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REVIEW

A Novel Integrative Methodology for Research on Pot-honey Variations During Post-harvest

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Abstract

This novel review of analytical methods for pot-honey research was intended to provide concise references to a 35-day post-harvest experiments at 30 °C, in an integrated study. Diverse methods were selected from specialized literature, from the AOAC (Association of Official Analytical Chemists), and the International Honey Commission. Besides the geographical and seasonal origin, the pot-honey I.D. consists of entomological and botanical identifications, the latter performed by acetolyzed or natural melissopalynology. The methods of this integrative study included: 1. Physicochemical analysis (Aw, color, moisture, pH, free acidity, lactone acidity, total acidity, hydroxymethylfurfural (HMF), and sugars by high-performance liquid chromatography HPLC), 2. Targeted proton nuclear magnetic resonance ^{1}H-NMR metabolomics (sugars, ethanol, HMF, aliphatic organic acids, amino acids, and botanical markers), 3. Biochemical composition (flavonoids, polyphenols), 4. Antioxidant activity (ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid-free radical scavenging assay, DPPH 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, ferric reduction assay FRAP), 5. Microbial counts (aerobic plate, yeast and mold, *Bacillus*, and lactic acid bacteria count), 6. Honey microbiome profiling via independent-culture method: high-throughput bacteria and fungi based on amplicon sequencing approaches, 7. Sensory evaluation (odor, aroma, taste, persistence), and 8. Honey authenticity and biosurfactant tests by an interphase emulsion. A further section was included to provide basic information on the results obtained using each method. This was needed to explain the interacting components derived from pot-honey processing within the stingless bee nest and post-harvest transformations.

Introduction

Stingless bees (tribe Meliponini) are a diverse lineage of highly eusocial bees, currently with more than 600 species distributed in the tropical and subtropical habitats of the globe. This diversity is not evenly distributed and the greatest concentration of species is found in South and Central America, accounting for more than 500 species. In the New World, stingless bees are found from Mexico to northern Argentina, while in the Old World they are found throughout sub-Saharan Africa and Madagascar, and throughout the Indian Subcontinent, southern and southeastern Asia, through the Indomalayan and Papuasian regions, and into Australia (Roubik & Vergara, 2021). Meliponini are ancient, with species scattered in various Cenozoic deposits throughout the world (Engel et al., 2021a), as well as a singular fossil from the end of the Cretaceous period (Engel, 2000). Accordingly, stingless bees have been producing honey for at least 70 million years and have evolved complex chemistries with associated microbiomes throughout this span of time, and through dramatic changes in global environments.

The common descriptor of "stingless" is a seemingly sensational moniker for any honey-making bees (Michener, 2013), and certainly, the usual method of stinging as a defense of the nest is hampered by the vestigial sclerites associated with the sting apparatus in Meliponini (Michener, 2007, 2013). Nonetheless, stingless bees are quite capable of defending themselves from predation, by the construction of inaccessible nests and sometimes alternative means of deterring predation (e.g., painful defensive secretions in species of *Oxytrigona* Cockerell) (Michener, 2007; Engel & Rasmussen, 2021; Melo, 2021). As honey-making bees, indigenous peoples have a long history of exploiting stingless bee nests and products, as well as their own forms of meliponiculture. Aside from the use of honey as food or for ethnomedicinal applications, various nest components such as resins and propolis are also employed in crafts (Ayala et al., 2013; Bhatta et al., 2020).

Stingless bees process and store their honey in cerumen pots rather than in the hexagonal prismatic waxen cells of honey bees (Michener, 1974), and the differences in honeys are not merely those of the architectural form of the storage media. Pot-honeys produced by stingless bees differ considerably in many properties from the comb-honey produced by honeybees (tribe Apini, extant sister group to Meliponini). This distinction was emphasized by Schwarz (1948), carefully explored by Gonnet et al. (1964), mentioned in diverse articles and reviews, and ultimately became the focus of a book where the term pot-honey was coined and highlighted (Vit et al., 2013).The effect of storage was studied on honey produced by Thai stingless bees (Chuttong et al., 2015).

In biological studies, specialized techniques are required for understanding the biological, botanical, chemical, microbial, and physical components of honey, and their interactions. Methods used for the determination of a given honey physicochemical characteristic (moisture, ash or electrical conductivity, free acidity, HMF, diastase activity, and sugars), microbiological counts (aerobic plate, yeast and mold, *Bacillus*, and lactic-acid bacteria), sensory evaluation (color, odor, aroma, taste, persistence), and melissopalynological composition for routine quality control are basically the same as those for any biological investigation. Post-harvest variations were monitored by routine analysis and further metabolites by HPLC, ^{1}H-NMR, and recent approaches to determine the honey-microbiome used to identify microbes associated with stingless bees, involved in the biotransformation of the honey, as well as an interphase emulsion test as a preliminary indicator of biosurfactant activity.

The scope of this article is to describe the analytical methods used in the integrated post-harvest study of pot-honey produced by *Tetragonula laeviceps* Smith 1857, and *Lepidotrigona flavibasis* (Cockerell 1929) from Chiang Mai, Thailand; *Geniotrigona thoracica* Moure 1961, and *Heterotrigona itama* (Cockerell 1918) from Selangor, Malaysia; *Lepidotrigona terminata* (Smith 1878) from Kubang Kerian, Kelantan, Malaysia; *Tetragonisca angustula* (Latreille 1811) from Costa Rica; and *Scaptotrigona vitorum* Engel 2022 honey from Ecuador. Variations in most parameters were monitored weekly at 7 day-intervals for 6 times (days: 0-7-14-21-28-35), or 5 times excluding the first measurement due to transportation to international laboratories.

The following methods presented here were required: 1. To identify the origin of the pot-honey, and 2. To describe the standard and innovative methods for pot-honey analysis, integrated to study post-harvest transformations. Sterile pot-honey sampling was needed for the microbiological quality and for the honey microbiome assessment. Therefore, a sterile sampling protocol was followed to collect one batch of honey produced by each stingless bee species, homogenized, and distributed laterto all participating laboratories.

1. Identification of the entomological and botanical origin of pot-honeys

The identification of the pot-honeys was done by entomological and botanical origins, the countries of origin were known, and the habitats and seasons were also recorded for the harvest date of year 2022. This I.D. of each honey was the starting point for the integrated multiparametric post-harvest study along 35-day storage at 30 °C.

1.1 Entomological identification of the stingless bees

A study of pot-honey characterization initiates with the mandatory identification of the taxon involved by an authority in this field. This is not a trivial effort as taxonomic research is a challenging and time-consuming process requiring considerable training and resources, ideally including the significant investment in an authoritatively identified reference collection. Accordingly, taxonomists must be acknowledged

in the Materials and Methods as well as Acknowledgements, their grants should be cited, and when developing grant proposals or projects it is ideal to incorporate the necessary funding to support the costly preparation and identification process. Furthermore, it is ideal to also invite taxonomists as coauthors given the responsibility, costs, and labor necessary for contributing to the identification of the stingless bees, as this is a basic component of the overall research. Their knowledge of species biology may also provide additional insights into comparative studies of pot-honey. Modern photographic technology and photographer expertise produce scientific details of taxonomic interest and natural beauty. For example, the face of *Scaptotrigona vitorum* Engel, 2022 in Fig 1 shows many features such as the pattern of integumental coloration; the color, density, length, and plumosity of the fine hairs and bristles; the relative breadth and width of the head; the length of the antennal scape and proportions of the flagellomeres; the dentition of the mandible; the relative length of the malar space; the distance between the antennal sockets; and the sculpturing of the integument (e.g., density of punctures, smooth versus patterned, shiny versus matte). Naturally, more information is required relative to other body structures, but the figure highlights a range of features that can be observed easily from sufficiently detailed photographs of particular views.

Stingless bee identification was undertaken by shipping specimens along with their associated collecting-event data (georeferenced coordinates, locality data, date of collection, elevation in m.a.s.l., collector, contact, and any ancillary data) to a research taxonomist. The specimens were then mounted, labeled, and digitized according to standard entomological procedures. The mounted specimens were then sorted initially into genus and morphotypes. The resulting morphotypes were then either run through existing identification keys in the case of those genera for which such keys exist or were point-by-point compared with authoritatively identified material in the bee research collections of the University of Kansas Biodiversity Institute, American Museum of Natural History, as well as against original and subsequent descriptions existing in the literature. Where needed, males were dissected to examine their hidden sterna and genitalia, and workers dissected to examine the vestigial sting sclerites. Ultimately, specimens were identified either as existing species known in the literature or were determined to represent taxa hitherto undescribed. When possible, formal taxonomic descriptions were prepared and published in order to provide a name for use in subsequent research articles on such species. The processes of preparation and taxonomic identification can take days to many months (and sometimes much longer if new species must be described) depending on the taxa involved, the number of specimens requiring identification, the condition of the material provided, the availability of funds for these tasks, and various other factors (e.g., teaching and curation responsibilities). The degree to which taxonomists are made full partners in research grants and publications can go a long way to easing the process of identification and supporting the science of taxonomy more broadly (Engel et al., 2021b).

Fig 1. *Scaptotrigona vitorum* Engel 2022, from El Oro, Ecuador, a protagonist of pot-honey making stingless bees. Photo: Michael S. Engel.

1.2 Botanical identification by melissopalynology

Melissopalynology is the palynological study of honey. Pollen grains are well preserved in honey, as such acetolysis is not required, but is often necessary for species identification. Acetolyzed pollen is used by some researchers who have a wider reference collection for ecological studies. Some preserved features in natural pollen techniques such as oils are used in the identification process (Haidamus et al., 2019). The diverse characteristics of the pollen exine are used in both methods to identify the botanical taxa to species, genus, or family visited by the honey-making bees. Pollen grains are quantified, and their percentage may be used for a unifloral protected denomination of origin (PDO).

1.2.1 Acetolyzed pollen

Residual pollen grains present in honey, to be analyzed and identified, require cleaning and preparation with chemical and physical methods. Without them, light microscopy, especially in species-rich botanical areas, has quite limited value. Fortunately, pollen coming directly from flowers or bee nests requires minimal treatment, compared to pollen in soil or other sediments (Brown, 1960; Kearns & Inouye, 1993). Because pollen is extremely resistant to strong acids (e.g. hydrofluoric acid, hydrochloric acid, sulfuric acid, acetic acid) while sensitive to oxidizing agents (nitric acid, potassium hydroxide, sodium hydroxide, and acetolysis, explained below, see Erdtman, 1952; Faegri & Iversen, 1950) we use a combination of chemicals and apply a special protocol to improve our identification of pollen. Spore tablets of *Lycopodium*, with a known number of spores per tablet

(Stockmarr, 1971), are sometimes added to samples for which the quantification of different species proportions is desired, especially among multiple microscope slide preparations from a given sample. This is a straightforward method to quantify the relative portion per weight or volume of each pollen species (Roubik & Moreno, 2009).

After the *Lycopodium* spores were included, the acetolysis method was the same as without those spores, as follows: 1. One *Lycopodium* tablet into a 10 to 50 cc of honey sample is dissolved in water and sieved with mesh (250 mm), to remove nest material, bees or large impurities. Next, samples were concentrated at 2,700 rpm for 5 min, and the supernatant was discarded. The honey was dissolved and a homogeneous sample - the residue - was obtained. 2. The residue was dried with glacial acetic acid and concentrated at 2,700 rpm for 5 min, and the supernatant was discarded. 3. An acetolysis solution (a mixture of 1:9 of sulfuric acid and acetic anhydride) was added and heated for 5 min to destroy all cellulose content. Samples were concentrated at 2,700 rpm for 5 min and the supernatant was discarded. Samples were washed twice with distilled water and the residue was concentrated. 4. Ethanol, used as a dehydrating agent, was added and samples were concentrated at 2,700 rpm for 5 min. Ethanol was discarded and some drops of glycerol were added. 5. The sediment was collected with a pipette and deposited on a slide. 6. Finally, permanent microscope preparations were prepared using glycerin jelly as the mounting medium and paraffin as the coverslip sealant.

To identify all pollen grain types (morphotypes, species, genera, or families), transects of slide preparations were examined at 400X magnification using a Nikon Eclipse-Ni binocular scope. Counts of grains were obtained at 400X magnification (Barth, 1970a,b). Biological daylight and differential interference contrast (DIC) microphotographs were obtained at 1000X magnification using a Nikon DS-Ri1 Camera System attached to the Nikon scope. Palynological descriptions were based on the terminology established by Punt et al. (2007). Botanical names were derived by comparisons with pollen atlases and collections (Moreno et al., 2014; Roubik & Moreno, 1991). The taxonomic status of botanical names was updated by consulting Tropicos (Missouri Botanical Garden online database). All work was carried out at the Center for Tropical Paleoecology and Archaeology (CTPA) of the Smithsonian Tropical Research Institute (STRI) in Panama.

1.2.2 Natural pollen

Zander (1935) published the first research on the natural pollen of honey, latter updated by Louveaux et al. (1978). It consisted of preparing a slide with the honey sediment by removing the sugars of honey. Besides pollen, this method permitted the detection of other elements of the sediment such as oils, fungi hyphae, yeast, insect fragments, and organic matter (Haidamus et al., 2019). Kerkvliet & Meijer (2000) observed unusual residual rings fingerprinting sugar cane origin in sediments of adulterated honey.

A honey dilution was prepared with 10 g of honey weighed into a 100 mL beaker, 20 mL distilled water was added, stirred with a glass rod, microwaved if needed for complete dilution, distributed in two conical 15 mL centrifuge tubes, centrifuged at 1,500 rpm for 15 min, the supernatant was discarded keeping the tube inverted for approximately 30 sec, 5 mL glycerin:distilled water (1:1) were added to resuspend the pellet, let 30 min, centrifuged again at 1,500 rpm for 15 min, the supernatant was discarded, draining the inverted tube by touching an absorbent paper, the sediment of each tube was used to prepare a microscope slide in a 2.4x2.4 cm^2 area, a drop of glycerine jelly was added, the slide heated gently to avoid bubbles, a coverslip on the mounted sediment was sealed with paraffin.

The honey sediment slides were kept in slide boxes. Microscopic observations always started at 100X, increased to 200X, and were done at 400X magnification, also at 1000X when needed (Barth et al., 2021). Reference pollen slides from regional or country vegetation and images of melissopalynology for natural (Barth, 1989; Vit, 2005) and acetolyzed pollen (Roubik & Moreno, 1991) were used for pollen grain identifications and further counts of 300 pollen per slide.

2. Standard and innovative methods for pot-honey analysis

A glass bottle (Duran) was selected to store the pot-honey at 30 °C in the dark. The size of the container was at least double the volume of the pot-honey we placed in it. A separate honey sample was organized for each day of analysis, taken bottle by bottle for the planned measurements every seven days until 35 days post-harvest.

2.1 Physicochemical analysis

All measurements were performed in triplicate for each pot-honey sample.

2.1.1 Water activity (Aw)

Aw was determined using a water activity meter (Alta Novasina Aw-center Novasina Aw-box, Switzerland). A total of 5 g of honey was filled into the chamber of the Aw-box and the value of water activity was read (Cavia et al., 2004).

2.1.2 Color (Pfund)

The color of honey was measured using a Pfund classifier. Homogeneous honey samples devoid of air bubbles were transferred into a cuvette with a 10 mm light path. The cuvette was inserted into a color photometer (HI 96785, Hanna Instruments, Cluj County, Romania). Color grades were expressed in millimeter (mm) Pfund scale as compared to an analytical-grade glycerol standard.

2.1.3 Moisture

Moisture content was determined using a portable digital refractometer (Atago PAL-2 model, Atago Co., LTD Tokyo, Japan) (AOAC, 2012) that could be thermostated at 20 °C, regularly calibrated with distilled water or with another certified reference material (Bogdanov, 2004).

2.1.4 pH, Free Acidity, Lactone Acidity, and Total Acidity

The determination of pH and acidity (free, lactone, and total) was performed according to the method described by AOAC (2012). Total acidity was obtained by examining free and lactone acidity. To analyze free acidity, 10 g of a sample was weighed and diluted in 75 mL of distilled water. The free acidity was then calculated by titration with 0.05 N NaOH until the solution reached a pH of 8.5. The lactone acidity was obtained by adding 10 mL of 0.05 N NaOH to the initial solution and titrating with 0.05 N HCl until the pH reached 8.3.

2.1.5 HMF by HPLC

Hydroxymethylfurfural (HMF) was determined by high-performance liquid chromatography (HPLC) Waters e2695 with Waters 2489 UV/Visible detector, following the International Honey Commission (2009) method. The column used was InertSustain C18 (4.6 x 150 mm, 3 µm) and operated at 40 °C. Using an isocratic method, the mobile phase was 10% methanol and 90% deionized water (v/v) with a flow rate of 1.0 mL/min. The solution was filtered through a 0.22 µm membrane filter before use. The detector was set at 280 nm wavelength. Samples were prepared using the solid phase extraction (SPE) method. We weighed 4 g of honey and diluted it with acidified water (HCl pH 2). Bond elut C18 column cartridge was used for sample purification and proceeded with pre-condition the cartridge with methanol and HCl (pH 2) two times. Then, the sample was loaded into the cartridge and released slowly. We added 100% methanol to wash the sediment absorbed in the sorbent bed (desired fraction). The collection fraction was dried using a vacuum pump (nitrogen). The dried sample was weighed and hydrated with 1 mL of 60% methanol. Then, the sample was transferred into HPLC vials and the injection volume used was 10 µL.

2.1.6 Sugars by HPLC

The sugar contents of fructose, glucose, sucrose, maltose, and trehalulose in honey were determined following Fletcher et al. (2020) with minor modifications using high-performance liquid chromatography (HPLC) Waters e2695 with Waters 2424 evaporative light scattering detector (ELSD). The column used was InertSustain NH2 (4.6 x 250 mm, 5 µm) and operated at 40 °C. Using an isocratic method, the mobile phase was 85% acetonitrile and 15% deionized water (v/v) with a flow rate of 2.5 mL/min. The solution was filtered through a 0.22 µm membrane filter before use. The ELSD was set at 60 °C for drift tube temperature, gain 1, and pressure at 60 psi. Sample preparation was done according to MS2683:2017 where 2 g of honey were mixed with deionized water (40 mL) and transferred into a 100 mL volumetric flask. Later 25 mL of methanol was added and deionized water was filled up to the 100 mL mark. The volumetric flask was shaken well to ensure the homogenization of the mixture solution. The honey stock solution was filtered into the vial using a nylon syringe filter 0.22 µm before injection.

2.2 Targeted NMR metabolomics

This is a multiparametric method. Identification and quantification of sugars, ethanol, HMF, aliphatic organic acids, amino acids, and botanical markers were done. In nuclear magnetic resonance (NMR) spectroscopy, the chemical shift is the resonant frequency of a nucleus relative to a standard in a magnetic field. Often the position and number of chemical shifts are diagnostic of the structure of a molecule. NMR spectral tables aid to remember structural positions according to the solvent. For Proton NMR, which is ^{1}H-NMR, the kinds of honey were diluted in deuterated water D_2O.

2.2.1 NMR reference sample and honey sample preparation

All chemicals used in NMR were of analytical grade (>99% purity). The Hl 44518 - QuantRefA-NMR-Tube 5 mm, 600 µL (Reference Sample for quantification in food applications) was used for the NMR mixture analysis. This reference sample was used with Wilmad 507-PP-7 NMR-tubes in combination with Bruker's Honey-Profiling. Deuterium oxide (D_2O) was supplied from Sigma-Aldrich (Steinheim, Germany), 2,2,3,3-d(4)-3-(trimethylsilyl)propionic acid sodium salt (TSP) by Alfa Aesar (Karlsruhe, Germany), potassium dihydrogen orthophosphate (KH_2PO_4), sodium hydroxide (NaOH), and hydrogen chloride (HCl) from Merck (Darmstadt, Germany), sodium azide (NaN_3) by Fluka (Steinheim, Germany), and deionized water was supplied by Th. Geyer (Renningen, Germany). The NMR profiles were obtained for functional groups of honey compounds: sugars, organic acids, amino acids, alkaloids, and alcohols. The pot-honey sample preparation method was adapted from BrukerBiospin (BrukerBiospin, Ettlingen, Germany). The homogenized honey samples (5 g) were diluted in 17.5 mL NMR-buffer (15.7 g KH_2PO_4, 0.05 g NaN_3 in 1L deionized water). For the neutralization of organic acids, the pH was adjusted to 3.1 with 1M HCl and 1M NaOH. Then, 900 µl of the homogenized honey solution were mixed with 100 µL standard solution (deuterium oxide D_2O containing 0.1% TSP 2,2,3,3-d(4)-3-(trimethylsilyl) propionic acid sodium salt), centrifuged at 14,000 rpm for 10 min, and 600 µL of supernatant were transferred into a 5 mm × 178 mm NMR-tube for direct measurement.

2.2.2 Targeted quantitative ^{1}H-NMR spectra acquisition and processing

All NMR spectra were acquired immediately after honey dilutions on a Bruker Ascend TM 400 MHz Food Screener (Bruker Biospin, Ettlingen, Germany) equipped with a

5 mm PA BBI 400SI H-BB-D-05 Z probe and using Bruker Sample Xpress (Bruker Biospin, Ettlingen, Germany) for automatic sample change. ^{1}H-NMR-spectra were recorded at 300 K using the pulse program noesygppr1d (1D spectra with water presaturation at 4.8 ppm) and jresgpprqf (2D J-resolved spectra, displaying chemical shift and spin-spin coupling information). For 1D spectra, 32 scans and 4 dummy scans of 64 k points were acquired with a spectral width of 20.55 ppm, a receiver gain of 16, and an acquisition time of 3.99 s. The 2D spectra were performed using 4 scans and 16 dummy scans of 8 k (F2-axis) and 40 k (F1-axis) points. The spectral widths were 16.7 ppm (F2) and 0.19 ppm (F1), receiver gain of 16, and acquisition times were 0.6 s (F2) and 0.3 s (F1). The NOESY (Nuclear Overhauser Effect Spectroscopy) spectra were used for quantification. The JRES (J-resolved spectrum) was used to verify the compound identifications. All spectra were standardized, automatically phased, baseline-corrected, and calibrated using 2,2,3,3-D4-3-(trimethylsilyl) propionic acid sodium salt (TSP) as a reference at 0.0 ppm. The compounds were quantified using the Honey-Profiling routine (release Version 3.0, BrukerBiospin, Ettlingen, Germany) for compound quantification by automatic integration of the peak area calculated with external standards (Spraul et al., 2009).

2.3 Biochemical analysis

2.3.1 Flavonoids by HPLC/PAD/ESI-MS

The pot-honey flavonoids were identified and quantified by High-Performance Liquid Chromatography/ Photodiode-Array Detection/Electrospray Ionization Ion Trap Mass Spectrometry (HPLC/PAD/ESI-MS), according to Truchado et al. (2015). Five grams. of pot-honey were dissolved with five parts of distilled water, and adjusted to pH 2 with HCl, up to a 50 mL volume, filtered through a Sep-Pak C18 cartridge, previously activated with methanol and water successively. The cartridge was washed with 10 mL distilled water and the phytochemical compounds were eluted with 2 mL methanol. The methanolic fraction was filtered through a 0.45 µm filter and stored at -20 °C until further analysis.

A LiChroCART column (250 × 4 mm, RP-18.5 µm particle size, LiChrospher®100 stationary phase, Merck, Darmstadt, Germany) protected with a LiChroCART guard column (4 × 4 mm, RP-18.5 µm particle size, Merck) was used for chromatographic analysis. The mobile phase consisted of two solvents: water/formic acid (1%) (A) and methanol (B), starting with 20% B, and using a gradient to reach 30% at 40 min, 60% at 45 min, 80% at 50 min. The flow rate was 1 mL/min, and the injection volume was 20 µL. Spectral data from all peaks were accumulated in the range of 240-400 nm, and the chromatograms were recorded at 280, 320, and 360 nm. The HPLC/PAD/ESI-MSn analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode-array detector and mass detector in series (Agilent Technologies, Waldron, Germany). The HPLC system consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode-array detector (model G1315B). The HPLC system was controlled by Chem Station software (version 08.03, Agilent). The mass detector used an ion trap spectrometer (model G2445A) with an ESI interface, controlled by LCMSD software (version 4.1, Agilent). Ionization was adjusted at 350 °C and 4 kV for capillary temperature and voltage. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min. The MS analyses covered the range m/z 100 up to 1500. MS data were acquired in the negative ionization mode. MSn was carried out automatically on the more abundant fragment ion in MS (n -1).

Fragment ions were assigned using the glycoconjugates nomenclature (Domon & Costello, 1988). Ions $^{k,l}X_j$, Y^n_j, Z^n_j represent those fragments still containing the flavonoid aglycone, where j is the number of the interglycosidic bond broken, counted from the aglycone, n represents the position where the oligosaccharide is attached to the aglycone, and k and l denote the cleavage within the carbohydrate rings.

2.3.2 Total flavonoid content

The total flavonoid content (TFC) was determined by the aluminum colorimetric method according to Hagr et al. (2017) and Tuksitha et al. (2018). Quercetin was used as a standard, with concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.125 ppm, 1.56 ppm, and 0.78 ppm. In total, 100 µL of the diluted honey sample was mixed with 100 µL 2% aluminum chloride ($AlCl_3$) in a 96-well plate. In total, 100 µL of each standard solution was combined with 2% $AlCl_3$. In total, 100 µL distilled water was mixed with 100 µL 2% $AlCl_3$, which acts as a negative control. In total, 200 µL ethanol was pipetted directly into a 96-well plate and served as a blank. The mixture was incubated for 10 min at room temperature. The absorbance of the mixture was measured at 450 nm using a Benchmark Plus Microplate (Bio-RAD170- 6930, Singapore). The results were expressed as micrograms of quercetin equivalents (QE) per gram of honey (mg QE/100 g honey).

2.3.3 Total phenolics

The total phenolic content was analyzed by the Follin-Ciocalteu method, according to Hagr et al. (2017). A 100 µL diluted honey volume was mixed with a 500 µL Follin-Ciocalteu reagent, and then vortexed for 30 s. In total, 400 µL 7.5% (w/v) aqueous sodium carbonate was added, and vortexed again. The mixture was allowed to stand for 1 h, and incubated at 40 °C in the dark. In total, 200 µL of the supernatant mixture was pipette out and loaded into a 96-well micro-plate. Methanol and distilled water acted as blank. The absorbance of the reaction mixture was measured at 765 nm using a micro-plate spectrophotometer (Bio-Rad, Hercules, CA, USA). Gallic acid was used as a standard, with concentrations of 400 ppm, 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, and 3.125 ppm. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of honey (mg GAE/100 g honey).

2.4 Antioxidant activity

2.4.1 $ABTS^+$ Free radical scavenging assay

The assay was done according to Ramlan et al. (2021). $ABTS^+$ radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid was prepared by mixing 3 mL of an aqueous 7 mM ABTS solution with 3 mL of an aqueous 2.5 mM solution of potassium persulfate. The reaction was kept in the dark for 16 h, and 4 mL of this solution was then dissolved in deionized water until it reached an absorbance of 0.700 ± 0.005 at 734 nm. The reaction consisted of mixing 20 µL of a sample with 200 µL of the final $ABTS^+$ solution. After 30 min, the absorbance of the reaction was measured at 734 nm using absolute ethanol and water as a blank. The $ABTS^+$ solution was prepared daily, and the reactions were carried out in the dark. All reactions were done in triplicate. The Trolox concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.125 ppm, 1.56 ppm, and 0.78 ppm were used as a standard. The percentage of the $ABTS^+$ radical scavenging activity was calculated as % Inhibition = [(blank absorbance - sample absorbance)/blank absorbance] × 100. The results were expressed as the % of $ABTS^+$ inhibition.

2.4.2 DPPH Radical scavenging assay

The antioxidant activity of the honey was analyzed using a DPPH 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, as described by Hagr et al. (2017). The Trolox concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.125 ppm, 1.56 ppm, and 0.78 ppm were used as a standard. In total, 50 µL diluted honey and standard Trolox were added to 195 µL DPPH reagent into a 96-well plate. In total, 50 µL methanol was also mixed with 195 µL DPPH reagent as a negative control. Those mixtures were incubated at room temperature in dark conditions and were allowed to stand for 1 h. The absorbance was measured at 540 nm using a microplate spectrophotometer (Bio-Rad, US), with methanol as a blank. The percentage of the radical scavenging activity was calculated as % Inhibition = [(blank absorbance - sample absorbance)/blank absorbance] × 100. The results were expressed as the % of DPPH inhibition.

2.4.3 FRAP Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) of each honey was analyzed using the method of Hagr et al. (2017) and Tuksitha et al. (2020). Ferrous sulfate ($FeSO_4$) was used as a standard, with concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.125 ppm, 1.56 ppm, and 0.78 ppm. In total, 20 µL diluted honey was mixed with 220 µL FRAP reagent. The FRAP reagent was prepared by mixing 40 mL acetate buffer with 4 mL 2, 4, 6-Tri (2-pyridyl)-striazine (TPTZ) solution and 4 mL ferric chloride (FeCl3) solution. In total, 20 µL distilled water was mixed with the FRAP reagent, and acted as a negative control, while 240 µL methanol was a blank. The mixture was pipetted into the 96-well plate, and the absorbance of the mixture was measured at 593 nm using a Benchmark Plus Microplate (Bio-RAD170-6930, Singapore). The results were expressed as micrograms of $FeSO_4$ equivalents per gram of honey (µmol $FeSO_4.7H_2O$/100 g honey).

2.5 Microbiological quality

The microbiological quality was examined following the study described by Lani et al., (2017).

2.5.1 Pot-honey sample preparation and storage

Pot-honey samples were collected using surgery gloves after piercing the honey pot and by suction with a sterile syringe, the needle was replaced by a rubber tube. The liquid honey was then forced into a centrifuge tube. Previously sterilized, blue-cap bottles were used to disperse a portion of pooled honey (~25 g/bottle). To avoid any honey degradation while being transported to and inside the lab, the honey samples were stored and protected from sunlight. Samples were stored at a constant temperature (30 ± 2°C) in the dark until analysis.

2.5.2 Aerobic Plate Count Agar (PCA)

In Merker (1998), an aerobic plate count was conducted in accordance with the guidelines in the Bacteriological Analytical Manual of the Food and Drug Administration. Five grams of pot-honey samples were thoroughly mixed with 45 mL of either 0.1% sterile buffered peptone water (Merck, Germany) in a stomacher bag (Huko, Seoul, Korea) using a sample mixer (3M, Wellington, New Zealand). Appropriate dilution 0.1 mL of $1x10^{-4}$ dilution was spread plated on Plate Count Agar (HiMedia, Mumbai, India) and incubated at 35 °C for 24 h. The morphology and Gram stain of each isolate was observed using a light microscope (Motic B Series, USA) at 400X after pure selected isolates were grown at 35 °C for 24 h.

2.5.3 Yeast and mold counts

Five grams of pot-honey samples were homogenized in a blue-capped bottle with 45 mL of 0.1% sterile buffered peptone water (Merck, Germany). Appropriate 0.1 mL of $1x10^{-4}$ dilution was spread plated on Yeast Malt Agar (HiMedia, Mumbai, India) and incubated at 30 °C for 24-48 h. A 48-h culture of each isolate was stained with lactophenol blue, and a light microscope (Motic B Series, USA) at 400X was used to observe the morphology.

2.5.4 *Bacillus* count

Five grams of each honey sample were diluted with 45 mL of 0.1% sterile buffered peptone water (Merck, Germany), 0.1 mL of $1x10^{-4}$ dilution was spread plated onto Mannitol Egg Yolk Polymyxin Agar (Oxoid, UK) and incubated at 35 °C for 24-48 h. A 48-h isolate culture was examined with a light microscope (Motic B Series, USA) at 400X.

2.5.5 Lactic acid bacteria (LAB) count

Following the procedure described by Aween et al. (2012), which included a pre-enrichment process before isolation on de Man, Rogosa, and Sharpe (MRS) Agar (Oxoid, UK), LAB were isolated from pot-honey samples. A five-gram sample of pot-honey was aseptically transferred to 45 mL of MRS broth (Oxoid, UK), and it was then incubated for 24 h at 30 °C in a CO_2 incubator (Model ICO150, Memmert, Germany). A 100 µL dilution in 0.1% sterile buffered peptone water (Merck, Germany) was spread on three different media MRS, MRS agar with 0.8% $CaCO_3$, and MRS agar with 1% glucose). The plates were then put in an anaerobic incubator (Model ICO150, Memmert, Germany) at 30 °C for 24-48 h. Hypothetical LAB were shown as a zone of clearance on MRS Agar with 0.8% $CaCO_3$. Twenty-four h pure cultures were gram stained, and then examined for catalase activity using 4% H_2O_2. LAB is a catalase-negative organism that prevents the cells from producing gas bubbles.

2.6 Honey-microbiome

2.6.1 Honey preparation for DNA extraction

To prepare the pot-honey sample for DNA extraction, approximately 5 mL of honey were diluted with 5 mL of distilled water in a 15 mL centrifuge tube. The mixture was stirred until the honey was completely dissolved and centrifuged for 5 minutes at 13,500 rpm in a (Witeg, CF-10, Germany) centrifuge to remove excess water. The supernatant was discarded, and the procedure was repeated three times, to obtain sufficient pellet from approximately 15 mL honey, based on optimized procedures.

2.6.2 DNA extraction, library preparation, and DNA sequencing

The total DNA was extracted using DNeasy Powersoil Pro Kit (QIAGEN, Germany) with a modified protocol as described by (Tay et al., 2021). Briefly, 250 mg of the collected pellet was transferred into Power Bead Pro Tube. 800 µL of Solution CD1 and 25 µL of Proteinase K were added into the tube and vortex briefly to mix. The subsequent extraction process was carried out in accordance with the manufacturer's protocol.

2.6.3 Library preparation

The library preparation for bacteria characterization was done by targeting the V3 region of the 16S rRNA gene using primers PRBA338fGC and PRUN518r (Castro-Mejía et al., 2020) in two-step Polymerase Chain Reaction (PCR) methods followed by 2×200 bp pair-ended Illumina iSeq 100 (Illumina, San Diego, CA) sequencing performed according to standard protocol.

For fungi characterization, the partial 18S and full length of ITS1-ITS2 region were amplified using ITS9NGS and ITS4 primers with Nanopore partial adapter on the primer 5' end (Tedersoo et al., 2015). 200 fmol of pooled barcoded amplicons were used for LSK110 library preparation (Oxford Nanopore, UK) and the library was sequenced on Nanopore Flongle Flowcell for 24 hours, as previously described (Tay et al., 2020).

2.6.4 QIIME2 Raw sequences data processing

The gene sequencing service is generally provided by private companies. The paired-end reads generated from sequencing were overlapped, error-corrected, and merged using fastp v0.21 (Chen et al., 2018). The reads were demultiplexed and the primers were removed using cutadapt v1.18 (Martin, 2011) before being imported into QIIME2 v.2021.4 (Bolyen et al., 2019) for denoising using dada 2 (Callahan et al., 2016). Taxonomic assignations of the amplicon sequence variant (ASV) were made using a q2-feature-classifier (Bokulich et al., 2018) trained on the latest GTDB release r202 16S rRNA database (trimmed to only retain the V3 hypervariable region) (Parks et al., 2020). ASVs with taxonomic assignment at least to the phylum level were selected for subsequent analysis.

The raw Nanopore reads were basecalled and demultiplexed using Guppy v5.0.7 (high accuracy mode). The raw reads containing primer ends were retained using cutadapt (Martin, 2011) and the identified primer sequences were removed using NanoClust (Rodríguez-Pérez et al., 2020). The taxonomic assignations of consensus non-chimeric ITS1-58S-ITS2 sequences were made using the q2-feature-classifier (Bokulich et al., 2018) against the latest UNITE ITS v8.3 database (Abarenkov et al., 2021; Nilsson et al., 2019). The 18S V9 region of the unsuccessful classified sequences was extracted using ITSx and searched against the SILVA 138 SSU Database (Quast et al., 2013) using QIIME2 v2021.4 classify-consensus-blast pipeline (Bolyen et al., 2019). Both the ASV table and OTU table generated from NanoClust cluster output were manually formatted to generate MicrobiomeAnalyst-compatible input (Chong et al., 2020; Dhariwal et al., 2017), with minor modifications according to Siew et al. (2022).

2.7 Sensory evaluation

Descriptive quantitative measurements were performed on odor, aroma, taste, and persistence (Vit, 2008). Each honey sample (5.0 ± 0.5 g) was presented in plastic capped cups, coded with three random digit numbers. Honey samples were served at room temperature in a tray with a plastic spoon, a glass of water, a serviette, a piece of green apple to bite at the end of each tasting, and water to prepare the oral cavity for the next tasting. These four actions were taken: 1. Follow the 18 tasting steps, 2. Select the sensory family for odor and aroma. If possible select the subfamily, and if the descriptor is identified select it too. If only a family such as Floral-Fruity is perceived, write it in the evaluation form, 3. In the nose is perceived the odor, and 4. In the mouth is perceived the flavor with tasting buds, and the aroma with retronasal perception.

The sensory families and sub-families used to identify odor perception in the nose and aroma perception in the mouth

are listed below as the Odor-Aroma (O-A) references for the eight sensory families (acronyms in bold) and corresponding sub-families. See the Table with sensory descriptors (Vit, 2008), which are needed to guide the sensory evaluation in related families. The expansion of sensory descriptors is desirable to identify different targets such as fruits and flowers familiar to particular countries but unknown to others.

The 18 steps below were followed in that order for the sensory evaluation of each pot-honey, to retrieve descriptors and their intensities:

This is the form used for the descriptive sensory evaluation of honey. The intensity of each attribute was perceived on a scale 0-3.

1FF Floral-Fruity: Floral, Citrus fruit, Fresh fruit, Processed fruit
2V Vegetable: Fresh, Dry, Aromatic
3F Fermented: Acetic, Alcoholic, Lactic
4W Woody: Wood, Resins, Spices
5N Nest: Stingless bees, *Apis mellifera*
6M Mellow: Sugary, Caramelized, Cakery
7P Primitive: Animal, Smoke, Wet, Sulfated, Mineral, Marine, Oily
8CI Chemical Industrial: Petrochemical, Medicinal.

- **In this test, the intensity of each odor, each flavor, each aroma, and persistence is evaluated. Mark with X.**
- **Odor and aroma are described in Table O-A for pot-honey.** Add descriptors as needed.
- **Select with a circle the relationship intensity odor/ aroma**
- **code sample**

The acceptance test was rated with icons for a 7-point hedonic scale anchored by: 1. 'Dislike it a lot', 2. 'Moderately dislike', 3. 'Slightly dislike'; 4. 'Indifferent, don't like it neither dislike it', 5. 'Slightly like'; 6. 'Moderately like', and 7. 'Like it a lot'.

2.8 Honey authenticity test by an interphase emulsion (HATIE)

This test is based on the number of phases observed after shaking a 1:1 honey:distilled water dilution with a double volume of ethanol, as described by Vit (1998). The two phases are a signature feature of fake honey. Genuine

scale	**0**	**1**	**2**	**3**
intensity	absent	mild	medium	strong

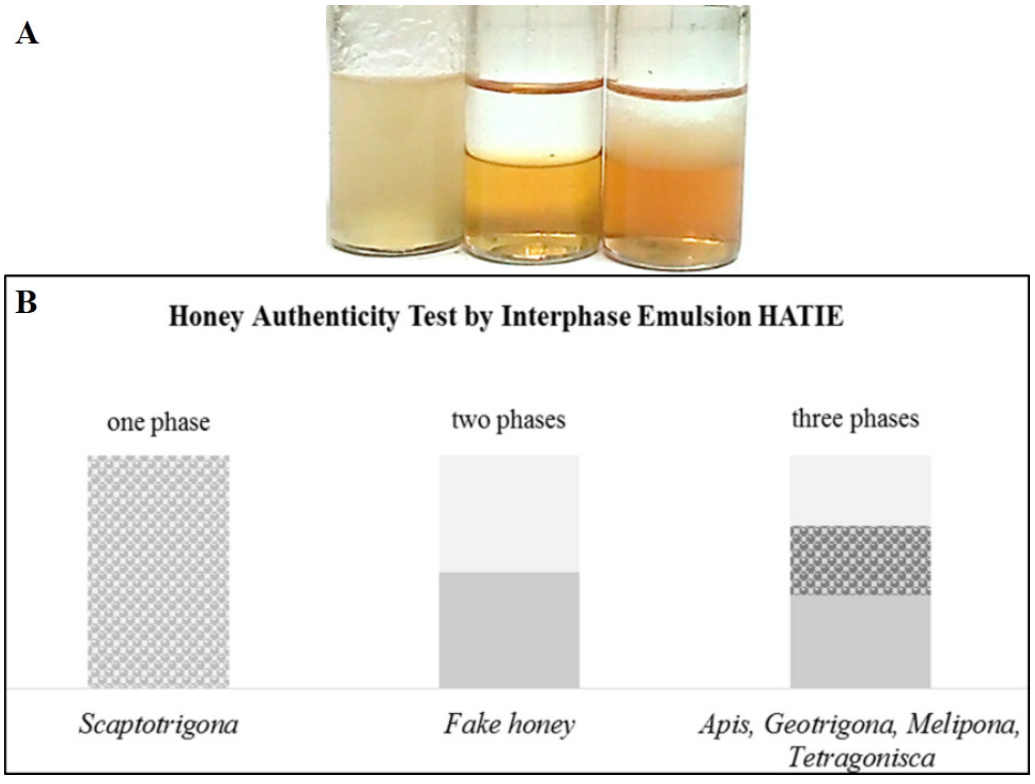
1 Uncap the plastic cup	expose honey volatiles
2 Mix honey with a plastic spoon	revolve honey in circles three times with the spoon
3 Smell Close your eyes	inhale the odorants of honey
4 annotate ODOR intensity	(use **intensityscale 0-3**)
5 Smell	inhale again (approximately 15 sec later)
6 annotate ODOR description	(use Table **O-A descriptors**)
7 smell it again if needed	
8 Tasting	take half tea spoon honey aprox. (0.5 g)
9	open and close your mandible 3 times, to dilute honey with saliva, rub your tongue against your palate
10 Close youre eyes	taste the admixture of honey and saliva
11	slowly swallow
12 Annotate intensity of each flavor	flavors have intensity (use **intensity scale 0-3**)
13 Annotate aroma intensity	aromas have intensity (use **intensity scale 0-3**)
14 Annotate aroma description	describe aromas (use Table **O-Adescriptors**)
15 Annotate intensity of persistence	aftertaste has intensity (use **intensity scale 0-3**)
16 Select relationship intensity (O-A) odor - aroma	higher intensity nose O > A equal intensity nose and mouth O = A higher intensity mouth A > O
17 taste it again if needed	
18 Clean the palate with a bite of green apple and a sip of water	

Evaluated attributes		Intensity scale			
		0	1	2	3
odor	intensity				
	description				
intensity of each flavor	sour				
	bitter				
	astringent				
	sweet				
	piquant				
	savory				
	umami				
aroma	intensity				
	description				
other perceptions in the mouth					
persistence	intensity				
Relationship intensity odor/aroma		odor < aroma	odor = aroma	odor > aroma	

honey was initially reported as a three-phase pattern, with a distinctive intermediate emulsion (Vit, 1998) caused by putatively minor protein and lipid contents of honey made by bees, not present in sugar-derived imitations. Recently, the third pattern of one phase was discovered for a type of honey with surfactant activity, lowering the interphase tension and distributing the intermediate emulsion in the reaction volume (Vit, 2022). In Fig 2, the number of phases is illustrated with an image and a diagram showing the three patterns observed for the HATIE, with genuine honey generating one and three phases, in contrast to fake honey with two phases.

2.9 Statistical analysis

Data were analyzed by one-way ANOVA, Principal Component Analysis (PCA), and Hierarchical Cluster Analysis (HCA), using version 26 IBM SPSS Statistics



Vit (2022)

Fig 2. Number of phases obtained after shaking a honey aqueous dilution with diethyl ether. One (left) phase for *Scaptotrigona vitorum* honey, three phases (right) for genuine honeys, and two phases in the center for fake honey. **A.** Image, and **B.** Diagram.

software (IBM Corp., 2019). Pearson correlation matrix ($P < 0.05$) and Principal component analysis (PCA) were conducted using XLSTAT Software Premium 2021. Microsoft Power Point 2018 was used to prepare, integrate, and edit the figures composed from multiple sources.

3. Particular information on the basics of each method, background arts

3.1 Identifications of the entomological and botanical origins of pot-honeys

3.1.1 Entomological identification of the stingless bee

Several articles on pot-honey lack entomological identification of the stingless bee that produced the analyzed honey, in the Materials and Methods section. Others use a generic name as an ethnic name, for example, the use of the name *Trigona* in some Indian contributions, without the identification by a trained entomologist taxonomist, and made worse by the fact that this genus does not exist in India, but solely in the Neotropics (Rasmussen & Cameron, 2008). Some important omissions of the stingless bee identification in pot-honey research are Guerrini et al. (2009) with an Ecuadorian stingless bee, published in Food Chemistry, and more recently Thomas & Kharnaior (2021) with an Indian stingless bee published in the Journal of Apicultural Research. A lack of precise identifications, despite the significant advances and taxonomic updates to stingless bees (Roubik, 2013; Melo, 2021) means that such published data are effectively useless as they are devoid of any link back to a biological entity and therefore the results cannot be repeated nor properly compared across the diverse fauna of stingless bees. The growing complexity of entomological identifications was clear for Peruvian stingless bees (Marconi et al., 2022). Authors and editors must be insistent on obtaining proper identifications as this is a mandatory requirement for any exploration of pot-honey in stingless bee research.

3.1.2 Botanical origin by melissopalynology

Because so many flowering plants are not observable - they are high aboveground in trees, epiphytes, and vines - despite the labors of field biologists, we are still woefully ignorant of which are most important to the honey-making social bees (Roubik, 1989). The honey and pollen samples collected from pots in tropical stingless bee nests offer the best option for confronting this challenge (Iwama & Melhem, 1979, Louveaux, 1968; Maurizio, 1975). The honey analysis is the most efficient in many ways. Honey carries not only the signature 'fingerprints' of nectar flowers but also the pollen signature of nectar-less flowering plant species, which are frequently found in the honey of tropical bees (Roubik & Moreno, 2013, 2018). The outer wall or exine of pollen grains contains sporopollenin. It is one of the most resistant organic compounds known and preserves pollen as a fossil. The exine is a distinctive structure observed by melissopalynologists for the thickness, surface, ornaments, aperture type, and number, and especially the shape of the grain that has been classified according to relationships between the polar axis and equatorial axis, in the equatorial view, spheric $P/E = 1$, prolate $P/E > 1$ (1.34-2.00) and oblate $P/E < 1$ (0.54-0.74) (Punt et al., 2007). Fatty acids provide different colors to pollen grains, they are observable by the naked eye, and in natural melissopalynology.

To assign a botanical origin of genuine honey, it is necessary to identify the types of pollen, represented by taxa at the level of family, genus, and even species for the best-known pollen grains. This is achieved with pollen reference collections, visual comparisons with photomicrographs from scientific literature (e.g. Barth, 1989, 2004; Roubik & Moreno, 1991; Vit, 2005; Moreno et al., 2014), and experience. It is also necessary to know the ecology, which plants are nectariferous and which are polliniferous, or if they offer both nectar and pollen rewards for pollinators. Some plants such as the *Clusia* genus offer floral resin. For example, if a high pollen count in honey is caused by contamination with pollen from nectarless flora, it should be removed since honey is made from nectar. In those cases, the following taxon is used to propose a botanical origin. Examples of polliniferous plants are *Mimosa caesalpiniifolia*, Melastomataceae, *Myrcia*, *Solanum*, *Borreria verticillata*, and *Piper*. Contamination with anemophilous pollen needs to be eliminated because it did not provide nectar for the analyzed honey. For example, some Asteraceae genera, *Casuarina*, *Cecropia*, *Celtis*, Chenopodiaceae, Cyperaceae, *Pinus*, Poaceae, *Podocarpus*, *Trema*. Another very important detail is the representation of pollen in the nectar of a certain taxon in honey, since there may be hypo- and hyper-represented pollen in honey. For example, the *Bombax ceiba* flower produces high quantities of nectar and its pollen is large, which causes low pollen counts in unifloral honey of Bombacaceae detected in Ecuador (Barth et al., 2015).

The fine-tuning of pollen analysis, using acetolyzed material, of course, comes with experience. Our experiences include the following, given here as general observations worthy of documentation: 1. Dye is not needed with acetolysis, the oxidative darkening of all surface features is sufficient, 2. Delicate exines of Marantaceae, Zingiberaceae, and Heliconiaceae are destroyed by acetolysis, 3. Tropical flora are composed of roughly 20% tricolporate, reticulate grains, of many families, 4. Therefore, pore and exine structures are of particular importance and require immersion oil magnification at 1000X, and 5. Good phenological data as to the flowering schedule can greatly improve possible identification.

The apertures of the exine allow the pollen tube to exit the pollen grain and transport the sperm to the egg in the pistil for germination. Apertures are thin or missing exine areas in pollen grains. According to their shape, pores and colpi are two types of apertures, and combinations of them. Some aperture features are annulate and aspidate. The surface

of the exine is very distinctive, it may vary from smooth or psilate to very ornamented types such as baculate, brochate, clavate, echinate, echinulate, eureticulate, fenestrate, foveolate, gemmate, granulate, microreticulate, punctitegillate, reticulate, rugulate, scabrate, striate, and verrucate (Vit, 2005).

Regarding the size, form, and fine surface structures of pollen grains of five botanical families in Fig 3, the two preparations of the honey sediment differ in color: 1. Acetolyzed pollen is amber, and 2. Natural pollen is light yellow or gray. Polar and equatorial views are taken as standards, focusing both on the equatorial or polar outlines, and the surface to have dual information. No.1 Amaranthaceae has panporate spherical pollen grains with no polarity, a thick exine, and numerous circular pores separated by a granulated exine surface. Both methodologies allow correct identification. No. 2 shows Anacardiaceae in equatorial view, with three long colpi (longitudinal apertures), each one containing a central and transversally elongate additional aperture, the surface is striate-reticulate, the yellowish natural pollen grains are more rounded and the apertures and surface features are less highlighted than the acetolyzed pollen. No. 3 Asteraceae in polar view possess three short colpi, hardly distinguishable in both methodologies of processing, due to the lack of spines. No. 4 Euphorbiaceae shows eight images. The two upper acetolyzed pollen grains are in equatorial view and the two below in polar view. Colpi have irregularly shaped

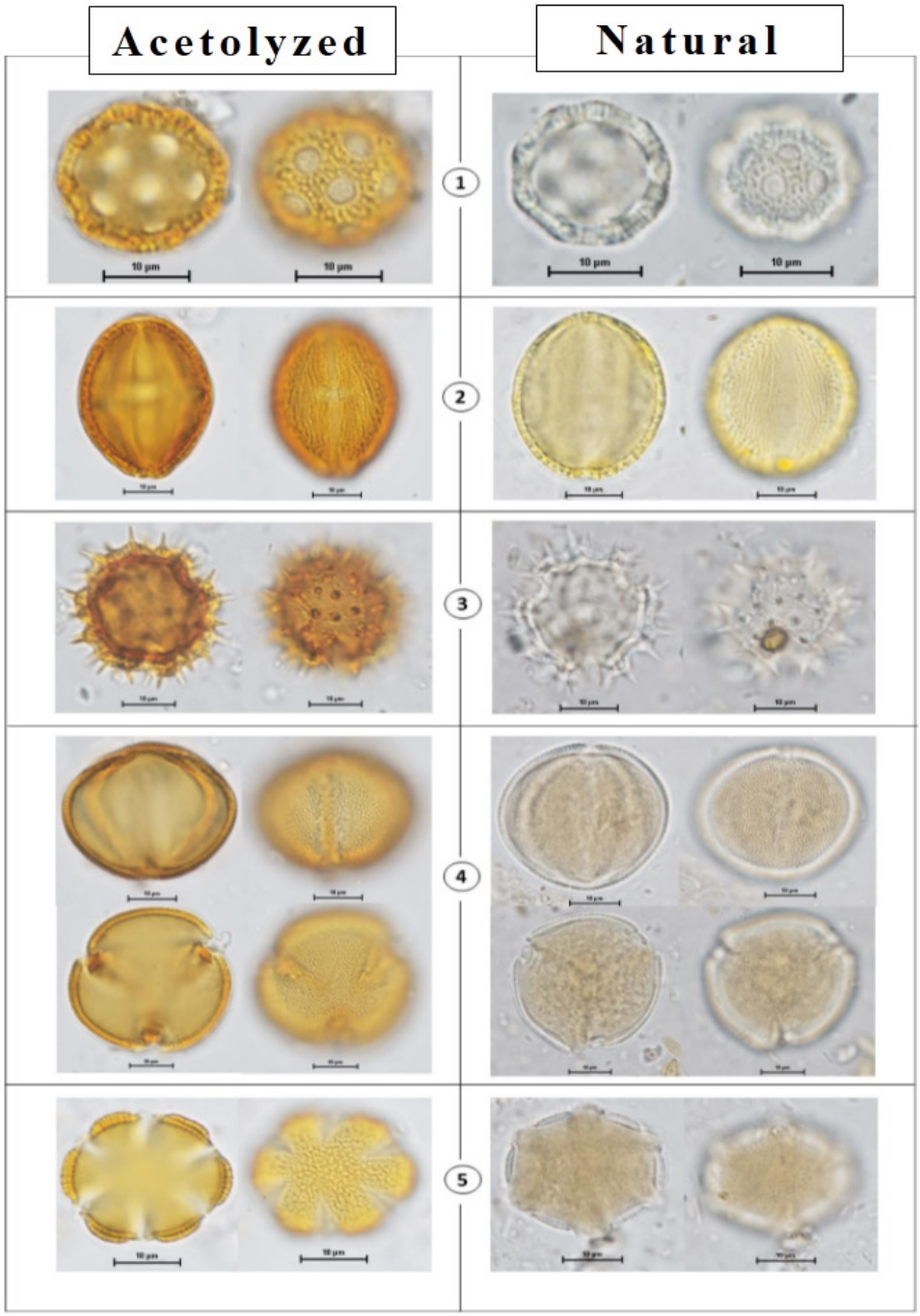


Fig 3. Photomicrographs of acetolyzed (left) and natural (right) pollen grains (1000x) of *Tetragonisca angustula* (Latreille, 1811) honey from Panama, of five botanical families: 1. Amaranthaceae, 2. Anacardiaceae, 3. Asteraceae, 4. Euphorbiaceae, and 5. Lamiaceae.

contours and the surface sculpture is finely reticulate. The form of the four natural pollen grains did not change, but the exine remained colorless because there was no acetolytic oxidation, and appears to be smoother than that of the acetolyzed pollen grains, the protoplasm was not destroyed; so the surface features are more or less indistinguishable. No. 5 Lamiaceae in polar view has six short well-defined colpi and a reticulate-granulate surface after acetolysis, similar in form and size to natural pollen grains, whereas its natural aspect is like that in No. 4. Nevertheless, the strongly hydrated protoplasm is protruding outside the apertures. The protoplasm inside the pollen grains of No. 1-3 is more or less translucent. With some practice in observing pollen grains prepared by both methodologies, it is possible to identify most of the bee plant families.

Photomicrographs of acetolyzed (amber color) and natural (grey color) Arecaceae pollen of *Trigona* pot-pollen from Panama were also observed at 200x and 1000x in Fig 4. This pollen pot was filled with one pollen type. Arecaceae is a family of perennial flowering plants known as palms. This family has unique pollen with trichotomosulcate or a single sulcus (a furrow, visible both in the acetolyzed and natural preparations), thus a monosulcate pollen type. The exine is tectate, and the sexine psilate. It is an oblate pollen with a P/E variable according to the preparation of the sample. Visually the acetolyzed pollen is more flattened with a P/E = 0.54 which is smaller than the natural pollen P/E = 0.60, measured in the 1000X images 1B and 2B, respectively. The presence of the protoplasmic membrane in natural grains allows the effect of turgidity. The use of glycerin jelly as mounting media is known to increase the volume of the pollen grains in both preparations. Here natural grains have a 20% larger equatorial axis (width of a pollen grain at the equator, representing the maximum measurable distance perpendicular to the polar axis) than acetolyzed pollen (natural = 60 µm, acetolyzed = 50 µm).

The protoplasm is the inner content of the pollen grain, and the pollenkit is the external component comprised of oils, proteins, etc. In the opinion of an acetolysis methodology expert, the melissopalynologist J.E. Moreno Patiño, both are present in natural pollen and may mask characteristics of apertures, sculpture, and structure of the exine, that are neat in acetolyzed pollen. Despite the pollen being mounted in water or glycerin, this does not clean or remove the protoplasm and pollenkit. That is why the acetolysis process helps to clean the grains and highlight their characteristics. Probably other chemical substances can help to achieve this goal (for example sodium hydroxide, potassium hydroxide, nitric acid) but never reach the efficiency of acetolysis since they are focused on destroying coarse organic fragments such as cuticles.

According to O.M. Barth, a natural melissopalynologist, pollen grains from Angiosperms are monocolpate or 1-colpate. Many colleagues call them monosulcate or 1-sulcate, just like fossils. O.M. Barth does not agree with that. The Monocotyledons are close to the Dicotyledons, and far from the Pteridophytes. In acetolyzed 1A and 1B images at 200X and 1000X respectively, pollen grains are very clean, translucent, and good for observing the exine structures, but pollen grains have a fossilized aspect. Inside the honey, they are preserved like living pollen grains and give other information, mainly about the quality of honey, without doing chemical analyses. Photomicrographs 2A and 2B are the natural pollen grains not acetolyzed. The granules visible at 1000X are lipids, some are inside the pollen grains, others outside. Sometimes they are indicators. The suggested palynological processing would be to prepare natural honey sediment and continue with acetolysis when the botanical origin of the pollen is not achieved.

These are the basic differences between the two avenues of melissopalynology based on acetolyzed and natural pollen preparations of the honey sediment. Classifying sporomorphs conservatively at the species, genus, or family level is important to ensure reproducible identifications between samples and between researchers (Mander & Punyasena, 2014).

3.2 Standard and innovative methods for pot-honey analysis

1. Physicochemical analysis

Moisture is estimated by a refractometric index as a physical measurement transformed into the water percentage of honey, which is a chemical component. Hydroxymethylfurfural (HMF) is the indicator of honey aging or heating. The pH and the free acidity are chemical indicators selected for quality control of a biological product like honey. The gravimetric method to estimate ash content was replaced by electrical conductivity, but for tropical honeys the correlation is not as good as for European honeys. The enzymes most frequently measured in honey are diastase and invertase. Their biochemical nature is not exactly physicochemical but this was the setup of the honey standards. The color is a physical property derived from the chemical nature of the honey matrix. It is an instrumental measurement in addition to sensory analysis. Aw is considered a physical property important to ascertain the water availability for microorganisms.

2. Targeted ^{1}H-NMR metabolomics

This is a multiparametric method. The maximum compound concentration measured in the profiler is 5000 mM (/www.chenomx.com/support/). Profiler works exclusively with clusters (groups of peaks), not individual peaks, it is possible to see cluster centers in ppm. There is no minimum level of absolute peak height that applies to every sample, it is fixed by the analyst. Identifications and quantifications of 36 organic metabolites were grouped into sugars, ethanol, HMF, aliphatic organic acids, amino acids, and botanical markers. Interpreting a proton NMR spectrum systematically starts with the knowledge of the molecule, how many different proton environments exist in the molecule, the chemical shift, how many protons exist in that environment, and the splitting pattern on the number of protons on carbons.

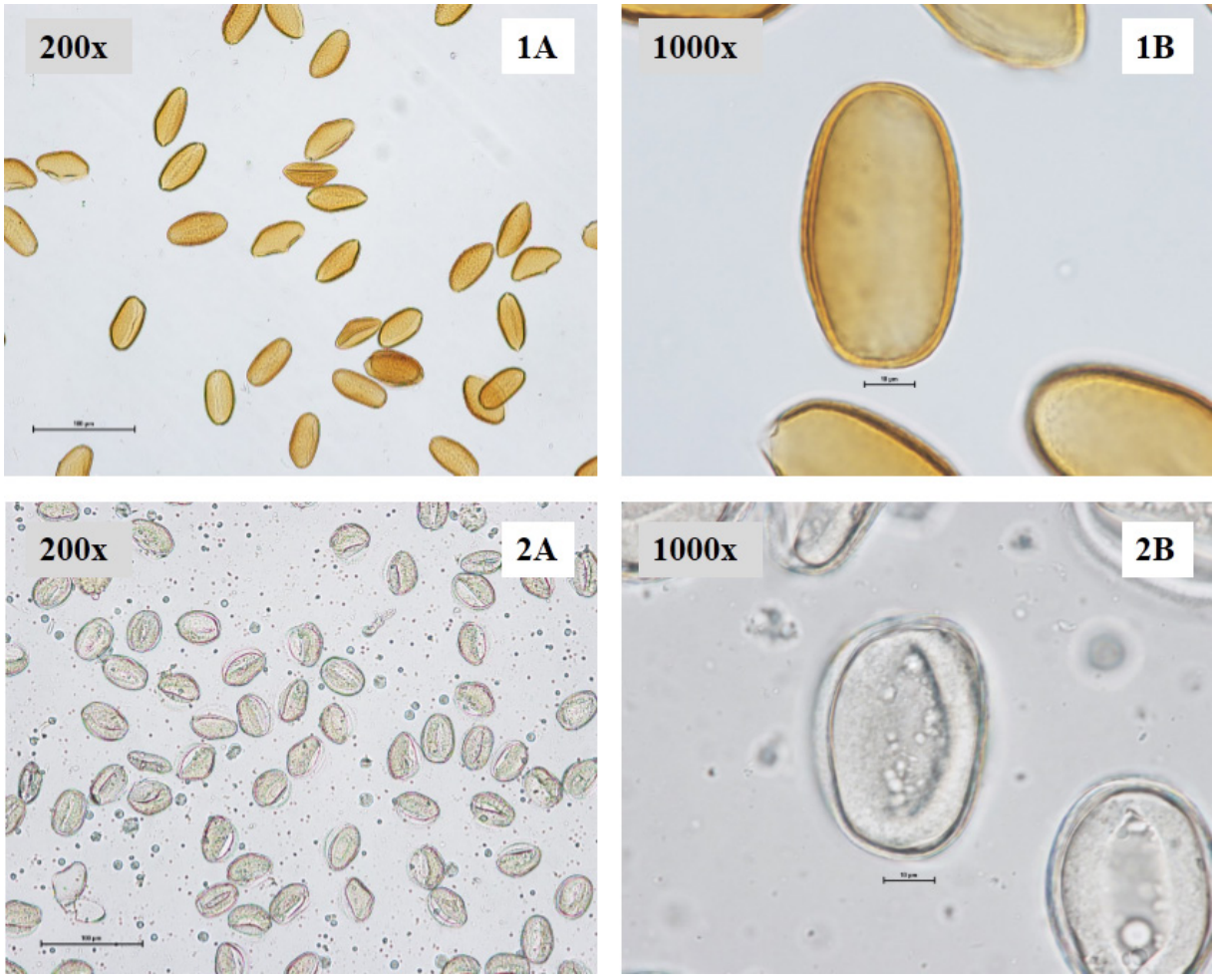


Fig 4. Acetolyzed (1) and natural (2) Arecaceae pollen at 200x (A) and 1000x (B).

The 36 metabolites were listed in Supplementary Table S1, with their molecular formula, chemical structure, ^{1}H-NMR ranges in ppm, and signal type. The NMR signals shift around depending on matrix, pH, solvent, temperature, etc. Therefore, shifts can be compared directly under absolutely the same conditions. This explains the differences observed between experimental honey (Ohmenhaeuser et al., 2013) and other matrices. Concerning the signal type, the multiplicity typically stays constant, apart from some cases where the peaks might shift into each other.

Few spectra of these metabolites are illustrated in Fig 5. The X-axis corresponds to the chemical shift (ppm), and the Y-axis is generally omitted, based on the intensity of the signals in arbitrary units. The ^{1}H-NMR spectra increase the chemical shift (ppm) from right to left. They need to be expanded for the identification of signal types that are otherwise overlapped in the main spectra (0-6 ppm). In the upper right corner, region 1.0-1.6 ppm, a) ethanol is the first metabolite with a triplet signal, and b) lactic acid is the second metabolite with a doublet signal. Below is the following region 2.6-3.0 ppm with c) acetic acid and d) citric acid. In the upper left corner, the following region, 4.6-5.3 ppm has the sugar e) glucose, and the last expanded spectra below 7.8-8.6 ppm have a singlet of the aliphatic organic acid f) formic acid.

2.1 Sugars

Ten sugars were identified and quantified by ^{1}H-NMR. A further fructose/glucose ratio and fructose +glucose addition were estimated (Pubchem, https://pubchem.ncbi.nlm.nih.gov/). **Fructose** $C_6H_{12}O_6$ is one of the three most common natural monosaccharides, a ketonic simple sugar found in fruits – originating its etymology –, some vegetables, and honey. High-fructose corn syrup (HFCS) is used to sweeten processed foods and beverages, and to adulterate honey. Fructose is a fructopyranose, a D-fructose, and a cyclic hemiketal. Fructose is a reducing sugar. Fructose bonded to glucose is a component of sucrose. **Glucose** $C_6H_{12}O_6$ (from Ancient Greek *γλῦκύς*, *glukús* "sweet") is one of the three most common natural monosaccharides, a simple sugar of six carbon atoms and an aldehyde group. Linear glucose is in equilibrium with the four cyclic forms. Glucose bonded to fructose is a component of sucrose. Glucose is a reducing sugar. **Sucrose** $C_{12}H_{22}O_{11}$ is a disaccharide of glucose and fructose, a glycosyl glycoside formed by glucose and fructose units joined by an acetal oxygen bridge from the hemiacetal of glucose to the hemiketal of fructose. **Gentiobiose** $C_{12}H_{22}O_{11}$ is a disaccharide of glucose, a natural sugar found in *Gentianopsis virgate* and *Gentianopsis crinit*, originating its etymology. Synonym 6-O-beta-D-glucopyranosyl-D-glucopyranose.

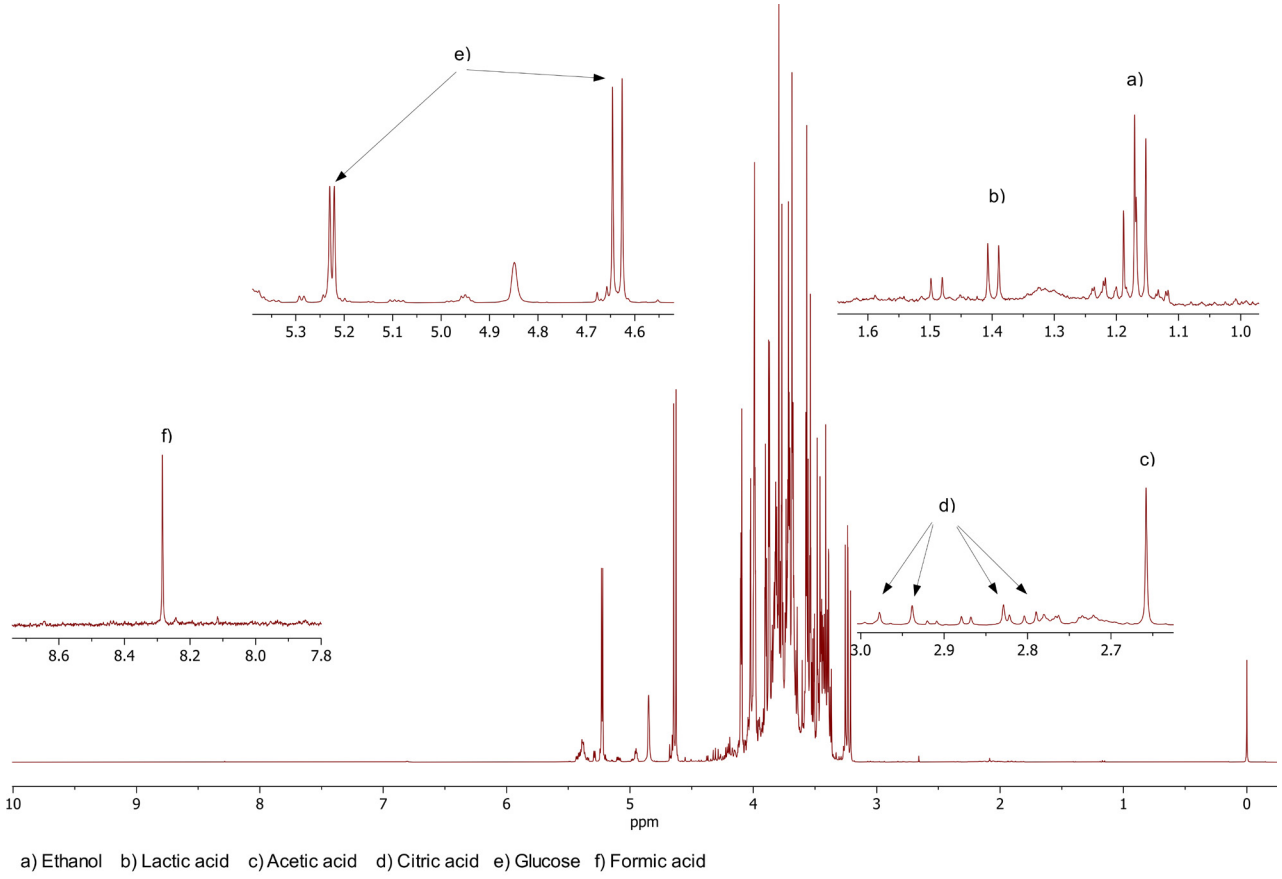


Fig 5. ^{1}H-NMR spectra of metabolites present in honey: sugar, aliphatic organic acids, ethanol.

Maltose $C_{12}H_{22}O_{11}$ is a disaccharide of glucose. Its degradation produces hydroxymethylfurfural. **Maltotriose** $C_{18}H_{32}O_{16}$ is a trisaccharide in which the glucose residue at the reducing end is in the pyranose ring form and has an alpha configuration at the anomeric carbon atom. It is a human metabolite. **Mannose** $C_6H_{12}O_6$ (from the Hebrew *manna*' miraculous food') is a monosaccharide with six carbon atoms and an aldehyde group. It is thus a D-aldohexose. **Melezitose** $C_{18}H_{32}O_{16}$ is a trisaccharide produced by insects such as aphids. It plays a role as an animal metabolite. **Raffinose** $C_{18}H_{32}O_{16}$ is a trisaccharide composed of galactose, glucose, and fructose. Glucose is a bridge between both α and β glycosidic bonds with galactose and fructose. It belongs to a family of oligosaccharides α-galactosyl derivatives of sucrose. It is found in asparagus, beans, broccoli, cabbage, celery, and radish. **Turanose** $C_{12}H_{22}O_{11}$ is a glycosylfructose disaccharide. **Fructose/Glucose** This ratio is used as a predictor of honey crystallization. **Fructose + Glucose**. This addition is the major sugar fraction in *Apis mellifera* honey but in certain pot-honey other major sugars such as trehalose are present.

2.2 HMF

The hydroxymethylfurfural (HMF) is a degradation product of fructose caused by heating and aging, and increases in acidic environments like that of honey.

2.3 Ethanol

The presence of ethanol in pot-honey derives from the alcoholic fermentation of diluted sugars by yeast enzymes. A balanced chemical equation for alcoholic fermentation converts one mole of glucose into two moles of liquid ethanol and two moles of volatile carbon dioxide, producing two moles of ATP in the process via two moles of pyruvate.

$$C_6H_{12}O_6 \text{ diluted in water} \rightarrow 2C_2H_5OH(\text{liquid}) + 2CO_2(\text{gas})$$

2.4 Aliphatic organic acids (AOA)

Most of these organic acids participate in the Krebs cycle, also called Tricarboxylic Acid Cycle (TAC) or Citric Acid Cycle. They are easily transformed into each other by electron transfer reactions. In metabocards (e.g., for acetic acid //hmdb.ca/metabolites/HMDB0000042), the AOA are present in bacteria, plants, animals, and humans, for their universal role in the organic reactions of life. They are commonly known as their salts or anionic forms: Acetate, citrate, formate, fumarate, lactate, malate, pyruvate, quinate, shikimate, and succinate. The AOA of honey was recently reviewed (Vit & Simova, unpublished data).

Acetic acid $C_2H_4O_2$ is a monocarboxylic acid CH_3-COOH, the smallest short-chain fatty acid (SCFA). It has a distinctive sour taste and acrid vinegar smell. It is the product of a two-stage fermentation: 1. Anaerobic fermentation,

the transformation of sugars into ethanol by yeasts, 2. Aerobic fermentation, is the oxidation of ethanol into acetic acid, by acetic acid bacteria (AAB). **Citric acid** $C_6H_8O_7$ is a tricarboxylic acid $HOC(COOH)(CH_2COOH)_2$ found in citrus fruits (grapefruit, lemon, orange, tangerine) and pineapple. **Formic acid** CH_2O_2 is a monocarboxylic acid HCOOH, the simplest carboxylic acid also called methanoic acid. Its presence in honey originates from the social chemistry of bee communication and defense. **Fumaric acid** $C_4H_4O_4$ is a dicarboxylic HO_2 acid $CCH=CHCO_2H$, a key intermediate metabolite in the TCA used to produce cellular energy as adenosine triphosphate (ATP). The fumarate salt has a fruit-like flavor and is useful as an acidity regulator, and tartness agent for food. **Lactic acid** $C_3H_6O_3$ is a monocarboxylic acid $CH_3CH(OH)COOH$ produced by lactic acid bacteria (LAB). It is found in fermented food such as buttermilk, kefir, kimchee, kombucha, sauerkraut, yogurt. The intense sourness of lactic acid at pH 3.5 is caused by 70% of undissociated acid compared with 30% of citric acid (Dziezak, 2016). **Malic acid** $C_4H_6O_5$ is a dicarboxylic acid $HOOCCH_2CH(OH)COOH$ tart-tasting. The tart taste of wine and honey has a malic acid component. Malate and fumarate are intermediates in the TCA cycle. Malate can be formed from pyruvate. Malic acid is both derived from food, like apples and pears, and is bio-synthesized by the TCA in the mitochondria. **Pyruvic acid** $C_3H_4O_3$ is a monocarboxylic acid $CH_3COCOOH$. **Quinic acid** $C_7H_{12}O_6$ is an astringent cyclic polyol from plant sources. Cyclic acids are not represented by the linear molecular formula. Quinic acid and shikimic acid are key intermediates in the biosynthesis of aromatic compounds in living systems, with important roles in the formation of phenolic compounds. Citric, malic, and quinic acids are the major AOA in coffee. The sour perception of coffee has a component related to quinic acid. **Shikimic acid** $C_7H_{10}O_5$ is a cyclohexene, a cyclitol, and a cyclohexanecarboxylic acid derived from phenolics. The shikimate biosynthesis pathway produces precursors for the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The physiology of the bee or metabolic associations with microbiota in the nest, also the bee diet, or their combination may originate shikimic acid in honey (Santos-Sánchez et al., 2019). **Succinic acid** $C_4H_6O_4$ is a dicarboxylic acid $(CH_2)_2(COOH)_2$, whose name denotes amber from Latin *succinum*. It is an electron donor in the TCA cycle. Succinate is a cell signaling molecule, that alters gene expression patterns, modulates epigenetics by hormone-like signaling functions, and is a flavor-enhancer (Dziezak, 2016).

2.5 Amino acids (AA)

Eight free amino acids were identified and quantified in pot-honey. **Alanine** $C_3H_7NO_2$ is a nonessential amino acid, biosynthesized from pyruvate by transamination. It is a glucogenic amino acid that constitutes a high percentage of the amino acids in most proteins. It contains an amine group and a carboxylic acid group, both attached to a carbon atom with a methyl group side chain. It is active in breaking down tryptophan and vitamin B-6. It is a source of energy for muscles and the central nervous system. It is active in the immune system. **Aspartic acid** $C_4H_7NO_4$ is a nonessential amino acid that plays an important role as general acids in enzyme active centers. **Glutamine** $C_5H_{10}N_2O_3$ is a charge-neutral, polar amino acid, with a side chain similar to that of glutamic acid, except that the carboxylic acid group is replaced by an amide. **Leucine** $C_6H_{13}NO_2$ is a non-polar aliphatic amino acid with a side chain isobutyl group. **Phenylalanine** $C_9H_{11}NO_2$ is an aromatic amino acid, like a benzyl group substituted for the methyl group of alanine, or a phenyl group in place of the terminal hydrogen of alanine. **Proline** $C_5H_9NO_2$ is a proteinogenic amino acid, without the amino group $-NH_2$, but is like a secondary amine with nitrogen in the protonated NH_2^+ form under biological conditions, while the carboxyl group is in the deprotonated $-COO^-$ form. **Tyrosine** $C_9H_{11}NO_3$ is a non-essential amino acid with a polar side group. L-Tyrosine or tyrosine or 4-hydroxyphenylalanine is a non-essential amino acid. **Valine** $C_5H_{11}NO_2$ is a non-polar aliphatic amino acid with an α-amino group, an α-carboxylic acid group, and a side chain isopropyl group, an essential amino acid in humans that must be obtained from the diet.

2.6 Botanical markers

An interest in discovering residual botanical markers of diverse chemical nature has attracted engaging research in the years. A recent review was conducted for botanical markers of twenty monofloral *Apis mellifera* honeys (Machado et al., 2020). For pot-honey, more recent contributions on entomological markers are those of the Tomás Barberán group on distinctive honey flavonoids of *Melipona favosa* from Venezuela (Truchado et al., 2011) and *Tetragonula carbonaria* from Australia (Truchado et al., 2015). From the six botanical markers included in the reference sample for quantification in food applications (QuantRefA-NMR-Tube), kynurenic acid was not detected.

Acetoin $C_4H_8O_2$ is formed by the decarboxylation of two pyruvic acids $C_3H_4O_3$. This natural volatile is used as a botanical marker for the Australian *Eucalyptus* species *E. leucoxylon* and *E. melliodora* (D'Arcy et al., 1997). **2,3-Butanediol** $C_4H_{10}O_2$ molecular formula $(CH_3CHOH)_2$ is classified as a vic-diol. It exists as three stereoisomers, a chiral pair, and ameso isomer. A fermentation pathway will ferment glucose and produce a 2,3 butanediol end product instead of organic acids. **Dihydroxyacetone** (DHA) $C_3H_6O_3$ is the precursor to methylglyoxal (Smallfield et al., 2018). It is obtained from plant sources such as sugar beets and sugar cane, and by the fermentation of glycerin. **Kynurenic acid** $C_{10}H_7NO_3$ is a metabolic derivative of the amino acid L-tryptophan. **Methylglyoxal** $C_3H_4O_2(MGO)CH_3CCHO$ is a reduced derivative of pyruvic acid. **3-Phenyllactic acid** $C_9H_{10}O_3$ is a lactic acid with a phenyl substituent, it belongs to the class of organic compounds known as phenylpropanoic acids.

3. Biochemical composition

3.1 Flavonoids

Flavonoids are secondary metabolites, a group of polyphenols biosynthesized by plants. Most flavonoids in edible plants are β-glycosides, bound to one or more sugar molecules which are detached by exposure to microbial β-glucosidases. The aglycones are not bound to sugars, and glycosylated flavonoids have a sugar substituent. Methoxylated forms also exist. According to their structure, flavonoids are divided into six major subclasses: 1. Anthocyanidins, 2. Flavanols, 3. Flavanones, 4. Flavones, 5. Flavonols, and 6. Isoflavones. The Linus Pauling Institute has a Micronutrient Information Center for the public, with useful information on flavonoids online.

Two measurements of flavonoids were done: 1. Total flavonoids by spectrophotometry, and 2. Flavonoid identification by High-Performance Liquid Chromatography/ Photodiode-Array Detection/Electrospray Ionization Ion Trap Mass Spectrometry (HPLC/DAD/ESI-MS). This advanced technique was needed for the study of flavonoids having diverse chemical natures, and present in a complex food matrix like honey. The monoglycosides/diglycosides/triglycosides and the position of the O-glycosylation and the sugar moiety were identified by the study of the MS data. Flavonoids have significant antioxidant properties, varying according to the chemical structure. Some of them like the aglycone quercetin are scavengers of Reactive Oxygen Species (ROS) such as superoxide radicals, and singlet oxygen, and also inhibit the formation of lipoid hydroperoxide radicals. In Fig 6 the chemical structures of the aglycone quercetin, and quercitrin, one of its glycosides, formed with the deoxy sugar rhamnose. The effects of quercitrin *in vivo* perhaps derive from the release of quercetin by intestinal microflora. Both quercetin and quercitrin attenuate LPS-induced macrophage inflammation and oxidative stress by anti-ROS effect (Tang et al., 2019). Their theoretical calculation illustrated that the oxygen atom on B rings suggests a possible mechanism for the anti-inflammatory and ROS scavenging effects.

3.2 Polyphenols

They are generally called phenolic compounds, and this term is accepted and widely used. A single phenolic such as hydroxybenzoic acid is not toxic at all. Tyrosine, and essential amino acid, is a single phenolic. However, phenol (hydroxy-benzene) is toxic. Polyphenols are metabolites that contain at least two phenolic rings in the structure as happens for flavonoids, stilbenes, lignans, hydrolyzable and condensed tannins, and some dimers of hydroxycinnamics (rosmarinic acid, curcumin). A more inclusive name to cover polyphenols and simple phenolics is (poly)phenols.

In the scientific literature, polyphenols are frequently wrongly named "phenols". This is a conceptual mistake considering that a polyphenol is healthy and a "phenol" is toxic. The plural of the toxic phenol molecule is not a synonym for polyphenol, meaning phenol structures inserted in one molecule, from the Greek *πολύς*, poly- is a prefix meaning "many".

4. Antioxidant activity

Three methods were selected to measure the antioxidant activity of pot-honey using spectrophotometric techniques: ABTS, DPPH, and FRAP. The $ABTS^{+}$and DPPH free radical scavenging assays are based on the quenching of stable colored radicals $ABTS^{\bullet+}$ or DPPH to show the radical scavenging ability of antioxidants in a complex matrix like honey. The FRAP ferric reducing antioxidant power is an antioxidant capacity assay that uses Trolox as a standard to measure the antioxidant capacity of honey because it contains polyphenols.

5. Microbiological quality

Pollen, nectar, the digestive tracts of bees, and the environment are the primary sources of microbial contamination in pre-harvest honey, which is very difficult to control. While post-harvest sources include air, and honey handling, and cross-contamination influences microorganisms' levels in honey. Honey's moisture and temperature influence microbial growth. Thus, low mixture has been considered

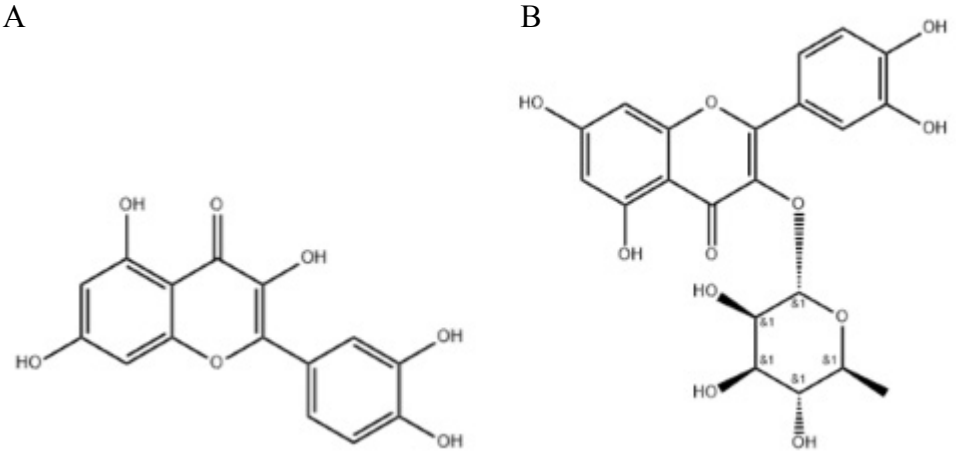


Fig 6. Chemical structures of **A.** the aglycone quercetin, and **B.** the glycosylated quercitrin with the deoxy sugar rhamnose. The B ring with the –OH in position 3, the flavonol signature.

to prevent honey spoilage for a long time. Yeast and spore-forming bacteria are commonly found in pot-honey. While these properties indicate safety and quality for commercial honey, the role of microorganisms in human illness is the most considered. For no particular reason, total plate counts from honey samples can range from zero to tens of thousands per gram. Yeasts can be found in most honey samples. Bacteria do not proliferate in honey, thus a high number of vegetative bacteria could indicate a recent secondary source of contamination. At low temperatures, specified vegetative bacteria can persist for years in honey. On the other hand, honey provides antimicrobial properties that prevent many microbes from growing or persisting. This was a classic approach to have counts on microbiological screening of total, yeast and mold, *Bacillus,* and lactic acid bacteria.

5.1 Aerobic Plate Count Agar (PCA)

The aerobic Plate Count Agar (PCA) is used to determine how many microorganisms are present in a product. It is also known as the standard plate count, mesophilic count, or total plate count. The test is predicated on the idea that when each cell is mixed with the necessary nutrient agar, it will form a visible colony. It is a generic test only for organisms that grow aerobically at mesophilic temperatures (25 to 40 °C) not for the entire bacterial population. APC does not discriminate among bacterial types however, it can be used to determine hygienic quality, organoleptic acceptability, and conformity with good manufacturing procedures and safety indications.

5.2 Yeast and mold counts

In this test, the total yeast and mold population is counted, whereas no identifications are made. Both yeasts and mold can produce various degrees of degradation and decomposition of honey. Yeasts found in honey can resist high concentrations of acids and sugar, they are osmotolerant. The number of yeasts increases in relation to the moisture of honey. Yeasts are classified as non-filamentous fungi, which require higher moisture than molds but less than bacteria for growth, and an optimum temperature range for growth is between 25 and 30 °C. They transform sugars into ethanol during fermentation with an ideal acid pH. Additionally, filamentous fungi (mold) can produce substances including enzymes and acids. Yeast and mold counts in many honey samples are below 100 colony-forming units per gram (cfu/g).

5.3 *Bacillus* count

Bacillus spp. are pore-forming bacteria frequent in honey, their count is an indicator of microbiological quality. Osmotolerant microbial contamination of honey has primary sources (pollen, digestive tracts of bees, dust, air, earth, nectar), and post-harvest secondary sources as in any food product (air, soil, water, food handlers, containers, cross-contamination, equipment, buildings). Primary sources of contamination are very difficult to control, whereas secondary sources are controlled by good manufacturing practices. Bacteria do not reproduce in honey, thus vegetative bacteria origin could be contaminated from a secondary source. The bee gut-microbiota contains 1% yeast, 27% Gram-positive bacteria including *Bacillus*, *Bacteridium*, *Streptococcus,* and *Clostridium* spp; 70% Gram negative or Gram variable bacteria including *Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus*, and *Pseudomonas* (Tysset et al., 1991).

5.4 Lactic Acid Bacteria (LAB) count

Acid-producing microbes are common in nature and can be found in honey. Lactic acid bacteria (LAB) are one of the most important types of acid-producing bacteria in the food industry. LAB counts are used to determine the total levels of bacteria in honey.

6. Honey-Microbiome

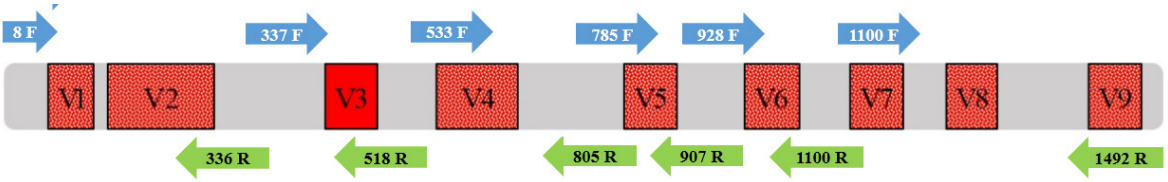
The Sanger method is a first-generation sequencing (FGS) technology. Recent culture-independent approaches for characterizing complex microbiome communities using next-generation sequencing (NGS) technologies have evolved over time. They provide fast and efficient identification of the microbial communities (Mardis, 2008), and permit phylogenetic studies. These methods are not based on growing microbes in culture media. For prokaryotes characterization, the V3-region of the 16S rRNA gene was selected as a marker for bacteria (Fig 7). It combines highly conserved regions useful as nearly universal prokaryotic primers, while at the same time it has maximum nucleotide heterogeneity and discriminatory power for capturing bacterial species due to the hypervariable V3-region (Castro-Mejía et al., 2020).

Although the V3-region of 16S rRNA was used to describe the prokaryote community, we applied the flexibility of the yeast ribosomal gene complex (Fig 8) to fungal markers for the detection of fungi and eukaryotic organisms. For example, the Internal Transcribed Spacer (ITS) regions were useful for the detection of the filamentous fungi and D1+D2 domain of Large Subunits (LSU) 16S ribosomal rDNA for yeasts, using the primers NL1 and NL4. Then, the species were identified by their GenBank accession number, after sequencing the D1+D2 domain of Large Subunits (LSU) ribosomal rDNA.

Microbial genomic DNA approaches for identifying the microbiome of honey-making bees and their products in the nest have increased. For instance, the bee gut microbiome of *Apis mellifera* has been reported to be a home for novel lactic acid bacteria (Olofsson & Vásquez, 2008). The lactic acid bacteria of bee pollen and bee bread were studied in *Apis mellifera* (Vásquez & Olofsson, 2009). Similarly, the bee gut microbiome has also been examined in both Apini and Meliponini (Vásquez et al., 2012), and in Kenyan stingless bees (Tola et al., 2021). The cuticle microbiome was also used

to compare sympatric Australian stingless bees (Leonhardt & Kaltenpoth, 2014) where a lack of Acetobacteriaceae in *Austroplebeia australis* clearly differentiated it from *Tetragonula carbonaria* and *Tetragonula hockingsi*. The latter two process their sour honey and pollen with AAB, and thus are rich in acetic acid (Massaro et al., 2018). The honey yeast microbiome was investigated in 17 species of Brazilian stingless bees, and their associations based on the entomological origin were confirmed (Echeverrigaray et al., 2021). These authors identified 16 species of yeasts from nine genera: *Candida*, *Debaryomyces*, *Moniliella*, *Rhynchogastrema*, *Starmerella*, *Torulaspora*, *Wickerhamiella*, *Wickerhamomyces*, and *Zygosaccharomyces*.

Generally, the alteration of the gut microbiota of honey bees is reportedly due to seasonal changes (Kešnerová et al., 2020) and also influenced by geography (Liu et al., 2021). However, differences in the hygienic habits of stingless bees were reflected in the bacteriome of the inner



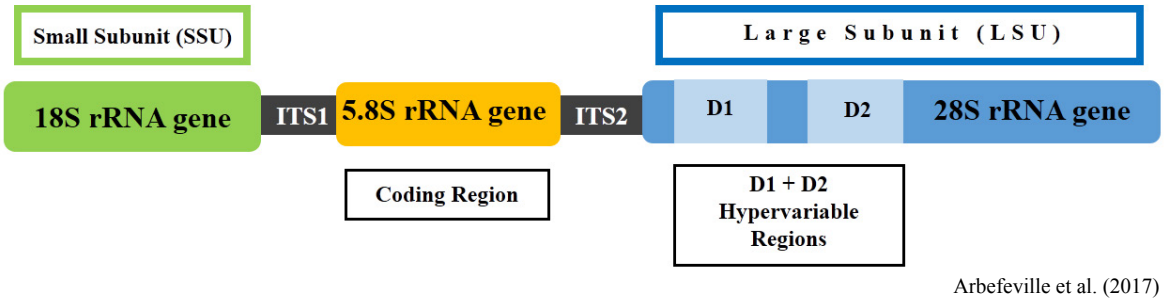
Forward Primers	Reverse Primers
8F – AGAGTTTGATCCTGGCTCAG	336R – ACTGCTGCSYCCCGTAGGAGTCT
27F – AGAGTTTGATCMTGGCTCAG	518R – GTATTACCGCGGCTGCTGG
337F – GACTCCTACGGGAGGCWGCAG	805R – GACTACHVGGGTATCTAATCC
533F – GTGCCAGCMGCCGCGGTAA	907R – CCGTCAATTCCTTTRAGTTT
785F – GGATTAGATACCCTGGTA	1100R – GGGTTGCGCTCGTTG
928F – TAAAACTYAAAKGAATTGACGGG	1492R – CGGTTACCTTGTTACGACTT
1100F – YAACGAGCGCAACCC	

Abellan-Schneyder et al. (2021)

Fig 7. Generalized linear diagram of a bacterial ribosomal gene complex (drawing not to scale). Sequence variation among bacterial 16S rRNA is known to be not uniformly distributed. Nine hypervariable regions (V1-V9) were identified among bacteria, tagged with the location of the genes of each region. Blue arrows denote the names of forward primers, and green arrows signify the names of reverse primers.

surface of nests. Several species of Brazilian stingless bees with distinctive habits for collecting materials to build their nests were investigated: 1. The microbiome of *Frieseomelitta varia* and *Tetragonisca angustula* were associated with *Pseudomonas* sp. and *Sphingomonas* sp. from the phylloplane (surface of leaves) and flowers because these bees collect plant materials, 2. The microbiome of *Trigona spinipes* was related to coliforms such as *Escherichia coli* and *Alcaligenes faecalis* that are abundant in mud and feces they collect, and 3. *Melipona quadrifasciata* combined microbiome of the previous two groups was caused by the collection of both plant resins and mud-feces (de Sousa, 2021).

Interestingly, another study showed that the diversity of the gut microbiome of the stingless bees was associated with host wing size (Fig 8) where the *T. carbonaria* bees with larger wings from more southern populations had higher microbial diversity in their guts (Liu et al., 2021). Moreover, the core gut microbiome of these Australian stingless bees consisted of a distinct group of bacterial taxa from that observed in honey bees and bumble bees such as Acetobacteraceae, *Bombella*, *Lactobacillus* sp., and *Snodgrassella*. The core microbiome of the stingless bee were *Aureobasidium pullulans*, Didymellaceae, *Malassezia globose*, *Malassezia restricta*, and *Monocilium mucidum*.



Arbefeville et al. (2017)

Fig 8. Generalized linear diagram of anuclear yeast ribosomal gene complex (drawing not to scale). The organization of this complex includes a sequence coding for the 18S rRNA gene, an internal transcribed region 1 (ITS1), the 5.8S rRNA gene coding region, another internal transcribed region 2 (ITS2), and the sequence coding for the 28S rRNA gene. The 28S rRNA is preceded by the D1+D2 hypervariable regions.

Dendrograms of microbial taxa associated with stingless bees show graphic evolutionary relationships, they are linear or circular graphics clustering more related species together. This microbial composition influences chemical profiles of honey processing and post-harvest transformations, as well as tracing back pollen, nectar, and other materials origins, defense, hygiene, phylloplane, bee, and plant pathogens. Host-specific stingless bee-AAB and -LAB associations may have phylogenetic and geographical components in their natural history, as observed for *Austroplebeia* and *Tetragonula* bees (Leonhardt et al., 2014; Leonhardt & Kaltenpoth, 2014), like fungi and yeasts.

7. Sensory evaluation

Descriptive quantitative measurements were done on odor, aroma, taste, and persistence of pot-honeys. Their histograms show similarities and differences between sensory family compositions for odor and aroma perceptions, compared between stingless bee species processing differently the honey in the cerumen pots with their associated microbiome. For example, see a *Melipona* honey in Fig 9.

"Fermentation produces the majority of flavor that we recognize as 'chocolate'. In addition to the production of acetic and lactic acid, ethanol, and minor sugars such as glycerol and mannitol, a variety of volatile compounds are also produced. More than 600 volatile compounds are found in fermented cacao." //thechocolatelife.com/factoid-fermentation-produces-the-majority-of-flavor-that-we-recognize-as-chocolate-in/ Similarly, in pot-honey, sensory complexity is added during the fermentation of thin honey in the warm and moist environment of the nest. Why a microbiota type is selected by some stingless bee species and not others? This question has not an immediate origin of bee vectoring yeasts from floral nectars and other sweet resources like honeydew. Evolutionary biology may fit micro-ecological pieces into place with important contributions to be deciphered from nests observed in the present. We could smell some of them from stingless bee colonies and their substrates but were not yet able to identify and explain these relationships or the reasons for their origins. For example, the blue cheese smell of the Ecuadorian *Scaptotrigona vitorum* nest was annotated as Roquefort in fieldwork forms (Vit, 2022).

From another perspective, honey bees changed the microbiota harbored in *Brassica* pollen collected and packed after adding regurgitated nectar to glue the pollen grains (Prado et al., 2022). The bacterial diversity of pollen increased with honey bee symbionts such as *Bombella*, *Frischella*, *Gilliamella,* and *Snodgrassella*, and nectar-dwelling *Lactobacillus* – having bee gut microbes as inoculum during corbicular pollen processing.

8. The honey authenticity test by interphase emulsion, became a biosurfactant test

Genuine pot-honeys tested before this integrated study had one, two, and three phases, as illustrated in Fig 2 in the previous section. In Fig 10, the result observed in the *Scaptotrigona vitorum* honey was interpreted as a Honey Biosurfactant Test (HBT), for the surfactant activity. This was a new Ecuadorian *Scaptotrigona* species described by Engel (2022).

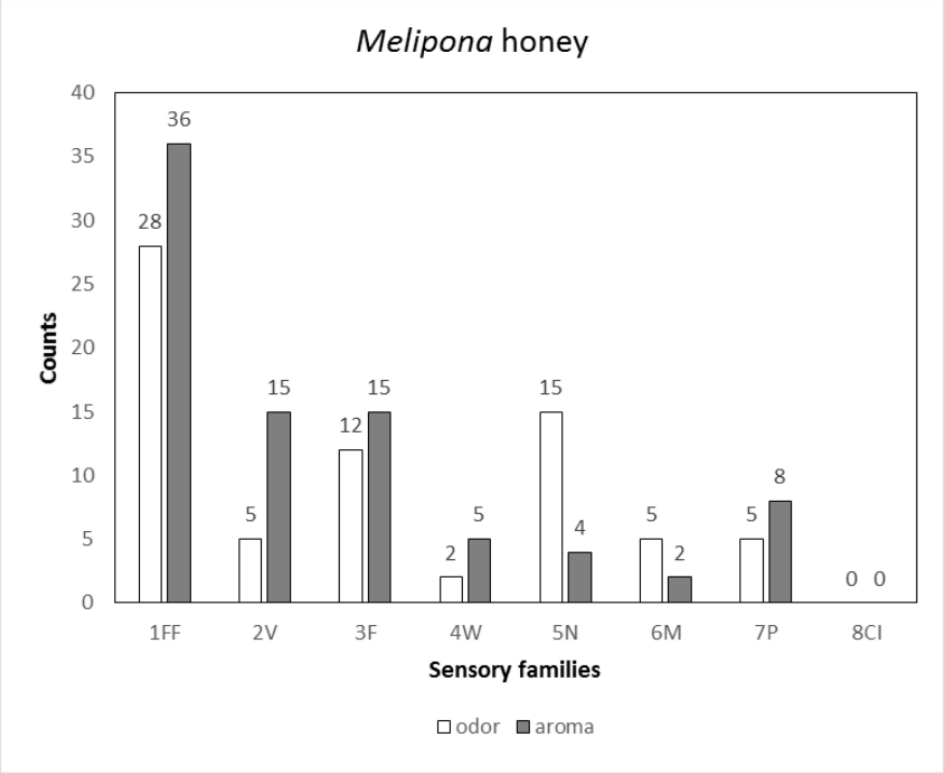


Fig 9. *Melipona* honey histograms for odor and aroma descriptors of sensory families 1FF Floral-fruity, 2V Vegetal, 3 F Fermented, 4 W Wood, 5 N Nest, 6 M Mellow, 7 P Primitive, and 8 CI Chemical Industrial.

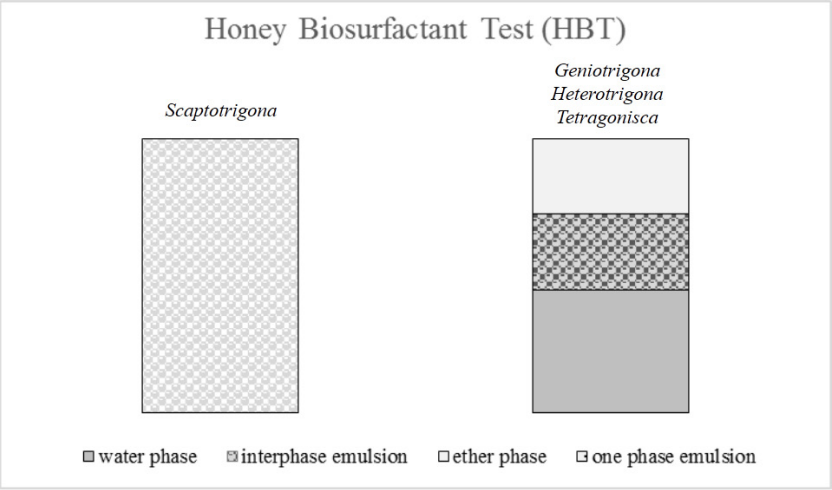


Fig 10. Diagram of the Honey Biosurfactant Test (HBT). To the left, the surfactant activity causes a cloudy emulsion in the reaction volume. To the right, the honey-based emulsion is an interphase between the water (lower) and the ether (upper) phases. This test was done by B. Chuttong and N. Zawawi for *Geniotrigona thoracica* and *Heterotrigona itama*, and P. Vit for *Scaptotrigona vitorum* and *Tetragonisca angustula*.

9. Final Remarks

These methods were used to analyze pot-honeys during post-harvest and to create a database to follow the pot-honey transformations along the established period of 35 days at 30 °C. The participating laboratories were led by experts in the field, who sometimes also coordinated the logistics for pot-honey harvest. Preliminary studies were based on multiparametric targeted ^{1}H-NMR of Ecuadorian pot-honey (Vit et al., 2022), but never compared before with classic microbiology and honey-microbiome. A missing data is on Volatile Organic Compounds VOCs measured with PTR-ToF-MS, a powerful method requiring a small quantity of honey for key information on odorants developed in biological processes. These integrated methods of analysis were proposed for the first time to follow a limited but substantial post-harvest period where major changes have been observed over the years by stingless bee keepers, consumers of their honey, and scientists. New data should provide a few answers and more questions for proposing future research avenues.

Supporting sustainable meliponiculture, pot-honey regulations, and quality schemes, start from the ID of stingless bee species. For example, about 10,000 *S. vitorum* Catiana hives are kept by communities of stingless bee keepers from El Oro and Loja provinces (S Loayza and D. Encalada, unpublished data). The approval of Ecuadorian standards for pot-honey (INEN, 2015), including *Scaptotrigona*, is imperative, and could be based on *S. vitorum* research.

The relevance of the entomological origins and ethnic names of pot-honey in a normative domain, would be also mandatory for Protected Designation of Origin (PDO) e.g. the *Scaptotrigona* honey called Catiana in Ecuador, and its Guaranteed Traditional Specialty (GTS) protecting the artisanal production method. The quality policy of the European Union (EU) focuses on protecting names of unique products. These EU strategies benefit producers and consumers, and would visualize the biodiversity of tropical stingless bee honey worldwide.

Authors' Contributions

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