly to antioxidant capacity

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https://doi.org/10.1016/j.lwt.2020.110737
Received 9 July 2020; Received in revised form 6 December 2020; Accepted 8 December 2020
Available online 10 December 2020

and powerful antimicrobial activity (Luchese, Prudencio, & Guerra, 2017). The phenolic compounds present in honey, together with other components such as amino acids, vitamin C, some carotenoids and reducing sugars, among other minor components, promote antioxidant activity (Santos-Buelga & González-Paramás, 2017). Honey antimicrobial activity is attributed to high sugar content, acidity, the presence of hydrogen peroxide, and the peptide defensin-1 (Proaño et al., 2021; Scepankova et al., 2017). The antimicrobial properties of certain stingless bee honeys have been described and studied, along with potential functional effects (Avila et al., 2018).

Pot honey is traditionally appreciated in Latin America, Africa and Australia, whereas honey from *A. mellifera* is mainly produced and distributed in Europe and Asia (Vit et al., 2018). Compared to honey from *A. mellifera,* the production and commerce of pot honey is extremely low, largely because of the low volumes produced by stingless bees, and thus has limited industrial production. In addition, there is little detailed knowledge about the product and to date, there are no approved international quality standards for stingless bee "pot honey" to regulate its quality and allow its commercialization to be formalized. In Latin America, stingless beekeeping, also known as meliponiculture or "*meliponicultura*", is relatively well established, primarily in Brazil, Costa Rica, Mexico and Venezuela (Vit et al., 2018), while in other countries, despite the presence of 100s of meliponine species, less development has been accomplished. In Ecuador, this activity is still domestic and utilizes common names, often erratically, and without distinction of genera or species (Vit, Pedro, Vergara, & Deliza, 2017; Vit, Vargas, López, & Maza, 2015). However, production is growing, mainly based on regional associations formed by the stingless beekeepers themselves, while in some parts of the Ecuadorian Amazon, pot honey is sold by indigenous people without particular classification. For instance, after harvesting honey of multiple species, the Achuar people preserve it in a mixture for nutritional and health purposes (Guerrini et al., 2009).

Ecuador has an exceptional diversity of stingless bee species throughout the different regions (Roubik, 2018; Vit et al., 2018), which can be used as an economic and socioenvironmental resource. Diverse climates and vegetation characterize the country, making stingless beekeeping possible, as well as the production and commercialization of honey with different characteristics. Although studies of the chemical composition, quality parameters and biological properties of pot honey from different regions of Ecuador have been reported (Guerrini et al., 2009; Vit et al., 2015), they are still insufficient and well below the levels of knowledge available in this field elsewhere. Against this backdrop, our study aimed to determine and to compare the physicochemical parameters, chemical composition, and antimicrobial properties of pot honey samples from different stingless bee species collected at the current production areas of this type of honey in Ecuador.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and solvents were of analytical grade. Milli-Q type 1 water was obtained from the Milli-Q® IQ 7003/05 system purchased from Millipore (Merck, Darmstadt, Germany). Solvents, chelex, Bradford reagent, ninhydrin, L-leucine (reagent grade, ≥98%), ascorbic acid (reagent grade, ≥99%), bovine serum albumin (reagent grade, ≥96%), metaphosphoric acid and KH_2PO_4 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). L-Proline (reagent grade, ≥99%) was purchased from AK Scientific (Union City, CA, USA). Potassium diacid phosphate, ICP multielement standard solution IV (23 elements, 1000 mg L^{-1}) (high-purity starting materials in 10% HNO_3) and ICP TraceCERT® were purchased from Merck. Glucose, fructose and sucrose (reagent grade, ≥99.5%), as well as oxalic acid, lactic acid, citric acid and acetic acid (reagent grade, ≥99.5%), were purchased from Chem Service (West Chester, PA, USA). Tryptic soy broth was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). *Staphylococcus aureus* MRSA 333 was isolated in the Research Laboratories of the Universidad de Las Américas, Quito, Ecuador, from nasal and pharyngeal sources of medical students in Ecuador after obtaining informed consent from the volunteers (Bastidas et al., 2019). *Klebsiella pneumoniae* KPC and *Pseudomonas aeruginosa* multiresistant P28 were donated from the collection of clinical isolates at Zurita & Zurita Clinical Laboratories (http://www.zuritalaboratorios.com) in Quito, Ecuador. The nonpathogenic bacteria, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 33495 and *Pseudomonas aeruginosa* ATCC 27853, were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA).

2.2. Honey and stingless bee samples

Twenty-six stingless bee honey samples were collected in 2018 directly from the artisanal hives of stingless beekeepers in the general pot honey production zones. These were in three geographical regions of mainland Ecuador: the Andean (6 samples), Amazonian (3 samples) and Coastal Pacific (17 samples) regions (Fig. 1S). Samples were donated by stingless beekeepers registered at the Ecuadorian Agency for Agricultural Quality Assurance (AGROCALIDAD, Ecuador). After collection, the honey samples were immediately stored in the dark at 4 °C in airtight sterile containers. In parallel, for each sampled hive, 10 stingless bee individuals were collected and stored in 95% ethanol for transportation and subsequent taxonomic identification.

A solution of artificial honey lacking H_2O_2, which is a normal product of glucose oxidation, consisted of 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose in 17 mL of deionized water. The latter was included as a control to evaluate the contribution of the predominant honey sugars to antimicrobial assay activity (Cooper, Molan, & Harding, 2002).

2.3. Phenotypical and molecular identification of stingless bee individuals

The identification of the stingless bee specimens was performed in parallel to phenotypical and molecular identification. Phenotypical identification was performed by coauthor David W. Roubik by comparing collected individuals to the identified reference specimens maintained at Smithsonian Tropical Research Institute, Panama, and at the Zoological Museum of the Pontifical Catholic University (MCAZ, PUCE) Quito, Ecuador. Voucher specimens are deposited there.

Molecular identification was performed by extracting the DNA of the collected samples using the chelex 10% protocol (Suenaga & Nakamura, 2005) and by PCR amplification of the 5' region of cytochrome oxidase subunit I using LCO1490 and HCO2198 primers (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). Amplification products were sequenced in a Genetic Analyzer 3130 (Applied Biosystems, Waltham, MA, USA) and aligned using the bioinformatics program Mega 7. Sequences were compared with the public *GenBank database* from NCBI (National Center for Biotechnology Information) using the Blast tool and with BOLDSYSTEMS (www.boldsystems.org) for species identification. Molecular and phenotypical identification were compared to achieve final taxonomic identification.

2.4. Physicochemical analysis

The honey polyfloral origins were confirmed following melissopalynological methods (Von Der Ohe, Persano Oddo, Piana, Morlot, & Martin, 2004). Honey was considered unifloral if the relative frequency of the pollen of one specific taxon exceeded 45% (Von Der Ohe et al., 2004). Physicochemical analysis for quality (ashes (g 100 g^{-1}), electrical conductivity (mS cm^{-1}), color (mm Pfund), pH, free acidity (meq kg^{-1}), moisture (g 100 g^{-1}), diastases index (°Gothe) and hydroxymethylfurfural (HMF) content (mg kg^{-1})) was determined by following the International Honey Commission's official methods (Bogdanov, 2009). Reducing sugars were determined by a colorimetric assay using 3,5-dinitrosalicylic acid (Fox, Gray, Dunn, & Marsden, 1984), and the

results are expressed as g 100 g^{-1} of honey.

2.5. Chemical composition of stingless bee honey

2.5.1. Total protein and free amino acid content

The protein content was determined following the Bradford method (Bradford, 1976). Bovine serum albumin was used for the calibration curve in a range of 0.25–14 μg mL^{-1} (y = 0.0382x + 0.0162, R^2 = 0.9921) in deionized water. The protein content of each honey sample was expressed as mg of bovine serum albumin (BSA) per gram of honey (mg BSA g^{-1} of honey).

The free amino acid content was determined following the Cd-ninhydrin method (Doi, Shibata, & Matoba, 1981). A sample of 0.1 g of honey was diluted in 1 mL of deionized water and mixed with 2 mL of the working solution (0.8 g of Ninhydrin, 80 mL of ethanol, 10 mL of acetic acid and 1 g of $CdCl_2$). The reaction mixture was incubated in a water bath at 84 °C for 5 min and then cooled in ice, and the absorbance was read spectrophotometrically at 507 nm using a Shimadzu UV 1240 UV–visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). L-Leucine was used for the preparation of a calibration curve in a range of 15–42 mg mL^{-1} (y = 0.0086x + 0.0217, R^2 = 0.9851), and L-proline was used for another calibration curve in a range of 0.001–1 mg mL^{-1} (y = 1.5359x + 0.0725, R^2 = 0.9028). The free amino acid content was expressed as mg of L-leucine (Leu) per 100 g of honey (mg Leu 100 g^{-1} of honey) and mg of L-proline (Prol) per kilogram of honey (mg Prol kg^{-1} of honey).

2.5.2. HPLC-DAD analysis for vitamin C content

Vitamin C content was determined as previously reported (Alvarez-Suarez, González-Paramás, Santos-Buelga, & Battino, 2010). Samples (1 g) were diluted in 5 mL of metaphosphoric acid (5 g 100 mL^{-1}), and 20 μL was injected onto an HPLC system consisting of a modular 1260 Agilent Technologies HPLC unit (CA, USA) equipped with a quaternary pump and DAD detector set at 245 nm. The stationary phase comprised an Agilent Eclipse Plus C18 (4.6 × 150 mm, 5 μm) (CA, USA) column. Elution was performed in an isocratic gradient with potassium diacid phosphate at a flow rate of 1 mL min^{-1} for 10 min. For quantitative analysis, a calibration curve was obtained using an external standard of ascorbic acid at a range of 5–50 mg L^{-1} (y = 370.65x + 153.23, R^2 = 0.998, LOD: 0.032 μg g^{-1} and LOQ: 0.107 μg g^{-1}), and the results are expressed as μg of Vit C per gram honey (μg Vit C g^{-1} of honey).

2.5.3. HPLC-RI analysis of sugar content

The sugar content was determined using an HPLC system with a refractive index detector (HPLC-RI Detector) (Doyon, Gaudreau, St-Gelais, Beaulieu, & Randall, 1991). Samples (500 mg) were diluted in 25 mL deionized water and filtered through a 45 mm Minisart syringe filter (0.26 mm) (Rephile Co., Hubei, China), and 20 μL was injected onto the HPLC system consisting of a modular 1260 Agilent Technologies HPLC unit (CA, USA) equipped with a quaternary pump and refractive index detector. The stationary phase involved a Zorbax NH2 (250 × 4.6 mm, 5 μm) column. Elution was performed with a solution of sulfuric acid (0.5 g L^{-1}) in an isocratic gradient at a flow rate of 1 mL min^{-1} for 55 min. For the quantitative analysis, a calibration curve was obtained using external standards for glucose (y = 4E-05x + 0.0019, R^2 = 0.9771), fructose (y = 56993x – 281.96, R^2 = 0.9606) and sucrose (y = 30514x + 201.38, R^2 = 0.9772) at a range of 0.005–0.070 mg mL^{-1}. The results are expressed as g 100 g^{-1} of honey.

2.5.4. HPLC analysis of organic acids

Organic acids were determined using an HPLC system (Nour, Trandafir, & Ionica, 2010). Samples (1 g) were diluted in 25 mL of distilled water, and 10 μL of this aqueous fraction was injected onto the HPLC system. This system consisted of a modular 1260 Agilent Technologies HPLC unit (CA, USA) with a quaternary pump and DAD detector set at 210 nm. The stationary phase involved a Zorbax Eclipse Plus C18 (4.6 × 250 mm, 5 μm) (Agilent, CA, USA) column. Elution was performed with potassium diacid phosphate (Merck, Germany) in an isocratic gradient at a flow rate of 0.7 mL min^{-1} for 15 min. For the quantitative analysis, a calibration curve was obtained using external standards for oxalic acid at a range of 5–75 mg L^{-1} (y = 8E-06x-0.0021, R^2 = 0.9994, LOD: 0.027 mg 100 g^{-1} and LOQ: 0.091 mg 100 g^{-1}), lactic acid at a range of 1–60 mg L^{-1} (y = 5429.4x+ 8.3139, R^2 = 0.9992, LOD: 0.008 mg 100 g^{-1} and LOQ: 0.026 mg 100 g^{-1}), acetic acid at a range of 1–60 mg L^{-1} (y = 4190.2x + 5.4518, R^2 = 0.9978, LOD: 0.007 mg 100 g^{-1} and LOQ: 0.024 mg 100 g^{-1}) and citric acid at a range of 1–60 mg L^{-1} (y = 14892x-26.278, R^2 = 0.9883, LOD: 0.004 mg 100 g^{-1} and LOQ: 0.014 mg 100 g^{-1}). The results are expressed as mg 100 g^{-1} honey.

2.5.5. Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis for mineral elements

For the analysis, sample digestion was performed following application note 43,060 of the ICP-OES system supplier (Price, 2012). Briefly, honey samples (0.5 g) were weighed into clean, dry Teflon microwave digestion vessels using a glass pipette to deposit honey directly onto the base of the digestion vessel. Then, 7 mL of nitric acid (>63%, trace metal grade) and 3 mL of hydrogen peroxide (>30% w/v, trace metal grade) were added and left uncovered for 15 min. The samples were sealed and digested in a closed MARS 6-Microwave Accelerated Reaction System (Smith Farm Road, Matthews, USA) via temperature ramping (ramped to 120 °C for 10 min, held for 5 min, then ramped to 200 °C for 10 min, then held for 15 min). A sample blank containing only nitric acid and hydrogen peroxide was prepared in the same way. To avoid any type of contamination (as in the case of aluminum), plastic or glass utensils were used throughout the processing of the samples (sampling, weighing and analysis).

The analyses were carried out using an ICP-OES system (iCAP™ 7400 Duo ICP-OES Analyzer, Thermo Scientific™, Germany) equipped with Thermo Scientific™ Qtegra™ Intelligent Scientific Data Solution™ (ISDS) software. Quality assurance and quality control were assessed using an ICP multi-element standard solution IV (23 elements: Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Ti, and Zn; 1000 mg L^{-1}). Periodic table mix 1 for ICP TraceCERT® was used as a standard. Eleven metals were detected and divided into three main groups: contaminants (Cu, Ni, Al and Pb), macronutrients (K, Mg and Ca) and micronutrients (Mn, Zn, Fe and Na). The results are expressed as mg g^{-1} of honey.

2.6. Antimicrobial activity

The minimal inhibitory concentration (MIC) of honey samples was determined according to the Clinical & Laboratory Standards Institute (CLSI) methods, A07-A10 guidance (Helyar et al., 2010). For susceptibility assays, the human multiresistant pathogens, *Staphylococcus aureus* MRSA 333 (gram-positive), *Klebsiella pneumoniae* KPC (gram-negative) and *Pseudomonas aeruginosa* multiresistant P28 (gram-negative), were used. The nonpathogenic bacteria, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 33495 and *Pseudomonas aeruginosa* ATCC 27853, were used as controls for each assay.

Bacterial strains were cultivated at 37 °C for 18 h in tryptic soy broth. Then, a 0.5 McFarland scale was made of each microorganism, and 10 μL of each bacterial dilution was added to 100 μL of each honey or artificial honey concentration (20, 15, 10, 8, 5, 3, 2, and 1 g 100 mL^{-1}) diluted in 50 mol L^{-1} (KH_2PO_4, pH 6.5 buffer) on microtitration plates (Nunclon™ Delta Surface, Thermo Scientific, Denmark) in triplicate. The plates were incubated at 37 °C for 18 h, and then complete growth inhibition was determined at 340 nm using a Synergy HT microplate reader (BioTek Instruments, VT, USA). The MIC was considered the lowest honey dilution at which microbial growth was completely inhibited, and the results were expressed as percentages of honey concentration that inhibited the total growth of bacteria.

2.7. Statistical analysis

The normality (Shapiro-Wilk) and homogeneity (Levene test) of the variances were verified for all variables. The results are presented as the mean ± standard error. One-way analysis of variance (ANOVA) with a Bonferroni post hoc test was used to determine significant differences ($P < 0.05$) between honey samples. Correlations between variables were calculated using Pearson's correlation test. All analyses were performed using the IBM SPSS Statistics 22.0.0 program (IBM, Armonk, NY, USA).

3. Results and discussion

3.1. Phenotypical and molecular identification of stingless bee specimens

Stingless bee specimens and honey (twenty-six multifloral honey samples) were collected from five Ecuadorian provinces from three geographical regions (Andean, Amazonian and Coastal Pacific) during 2018. Table 1S shows the geographic origin and taxonomic identification. A total of 8 genera and 12 species were identified (*Cephalotrigona* sp., *Melipona indecisa*, *Melipona cramptoni, Melipona mimetica, Melipona grandis, Melipona* sp., *Nannotrigona chapadana, Oxytrigona mellaria, Paratrigona* sp., *Scaptotrigona polysticta*, *Tetragonisca angustula* and *Trigona silvestriana*). El Oro Province provided most of the sampled species, specifically nine of the 12 that were assayed. Although Ecuador certainly has over 100 honey-producing stingless bee species and 24 genera (Roubik, 2018), the most common bees used by beekeepers in our study were *Melipona* spp. and *Scaptotrigona* spp., in agreement with previous studies on stingless bee honey from the Ecuadorian Amazon (Guerrini et al., 2009).

3.2. Physicochemical parameters

Table 1 presents the physicochemical parameters of the honey samples produced by the different stingless bee species studied. Compared to *Apis mellifera* honey, where quality criteria are well defined in international legislation (European Commission, 2002), at present, there are no international standards to regulate the quality parameters of stingless bee honey. There is only one proposal for a standard (Vit, Medina, & Enríquez, 2004), which is used as a reference, alongside the published results of the different investigations of Meliponini honey. Compared to that of *A. mellifera,* the authors provide comparable data that have allowed a definition of distinctive characteristics of this honey. According to the proposed standard, the maximum water content of honey stored by Meliponini can be up to 30 g 100 g^{-1} (Vit et al., 2004). This high content has been directly related to the humid tropical environment these species inhabit, as well as nectar collections from flowers in the forest undergrowth and ripe fruits, the latter being richer in water. Other contributing factors are climatic conditions, the degree of nectar ripening reached in the bee nest, handling during the harvest period, storage conditions (including the antimicrobial properties of honey and resident microbes), and the different flora visited by bees (Vit et al., 2018). The water content in our honey samples ranged from 22.8 g 100 g^{-1} (*S. polysticta*) to 30 g 100 g^{-1} (*Paratrigona* sp., *Cephalotrigona* sp., *T. silvestriana* and *O. mellaria*) (Table 1). This finding is in agreement with Guerrini et al. (2009), who reported fluid consistency and a high water content (34 g 100 g^{-1}) in stingless bee honeys from the Ecuadorian Amazon region. Stingless bee honey from other geographic regions and species has also been found to have a high water content, ranging from 13.3 to 56.3 g 100 g^{-1} (Avila et al., 2018). Interestingly, we found a significant negative correlation ($P \leq 0.01$) between moisture and free amino acids (Leu, $r = -0.547$ and Prol, $r = -0.416$) and organic acids (LacA, $r = -0.663$; AcA, $r = -0.470$ and CitA, $r = -0.534$) (Table 2).

Excess water in honey can be a negative quality attribute since it creates a high risk of inducing fermentative processes and subsequently altering organoleptic properties, physicochemical parameters, chemical composition and functional characteristics. Stingless bee honey has been characterized as being more acidic than *A. mellifera* honey (Alvarez-Suarez et al., 2018; Chuttong, Chanbang, Sringarm, & Burgett, 2016; Vit et al., 2004). We found acidic pH values for all the analyzed honey samples. They ranged from 3.08 (*M. cramptoni*) to 3.58 (*Paratrigona* sp.) (Table 1). Our results are within the range reported for stingless bee honey from different geographical regions and species (Avila et al., 2018). In parallel to pH, free acidity is another parameter that plays an important role in honey quality and freshness. The suggested standard

Table 1
Average values of data obtained in physicochemical parameters analyzed in stingless bee honey from Ecuador.

Stingless bee species	n	Ashes (g 100 g^{-1})	Electrical conductivity (mS cm^{-1})	Color (mm Pfund)	pH	Free Acidity (meq kg^{-1})	Moisture (g 100 g^{-1})	Diastase index (°Gothe)	HMF (mg kg^{-1})	Reducing sugars (g 100 g^{-1})
S. polysticta	7	0.84 ± 0.07^{a}	0.23 ± 0.01^{a}	52.74 ± 1.96^{a}	3.55 ± 0.09^{a}	63.36 ± 4.17^{a}	22.00 ± 0.01^{a}	17.50 ± 1.96^{a}	30.48 ± 4.15^{a}	73.11 ± 1.32^{a}
M. indecisa	6	0.40 ± 0.04^{c}	0.14 ± 0.01^{b}	19.83 ± 1.53^{b}	3.32 ± 0.06^{b}	44.21 ± 4.42^{b}	27.33 ± 0.23^{b}	17.44 ± 1.71^{a}	18.16 ± 3.82^{b}	73.67 ± 1.02^{a}
M. cramptoni	3	0.40 ± 0.03^{c}	0.13 ± 0.01^{b}	16.30 ± 1.17^{c}	3.08 ± 0.01^{c}	40.46 ± 3.03^{b}	26.33 ± 0.44^{b}	16.44 ± 3.46^{a}	24.06 ± 6.32^{a}	81.67 ± 4.56^{b}
Paratrigona sp.	1	0.11 ± 0.00^{b}	0.16 ± 0.00^{b}	24.29 ± 2.54^{b}	3.58 ± 0.01^{a}	46.53 ± 0.01^{b}	27.00 ± 0.00^{b}	8.33 ± 0.00^{b}	3.00 ± 0.00^{c}	70.90 ± 1.52^{a}
Melipona sp.	1	0.29 ± 0.01^{c}	0.11 ± 0.00^{b}	13.97 ± 0.00^{c}	3.33 ± 0.03^{b}	32.08 ± 0.01^{c}	27.00 ± 0.00^{b}	30.00 ± 0.00^{c}	7.48 ± 0.00^{d}	81.08 ± 0.35^{b}
Cephalotrigona sp.	1	0.35 ± 0.00^{c}	0.21 ± 0.00^{a}	55.11 ± 0.11^{a}	3.34 ± 0.07^{b}	116.47 ± 0.20^{d}	30.00 ± 0.00^{b}	10.71 ± 0.00^{b}	50.40 ± 0.50^{e}	67.17 ± 0.56^{a}
M. mimetica	1	0.41 ± 0.01^{c}	0.38 ± 0.28^{c}	47.24 ± 0.33^{a}	3.45 ± 0.00^{d}	20.26 ± 0.20^{e}	27.00 ± 0.00^{b}	17.65 ± 0.00^{a}	32.43 ± 0.50^{a}	71.94 ± 0.27^{a}
T. angustula	3	0.69 ± 0.21^{e}	0.31 ± 0.08^{c}	43.41 ± 0.87^{d}	3.51 ± 0.17^{a}	70.55 ± 1.01^{f}	25.50 ± 1.12^{b}	40.00 ± 8.94^{d}	27.70 ± 0.28^{a}	67.40 ± 1.09^{a}
T. silvestriana	1	0.51 ± 0.01^{c}	0.12 ± 0.00^{b}	65.10 ± 0.11^{e}	3.41 ± 0.05^{d}	61.06 ± 0.04^{a}	30.00 ± 0.00^{b}	13.04 ± 0.00^{b}	7.65 ± 0.17^{d}	66.29 ± 1.18^{a}
O. mellaria	1	0.52 ± 0.01^{c}	0.14 ± 0.00^{b}	43.25 ± 0.19^{d}	3.11 ± 0.00^{e}	58.84 ± 0.04^{a}	30.00 ± 0.00^{b}	15.00 ± 0.00^{b}	5.99 ± 0.00^{d}	62.62 ± 1.23^{b}
N. chapadana	1	0.40 ± 0.00^{c}	0.10 ± 0.00^{b}	52.79 ± 0.11^{a}	3.18 ± 0.00^{e}	42.07 ± 0.03^{b}	24.00 ± 0.00^{a}	12.00 ± 0.00^{b}	10.98 ± 0.50^{d}	77.11 ± 1.01^{b}
M. grandis	1	0.30 ± 0.00^{d}	0.18 ± 0.01^{a}	42.14 ± 0.29^{d}	3.12 ± 0.01^{e}	60.35 ± 0.03^{a}	25.00 ± 0.00^{a}	12.00 ± 0.00^{b}	56.72 ± 0.17^{e}	82.63 ± 0.55^{b}

Samples were analyzed in triplicate and data are presented as means ± standard error. Mean values within a column with different letters are significantly different for $P < 0.05$.

Table 2
Correlation matrix (Pearson's correlation coefficients) for quantitative determinations in the different stingless bee honey from Ecuador.[a]

	pH	FA	Ash	Clr	EC	Moist	Dse	HMF	RS	TProt	Leu	Prol	VitC	TSgr	OxA	LacA	AcA	CitA
pH	–	-.139	.719**	.318**	.386**	-.401**	-.086	−0.178	-.267*	.007	.484**	.474**	-.180	-.033	-.065	.229*	.407**	.557**
FA	–	–	.030	.436**	.206	-.153	.085	.301**	-.302**	.266*	.031	-.043	.037	.153	.082	.494**	.197	.338**
Ash	–	–	–	.412**	.381**	-.645**	.029	.102	-.218	.015	.519**	.523**	-.017	-.057	.045	.486**	.521**	.624**
Clr	–	–	–	–	.294**	-.505**	-.118	.208	-.380**	.632**	.287*	.229*	.039	.008	.346**	.409**	.255*	.490**
EC	–	–	–	–	–	-.325**	-.015	.145	-.221	.056	.140	.109	-.081	-.056	-.042	.473**	.527**	.448**
Moist	–	–	–	–	–	–	.115	-.148	-.194	.027	-.547**	-.416**	.224*	.086	.167	-.663**	-.470**	-.534**
Dse	–	–	–	–	–	–	–	-.171	.028	.181	.048	.064	-.071	.335**	.118	.023	-.071	-.180
HMF	–	–	–	–	–	–	–	–	-.060	.012	-.015	-.003	-.164	-.155	.039	.071	.138	.182
RS	–	–	–	–	–	–	–	–	–	-.437**	.104	.057	-.276*	-.017	-.303**	-.098	-.129	-.165
TProt	–	–	–	–	–	–	–	–	–	–	-.138	-.135	.296**	.019	.609**	.079	-.073	-.047
Leu	–	–	–	–	–	–	–	–	–	–	–	.963**	-.118	.345**	-.325**	.368**	.159	.462**
Prol	–	–	–	–	–	–	–	–	–	–	–	–	-.095	.373**	-.270*	.249*	.134	.461**
VitC	–	–	–	–	–	–	–	–	–	–	–	–	–	.073	.312**	-.028	-.080	-.095
TSgr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.018	-.208	-.216	.094
OxA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	-.127	.043	-.155
LacA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.685**	.489**
AcA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.692**
CitA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Except in the diagonal, the values at the level of significance are reported. FA, free acidity; Clr, Color; EC, electrical conductivity; Moist, moisture; Dse, Diastase activity; HMF, hydroxymethylfurfural; RS, reducing sugars; TProt, total proteins; Leu, leucine; Prol, proline; VitC, vitamin C content; DPPH, DPPH assay for total antioxidant capacity; TSgr, total sugar content; OxA, oxalic acid; LacA, lactic acid; AcA, acetic acid; CitA, citric acid. a 95% confidence interval. * Significant at $P \leq 0.05$; ** Significant at $P \leq 0.01$.

for free acidity in stingless bee honey is a maximum of 85 meq 100 g^{-1} (Vit et al., 2004). Our results showed that except for *Cephalotrigona* sp. honey (116.47 meq 100 g^{-1}), all honeys were below the recommended limit, with average values ranging between 20.26 and 70.55 meq 100 g^{-1} (Table 1). This is in agreement with previous studies, which proposed that free acidity in stingless bee honey varies according to species and geographic origin (Vit et al., 2018). Guerrini et al. (2009) reported values in Ecuadorian stingless bee honey that were on average 31.8 meq 100 g^{-1}, while in samples from other geographic regions and stingless bee species, the determined values ranged from 19.90 to 139 meq kg^{-1} (Avila et al., 2018). The acid-to-base comparative pH showed a high correlation ($P \leq 0.01$) with ash ($r = 0.719$), color (Clr, $r = 0.318$), electrical conductivity (EC, $r = 0.386$), free amino acids (Leu, $r = 0.484$ and Prol, $r = 0.474$), and organic acids (LacA, $r = 0.229$; AcA, $r = 0.407$; CitA, $r = 0.557$). In addition, a positive correlation ($P \leq 0.01$) was found between free acidity and color ($r = 0.436$), HMF ($r = 0.301$) and the organic acids LacA ($r = 0.494$) and CitA ($r = 0.338$), while a negative correlation was found with reducing sugars (RS, $r = -0.302$, $P \leq 0.01$) (Table 2).

Acidity in honey, as well as its increase during fermentation, is associated with the transformation of honey sugars and alcohols into acids by honey osmophilic yeasts. When honey moisture content is high enough, the yeast will grow, thereby fermenting sugars and making more yeast, alcohol, carbon dioxide and acetic acid, all of which affect honey flavor over time (Aina et al., 2020). A high concentration of acetic acid can result in an undesirable vinegary flavor in honey. Lactic acid bacteria (LAB) influence acidity and fermentation in honey and have even been related to their antimicrobial activity (Aween et al., 2012; Bulgasem, Lani, Hassan, Wan Yusoff, & Fnaish, 2016; Mahnot, Saikia, & Mahanta, 2019; Olofsson et al., 2016). In fact, researchers have reported 13 taxonomically well-defined *Lactobacillus* (9 spp.) and *Bifidobacterium* (4 spp.) (13 LAB symbionts) from the honey stomach of honeybees in large concentrations in fresh honey (Olofsson et al., 2016). Furthermore, a recent study of honey from several stingless bees (*Heterotrigona itama, H. erythrogastra, Tetrigona apicalis, T. melanoleuca, T. binghami, Lepidotrigona terminata, Geniotrigona thoracica* and *Homotrigona fimbriata*) reported that the seven main bacteria in honey were *Lactobacillus*, with *L. malefermentans* being the most abundant. Other bacteria identified were *L. johnsonii, L. wasatchensis, L. amylovorus, L. pentosiphilus* and *L. salivarius* (Rosli et al., 2020). These LAB produce common metabolites, such as formic acid and lactic acid, which directly affect honey acidity and are even used as indicators of fermentation (Mato, Huidobro, Simal-Lozano, & Sancho, 2006). Given the direct contribution of organic acids to honey pH and quality, the profile and content of selected organic acids in honey were of interest (Table 3). Fig. S2 A (supplementary material) shows a representative chromatogram of the organic acid profile in the studied honeys. Although there are reports of free acidity in stingless bee honey, few studies have examined the profile and content of organic acids (da S. Sant'ana, de Carvalho, OdaSouza, de A. Souza, & de S. Dias, 2020). In our samples, oxalic acid ranged from 0.24 mg 100 g^{-1} (*M. cramptoni*) to 0.73 mg 100 g^{-1} (*M. grandis*). Lactic acid was not detected in *Paratrigona* sp., *Melipona* sp. or *M. mimetica* honey, and acetic acid was not detected in *M. mimetica*. The highest concentrations of acetic acid (1.19 mg 100 g^{-1}) and lactic acid (0.72 mg 100 g^{-1}) were detected in *T. angustula* honey, while the highest values of citric acid were found in *Cephalotrigona* sp. honey (0.68 mg 100 g^{-1}). Regarding the total content of organic acids, *T. angustula* honey showed the highest concentration, followed by *S. polysticta*, *N. chapadana*, *Cephalotrigona* sp. and *T. silvestriana*. These honeys also had higher values of free acidity, whereby there was a significant correlation between free acidity and total organic acid content ($r = 0.4915$, $P \leq 0.01$).

Hydroxymethylfurfural (HMF) and diastase are also important indicators of honey quality and freshness. Under normal conditions, HMF is absent in honey, yet conditions such as processing or aging, mainly influenced by temperature fluctuation, pH, storage conditions and floral origin, may help bring about its presence (Machado De-Melo,

Table 3
Total organic acids content in stingless bee honey from Ecuador.

Singless bee species	n	Organic acids (mg 100 g^{-1})				
		Oxalic acid	Lactic acid	Acetic acid	Citric acid	Total
S. polysticta	7	0.31 ± 0.03^{a}	0.69 ± 0.07^{a}	0.41 ± 0.04^{a}	0.40 ± 0.05^{a}	1.81 ± 0.22^{a}
M. indecisa	6	0.30 ± 0.03^{a}	0.13 ± 0.05^{b}	0.18 ± 0.05^{b}	0.10 ± 0.03^{b}	0.71 ± 0.04^{b}
M. cramptoni	3	0.24 ± 0.00^{a}	0.09 ± 0.04^{bc}	0.09 ± 0.02^{c}	0.12 ± 0.02^{b}	0.54 ± 0.03^{b}
Paratrigona sp.	1	0.40 ± 0.01^{b}	*ND*	0.06 ± 0.01^{c}	0.13 ± 0.01^{b}	0.59 ± 0.06^{b}
Melipona sp.	1	0.50 ± 0.01^{b}	*ND*	0.06 ± 0.00^{c}	*ND*	0.56 ± 0.00^{b}
Cephalotrigona sp.	1	0.44 ± 0.00^{b}	0.04 ± 0.01^{c}	0.06 ± 0.00^{c}	0.68 ± 0.00^{c}	1.22 ± 0.03^{c}
M. mimetica	1	0.37 ± 0.05^{b}	*ND*	*ND*	0.07 ± 0.01^{b}	0.44 ± 0.00^{b}
T. angustula	3	0.65 ± 0.02^{c}	0.72 ± 0.28^{a}	1.19 ± 0.51^{d}	0.44 ± 0.19^{a}	3.00 ± 0.02^{d}
T. silvestriana	1	0.69 ± 0.02^{c}	0.16 ± 0.01^{b}	0.24 ± 0.01^{b}	0.13 ± 0.01^{b}	1.22 ± 0.01^{c}
O. mellaria	1	0.72 ± 0.01^{c}	0.28 ± 0.01^{d}	0.06 ± 0.01^{c}	0.12 ± 0.01^{b}	1.18 ± 0.02^{c}
N. chapadana	1	0.63 ± 0.10^{c}	0.33 ± 0.00^{d}	0.19 ± 0.01^{b}	0.12 ± 0.01^{b}	1.27 ± 0.00^{c}
M. grandis	1	0.73 ± 0.02^{c}	0.17 ± 0.01^{b}	0.15 ± 0.00^{b}	0.03 ± 0.01^{d}	1.08 ± 0.04^{e}

Samples were analyzed in triplicate and data are presented as means ± standard error. Mean values within a column with different letters are significantly different for $P < 0.05$. ND, non-detected.

Almeida-Muradian, Sancho, & Pascual-Maté, 2017). The HMF values in the honey analyzed ranged from 56.72 mg kg^{-1} in *M. grandis* to 3 mg kg^{-1} of honey in *Paratrigona* sp., with an average of 24 mg kg^{-1}. According to the recommended limit proposed, the maximum recommended HMF value for stingless bee honey is 40 mg kg^{-1} (Shapla, Solayman, Alam, Khalil, & Gan, 2018; Thrasyvoulou et al., 2018; Vit et al., 2004). In our results, only the honey from *Cephalotrigona* sp. (50.40 mg kg^{-1}) and *M. grandis* (56.72 mg kg^{-1}) exceeded the recommended limit proposed for this type of honey (40 mg kg^{-1}), while the rest were within the recommended parameters, similar to other studies that also found values within the suggested quality range (Avila et al., 2018). Although in some honey samples HMF levels were found to be outside the allowed range (<40 mg kg $^{-1}$, in two honeys), these still represent low values. They are below the accepted limits for A. mellifera tropical honeys (80 mg kg $^{-1}$). Therefore, they are still within the recommended limits so as not to cause toxicity in consumers. Meanwhile, the results obtained from diastase activity showed that all the honey analyzed was within the recommended range, i.e., a minimum of 7 °Gothe (Vit et al., 2004), varying from 8.33 °Gothe in *Paratrigona* sp. to 40 °Gothe in *T. angustula*, with a mean value of 18.28 °Gothe. The diastase index, previously reported in stingless honey from different geographical and species origins, has values from 1.3 to 49.60 °Gothe (Avila et al., 2018). Like HMF, diastase activity can also be affected by factors such as processing, aging, temperature, storage conditions, and floral origin. Hence, the wide variability in the values found herein, as well as those reported by other authors, could be related to one or more of these factors. Nonetheless, the diastase activity values found in our study allowed us to evaluate the quality and freshness of the honey samples.

Ash content (mineral content) is an important quality parameter to be considered in honey. Blossom honey from *A. mellifera* should have a maximum mineral content of 0.6 g 100 g^{-1}, according to the recommended standards that suggest that honey within these values has a nectar origin (European Commission, 2002). Stingless bee honey should have the same value according to a suggested standard (Vit et al., 2004). In our study, the ash content ranged from 0.12 g 100 g^{-1} (*Paratrigona* sp.) to 0.84 g 100 g^{-1} (*S. polysticta*), with a mean value of 0.53 g 100 g^{-1}. Ash contents previously reported for Ecuadorian stingless bee honey showed an average of 0.28 g 100 g^{-1} (Guerrini et al., 2009), while other studies from different geographic regions and stingless bee species ranged between 0.07 and 3.10 g 100 g^{-1} (Avila et al., 2018). In our study, honey from *S. polysticta* and *T. angustula* exceeded the recommended value. Usually, ash content in honey is low and is related to the kind of plant and the soil where the plant was grown, predisposing a wide variability in ash levels (Andrade et al., 1999). Some studies also suggest that the variability in ash content of stingless bee honey could be related to the bee species (Vit, 2013) as well as harvesting practices; thus, this parameter can be useful for determining good manufacturing practices (Bogdanov, 2009). The ash content in honey has been directly associated with electrical conductivity (EC), which reveals the presence of ions, organic acids and proteins (Machado De-Melo et al., 2017). This parameter has been used as a honey quality indicator and a criterion for confirming floral origin (Bogdanov, 2009). EC in the analyzed honey showed great variability among the species, with values ranging from 0.10 mS cm^{-1} (*N. chapadana*) to 0.38 mS cm^{-1} (*M. mimetica*). All of the values were below the limit required for *A. mellifera* (European Commission, 2002), established at no more than 0.80 mS cm^{-1}, suggesting that the studied honeys have a nectar origin. Similar to a previous study (Alvarez-Suarez, Tulipani, et al., 2010), ash content was significantly correlated ($P \leq 0.01$) with color (Clr, $r = 0.412$), although a positive correlation was also found with EC ($r = 0.381$), free amino acids (Leu, $r = 0.519$ and Prol, $r = 0.523$) and organic acids (LacA, $r = 0.486$; AcA, $r = 0.521$ and CitA, $r = 0.624$), while a negative correlation ($P \leq 0.01$) was observed between ash and moisture (Moist, $r = -0.645$) ($P \leq 0.01$).

Color is a physical property of honey that is mostly related to botanical origin, mineral content, phenolic content, antioxidant properties, storage temperature and storage time (Bertoncelj, Doberšek, Jamnik, & Golob, 2007). The floral origin of honey largely determines its physical and chemical properties, which is sometimes used in monofloral honey for the establishment of a range of colors deemed diagnostic of a specific floral origin (Machado De-Melo et al., 2017). However, in multifloral honey, this relationship becomes more complex since there is no predominance of a specific floral species. On a color basis, our study showed great variability among species, as they were classified from light amber in *T. silvestriana* (65.10 mm Pfund) to extra white in *Melipona* sp. (13.97 mm Pfund). Stingless bee honey is usually classified as multifloral, so only a few studies have reported the color of honey, e.g., a report on Cuban stingless bee honey (41.65 mm Pfund, extra light amber) (Alvarez-Suarez et al., 2018) and one on Ecuadorian honey (150 mm Pfund, dark amber) (Guerrini et al., 2009). Color was significantly correlated ($P \leq 0.01$) with EC ($r = 0.294$), moisture (Moist, $r = -0.505$), total protein content (TProt, $r = 632$), and the organic acids, OxA, LacA and CitA ($r = 0.346$, $r = 0.409$ and $r = 0.490$, respectively).

Sugars are the principal components of stingless bee honey and depend mostly on the floral resources used by bees to make their honey (Biluca, Braghini, Gonzaga, Costa, & Fett, 2016). The main sugars in honey are fructose and glucose (Machado De-Melo et al., 2017). The recommended standard proposed for reducing sugar content in stingless bee honey is a minimum of 50 g 100 g^{-1} (Vit et al., 2004). The reducing sugar content in our samples ranged from 62.62 g 100 g^{-1} (*O. mellaria*) to 82.63 g 100 g^{-1} (*M. grandis*), thus fulfilling the recommended standard. Since stingless bee honey is commonly multifloral, the variation in this parameter depends on natural sources, and it is, therefore, difficult to consider it a differentiating factor for types of honey or the producing species. On the other hand, the nature and characteristics of the methods used must also be considered. The dinitrosalicylic acid (DNS) method used here gives a rapid and simple estimation of the extent of saccharification by measuring the total amount of reducing sugars in the sample. However, it is subject to interference by citrate buffer and other substances, such as amino acids, and by the differing reactivities of the various reducing sugars. In fact, it has been reported that amino acid interference in the quantification of reducing sugars using the 3,

5-dinitrosalicylic acid assay misleads carbohydrase activity measurements (Teixeira, Da Silva, Ferreira-Leitão, & Da Silva Bon, 2012). Moreover, in this method, a reaction by temperature change is used, which can also affect the results. All these interferences become more apparent upon examining complex substrates, such as honey.

3.3. Chemical composition

The contents of amino acids and proteins, vitamin C and sugars (Table 4) were also analyzed in the honey of the different stingless bees, as well as those of mineral contaminants, macronutrients and micronutrients (Table 5).

Total protein contents varied from 0.02 mg g^{-1} (*M. cramptoni*) to 0.37 mg g^{-1} (*O. mellaria),* with a mean of 0.16 mg BSA g^{-1}. Avila et al. (2018), in a study of stingless bee honey from different geographical regions, also found a great variety in total protein content (0.12–3.10 mg g^{-1}), which was attributed to the differences between bee species and pollen content. On the other hand, amino acids constitute a low percentage of honey (Hermosín, Chicón, & Cabezudo, 2003), with proline being the most abundant amino acid in honey and pollen. In *A. mellifera* honey, this content has been used to evaluate honey maturation and, in some cases, adulteration with sugar (Iglesias et al., 2006); however, in stingless bee honey, this has not been explored. In the analyzed samples, proline levels ranged from 84.56 mg kg^{-1} (*T. angustula)* to 5132.71 mg kg^{-1} (*Melipona* sp.), with an average of 1726.70 mg kg^{-1}. In addition to proline, there is a small fraction of other amino acids present in honey, with leucine as the most common amino acid found in this fraction (Machado De-Melo et al., 2017). Similar to proline, leucine content varied greatly: from 6.45 mg 100 g^{-1} (*T. angustula*) to 150.73 mg 100 g^{-1} (*Melipona* sp.), with a mean of 50 mg 100 g^{-1} of honey. Protein and amino acids in honey are attributed to fluid secretions of honey bee salivary glands and pharynx and to pollen, which is considered the main floral source of these components (Machado De-Melo et al., 2017). In both cases, the variability could be associated with and validated by analysis of these factors.

Present in a small but important fraction, vitamins in honey come from the pollen of the flowers visited by bees (Machado De-Melo et al., 2017). Vitamin C content in honey is not usually assessed because of its low concentration and instability (Lešková et al., 2006). Nonetheless, previous studies have reported its presence in stingless bee honey (Alvarez-Suarez et al., 2018; Selvaraju, Vikram, Soon, Krishnan, & Mohammed, 2019). In our samples, vitamin C was detected only in five of the 12 honey samples studied, and the highest content was found in honey from *O. mellaria* (63754.04 μg Vit C g^{-1}). Fig. S2 B (supplementary material) shows a chromatogram of the vitamin C analysis of this honey type. Considering these results, further studies are necessary to determine the possible factors that may affect vitamin C content in honey, such as high temperature, sunlight, maturity level and floral origin.

In addition to the reducing sugar content reported within the physical-chemical parameters in the honey under study, we also determined the total sugar content and profile of the main types of sugar in the samples (glucose, fructose and sucrose) (Table 4). Fig. S2 C (supplementary material) shows a representative chromatogram of the sugar profile in the studied honey. The total sugar content ranged from 67.70 g 100 g^{-1} honey (*M. mimetica*) to 85.74 g 100 g^{-1} honey (*Melipona* sp.), with an average of 76.65 g 100 g^{-1} honey, which is in agreement with the recommended limits for blossom honey (European Commission, 2002). The most prominent type of sugar was fructose, followed by glucose, complying with the recommended proportions for floral honey (European Commission, 2002). The values reported here were higher than those previously reported in stingless bee honey from Ecuador (Guerrini et al., 2009) but similar to those reported for Brazil (de Sousa et al., 2016). The sucrose content was lower than glucose and fructose, indicating that these honey have not been altered by the addition of commercial sugar (de Sousa et al., 2016).

The results of the analysis of contaminants, macronutrients, and micronutrients are shown in Table 5. The minerals contained in stingless bee honey have rarely been studied. To the best of our knowledge, this is the first study showing the mineral content of stingless bee honey from Ecuador. Cu, Pb, Ni, Mn, Zn and Fe were below the limits of quantification for the method used (<LQ), while the Al content was low, with an average of 0.06 mg g^{-1} honey. Among macronutrients, potassium was the highest in concentration, ranging from 0.07 to 1.33 mg g^{-1}, with an

Table 4
Average values of data obtained in the proteins and amino acids, vitamin C and sugar analysis of stingless bee honey from Ecuador.

Stingless bee species	Proteins and amino acids			Bioactive compounds	Sugar content (g 100 g^{-1} of honey)			
	Leucine (mg LE 100 g^{-1})	Proline (mg Prol kg^{-1})	Total protein content (mg BSA g^{-1})	Vitamin C content (μg Vit C g^{-1})	Glucose	Fructose	Sucrose	Total sugar content
S. polysticta	125.70 ± 15.29^{a}	4179.00 ± 756.95^{a}	0.18 ± 0.02^{a}	13.63 ± 4.35^{a}	32.07 ± 0.40^{a}	40.54 ± 0.47^{a}	4.63 ± 0.11^{a}	77.25 ± 0.62^{a}
M. indecisa	15.47 ± 4.09^{b}	584.99 ± 218.97^{b}	0.06 ± 0.02^{b}	1.161 ± 0.62^{b}	31.23 ± 0.14^{a}	41.34 ± 0.39^{a}	4.02 ± 0.14^{b}	76.60 ± 0.56^{a}
M. cramptoni	18.67 ± 2.13^{b}	781.77 ± 100.51^{c}	0.02 ± 0.00^{b}	77.64 ± 19.45^{c}	29.54 ± 1.35^{a}	40.24 ± 0.75^{a}	5.00 ± 0.08^{a}	74.77 ± 1.61^{a}
Paratrigona sp.	10.42 ± 2.4^{c}	383.05 ± 9.46^{d}	0.23 ± 0.01^{c}	*ND*	26.62 ± 0.10^{b}	44.57 ± 0.13^{a}	4.90 ± 0.02^{a}	76.10 ± 0.05^{a}
Melipona sp.	150.73 ± 2.71^{d}	5132.71 ± 151.92^{a}	0.17 ± 0.00^{a}	*ND*	38.26 ± 0.02^{c}	42.33 ± 0.13^{a}	5.14 ± 0.16^{a}	85.74 ± 0.32^{b}
Cephalotrigona sp.	31.97 ± 2.62^{e}	1459.51 ± 146.57^{e}	0.22 ± 0.05^{c}	*ND*	35.60 ± 0.11^{c}	43.42 ± 0.08^{a}	3.09 ± 0.07^{c}	82.11 ± 0.11^{b}
M. mimetica	9.34 ± 3.25^{c}	482.89 ± 20.70^{b}	0.30 ± 0.03^{d}	*ND*	30.28 ± 3.14^{a}	34.77 ± 3.49^{b}	2.63 ± 0.33^{d}	67.70 ± 6.96^{c}
T. angustula	6.45 ± 0.66^{f}	84.56 ± 33.14^{g}	0.27 ± 0.06^{d}	73.76 ± 33.01^{c}	30.48 ± 2.99^{a}	41.86 ± 0.18^{a}	3.70 ± 0.26^{b}	76.03 ± 2.93^{a}
T. silvestriana	18.60 ± 2.67^{b}	844.10 ± 22.83^{c}	0.34 ± 0.03^{e}	*ND*	34.38 ± 0.02^{c}	39.70 ± 0.02^{ab}	3.11 ± 0.15^{c}	77.18 ± 0.15^{a}
O. mellaria	12.05 ± 2.16^{c}	443.99 ± 48.43^{d}	0.37 ± 0.01^{f}	63754.04 ± 140.41^{d}	32.69 ± 0.02^{a}	42.09 ± 0.01^{a}	3.72 ± 0.02^{b}	78.50 ± 0.02^{a}
N. chapadana	16.12 ± 2.38^{b}	671.87 ± 46.90^{c}	0.32 ± 0.03^{e}	*ND*	33.03 ± 0.03^{a}	41.25 ± 0.42^{a}	4.75 ± 0.01^{a}	79.03 ± 0.44^{a}
M. grandis	9.45 ± 0.47^{c}	198.58 ± 26.04^{f}	0.09 ± 0.1^{g}	*ND*	26.00 ± 0.40^{b}	39.74 ± 0.16^{ab}	4.02 ± 0.3b	69.77 ± 0.28^{c}

Samples were analyzed in triplicate and data are presented as means ± standard error. Mean values within a column with different letters are significantly different for $P < 0.05$. *ND*, non-detected.

Table 5
Average values of data obtained in measurement of quantity of different mineral elements contained in stingless bee honey from Ecuador (mg g^{-1} of honey).

Stingless bee species	n	Contaminants				Macronutrients			Micronutrients			
		Cu	Ni	Al	Pb	Mg	Ca	K	Mn	Zn	Fe	Na
S. polysticta	7	*ND*	*ND*	0.07 ± 0.01[a]	*ND*	0.09 ± 0.00[a]	0.24 ± 0.01[a]	1.03 ± 0.10[a]	*ND*	*ND*	*ND*	0.15 ± 0.00[a]
M. indecisa	6	*ND*	*ND*	0.07 ± 0.00[a]	*ND*	0.05 ± 0.00[b]	0.18 ± 0.01[b]	0.18 ± 0.02[b]	*ND*	*ND*	*ND*	0.15 ± 0.01[a]
M. cramptoni	3	*ND*	*ND*	0.06 ± 0.00[a]	*ND*	0.04 ± 0.00[b]	0.16 ± 0.01[b]	0.07 ± 0.01[c]	*ND*	*ND*	*ND*	0.15 ± 0.01[a]
Paratrigona sp.	1	*ND*	*ND*	0.07 ± 0.00[a]	*ND*	0.05 ± 0.00[b]	0.17 ± 0.00[b]	0.23 ± 0.00[b]	*ND*	*ND*	*ND*	0.09 ± 0.00[b]
Melipona sp.	1	*ND*	*ND*	0.05 ± 0.00[a]	*ND*	0.04 ± 0.00[b]	0.15 ± 0.00[b]	0.07 ± 0.00[c]	*ND*	*ND*	*ND*	0.12 ± 0.00[a]
Cephalotrigona sp.	1	*ND*	*ND*	0.05 ± 0.00[a]	*ND*	0.04 ± 0.00[b]	0.18 ± 0.00[b]	0.30 ± 0.00[d]	*ND*	*ND*	*ND*	0.14 ± 0.00[a]
M. mimetica	1	*ND*	*ND*	0.06 ± 0.00[a]	*ND*	0.05 ± 0.00[b]	0.18 ± 0.00[b]	0.27 ± 0.00[d]	*ND*	*ND*	*ND*	0.15 ± 0.00[a]
T. angustula	3	*ND*	*ND*	0.08 ± 0.00[a]	*ND*	0.11 ± 0.02[a]	0.31 ± 0.03[c]	1.33 ± 0.49[e]	*ND*	*ND*	*ND*	0.23 ± 0.03[c]
T. silvestriana	1	*ND*	*ND*	0.06 ± 0.00[a]	*ND*	0.05 ± 0.00[b]	0.19 ± 0.00[b]	0.27 ± 0.00[b]	*ND*	*ND*	*ND*	0.13 ± 0.00a
O. mellaria	1	*ND*	*ND*	0.06 ± 0.00[a]	*ND*	0.06 ± 0.00b	0.20 ± 0.00[b]	0.23 ± 0.00[bc]	*ND*	*ND*	*ND*	0.14 ± 0.00[a]
N. chapadana	1	*ND*	*ND*	0.09 ± 0.00[a]	*ND*	0.06 ± 0.00b	0.23 ± 0.00[a]	0.25 ± 0.00[b]	*ND*	*ND*	*ND*	0.18 ± 0.00[d]
M. grandis	1	*ND*	*ND*	0.07 ± 0.00[a]	*ND*	0.06 ± 0.00b	0.24 ± 0.00[a]	0.36 ± 0.00[d]	*ND*	*ND*	*ND*	0.16 ± 0.00[d]
LOD (mg g^{-1})		1×10^{-5}	1×10^{-5}	1×10^{-5}	1×10^{-5}	2×10^{-5}	3×10^{-5}	3×10^{-5}	1×10^{-5}	2×10^{-5}	1×10^{-5}	2×10^{-5}
LOQ (mg g^{-1})		3×10^{-5}	2×10^{-5}	4×10^{-5}	3×10^{-5}	5×10^{-5}	1×10^{-4}	1.2×10^{-4}	2×10^{-5}	5×10^{-5}	2×10^{-5}	5×10^{-5}

Sample was analyzed in triplicate and data are presented as means ± standard error of the mean. Mean values within a column with different letters are significantly different for $P < 0.05$. *ND*, not-detected.

average of 0.40 mg g^{-1} honey. The second most abundant mineral in all samples was Ca, ranging between 0.15 and 0.31 mg g^{-1} honey, with an average of 0.20 mg g^{-1}. These findings are consistent with previous research on stingless bee honey from Brazil, which found these minerals to be the most quantitatively important (Biluca et al., 2016).

3.4. Antibacterial activity

Among the beneficial effects of honey on human health, antimicrobial activity is considered one of the most relevant (Machado De-Melo et al., 2017). The antimicrobial activity of stingless bee honey from various geographic regions and species against different pathogens has been previously reported (Avila et al., 2018); however, despite Ecuador's variety of species and floral richness, the antimicrobial activity of pot honey in this country has not yet been adequately studied. The effectiveness of the 26 stingless bee honey samples against three species of multiresistant pathogenic bacteria was tested (Table 6). With different levels of efficiency, the results demonstrated the inhibition of the gram-positive bacterium *S. aureus* in all samples and at many concentrations, with MIC values ranging between 20 g 100 mL^{-1} (*M. grandis*) and 4.33 g 100 mL^{-1} honey (*T. silvestriana* and *O. mellaria*). Similarly, honey samples were effective against the gram-negative bacteria *P. aeruginosa* P28 in an MIC range from 11.67 g 100 mL^{-1} (*Paratrigona* sp.) to 3 g 100 mL^{-1} (*T. silvestriana* and *O. mellaria*) and against *K. pneumoniae* KPC, with an MIC between 20 g 100 mL^{-1} (*Paratrigona* sp., *M. mimetica*, *N. chapadana*) and 3.66 g 100 mL^{-1} (*T. silvestriana*), with *T. silvestriana* honey being the most effective against the pathogens. Particularly interesting is the low MIC value of *T. silvestriana* and *O. mellaria* honey against *P. aeruginosa* and of *T. silvestriana* honey against *K. pneumoniae*. Gram-negative bacteria are generally more resistant than gram-positive bacteria; however, in the studied samples, the MIC values for these pathogens were at the same level as the most effective MIC values against *S. aureus*. The antibacterial activity of stingless bee honey has been previously reported in honey from different native species of bees and geographic origins, suggesting the effectiveness of pot honey against multiple pathogens (Avila et al., 2018). Several factors have been attributed to honey antibacterial activity, such as osmolarity, acidity, hydrogen peroxide and factors not dependent on peroxide (including bioactive peptides, polyphenols, and enzymes) (Machado De-Melo et al., 2017). In our opinion, in the case of stingless honey, given its acidic character, acidity seems to play a

Table 6
Minimal Inhibitory Concentration (MIC) of honey (g 100 mL^{-1}) from the different stingless bees against the multi-resistant bacteria species.

	Multirresistant bacteria species		
Meliponinae species	*S.aureus* MRSA	*K. pneumoniae* KPC	*P. aeruginosa* P28
S. polysticta	10.14 ± 1.36[a]	10 ± 0.95[a]	7.76 ± 1.18[a]
M. indecisa	14.50 ± 1.10[a]	19.17 ± 0.45[b]	9.89 ± 1.35[a]
M. cramptoni	16.67 ± 1.19[b]	18.33 ± 10.83[b]	11.00 ± 1.93[a]
Paratrigona sp.	18.33 ± 1.67[b]	20.00 ± 0.00[b]	11.67 ± 1.67[a]
Melipona sp.	9.33 ± 0.67[c]	6.67 ± 1.67[c]	3.67 ± 0.67[b]
Cephalotrigona sp.	7.00 ± 1.00[c]	8.67 ± 0.67[c]	3.67 ± 0.67[b]
M. mimetica	18.33 ± 1.67[b]	20.00 ± 0.00[b]	8.67 ± 0.67[a]
T. angustula	11.33 ± 3.23[a]	11.00 ± 3.38[d]	3.33 ± 0.33[b]
T. silvestriana	4.33 ± 0.67[d]	3.66±0.67[3]	3.00 ± 0.00[b]
O. mellaria	4.33 ± 0.67[d]	8.67 ± 0.67[c]	3.00 ± 0.00[b]
N. chapadana	16.67 ± 1.67[b]	20.00 ± 0.00[b]	8.67 ± 0.67[a]
M. grandis	20.00 ± 0.00[e]	13.33 ± 1.66[d]	3.67 ± 0.67[b]
Artificial honey	≥20.00 ± 0.00[e]	≥20.00 ± 0.00[b]	≥20.00 ± 0.00[c]

Results are expressed as the honey concentration that inhibits total growth of bacteria. Honey samples were analyzed in triplicate for each bacterium and data are presented as means of g/100 mL honey ±standard error of the mean. Mean values within a column with different letters are significantly different for $P < 0.05$.
S. aureus MRSA333 resistant to penicillin and oxacillin.
P. aeruginosa P28 resistant to amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin and trimethoprim/sulfamethoxazole.
K. pneumoniae KPC resistant to imipenem and ertapenem.

fundamental role in this activity; however, more studies are necessary to delineate the true mechanisms of its antibacterial action.

4. Conclusions

Based on the results of the physicochemical and chemical analysis, this report, which aimed to cover a wide variety of stingless bee species from different geographic regions of Ecuador, revealed many similarities with previously evaluated Central and South American honey. The moisture content and acidity results highlight the high water content in stingless bee honey as well as its acidic character.

From our results, we can also conclude that stingless bee honey contains mineral elements, with potassium and calcium predominating all the analyzed honey. Some qualities, such as the presence of vitamin C and the profile of organic acids described here, with significant variation between samples, constitute a new contribution to the study of native bee honey in the region, until now unreported. These findings add knowledge of honey as a source of certain bioactive compounds (in addition to the previously reported polyphenols) and their possible use as a food source of interest. The relevant results, shown by *in vitro* tests of antibacterial activity, reflect this honey's potential for treating infections caused by both gram-positive and gram-negative pathogenic bacteria. Honey from *Oxytrigona mellaria* and *Trigona silvestriana* were particularly effective in this regard, yet many more species and genera remain to be studied in Ecuador. Despite these results, the study did not assess the flora and potential preferences of bee species that contribute to Ecuadorian honey. Thus, further studies should be carried out to investigate and to emphasize the particular differences of each honey and of each species.

CRediT authorship contribution statement

Irina Villacrés-Granda: Formal analysis, Investigation, Writing - original draft. **Dayana Coello:** Formal analysis. **Adrián Proaño:** Formal analysis. **Isabel Ballesteros:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **David W. Roubik:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Gabriela Jijón:** Formal analysis. **Genoveva Granda-Albuja:** Formal analysis, Data curation. **Silvana Granda-Albuja:** Formal analysis, Data curation. **Reinier Abreu-Naranjo:** Formal analysis, Data curation. **Favian Maza:** Formal analysis. **Eduardo Tejera:** Validation, Formal analysis, Investigation, Writing - review & editing. **Ana M. González-Paramás:** Methodology, Validation, Writing - review & editing. **Pedro Bullón:** Methodology, Validation, Writing - review & editing. **José M. Alvarez-Suarez:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was funded by the Universidad de Las Américas (UDLA) (grant number AGR.JMA.19.02). Access to genetic resources was granted by means of the framework agreement "MAE-DNB-VM-2019-0110" celebrated between the Environment Ministry of Ecuador (MAE) and the Universidad de Las Américas (UDLA), Ecuador. The GIP-USAL was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) through the project AGL 2015-64522-C2-2R. The authors would like to thank Professor Celestino Santos-Buelga for his critical review of the manuscript and Ms. Helen Pugh for extensive proofreading of the manuscript. The authors also thank Gerardo Espinosa Brborich, Digital Animator, for preparing the figures for this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110737.

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