A HONEY AUTHENTICITY TEST BY INTERPHASE EMULSION REVEALS BIOSURFACTANT ACTIVITY AND BIOTECHNOLOGY IN THE STINGLESS BEE NEST OF *Scaptotrigona vitorum* 'CATIANA' FROM ECUADOR

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SUMMARY

Bees are valuable pollinators of fruit crops and other flowering plants. Honey is a medicinal food of plant and animal origin, with a social impact on the wellbeing of bee keepers. The Neotropical apifauna has about 500 species of stingless bees (Meliponini). Commercial bee-keeping with Apis mellifera *underestimates the cultural legacy of meliponiculture, and both are affected by the presence of fake honeys in the market. The alternative technique Honey Authenticity Test by Interphase Emulsion (HATIE) was investigated to detect false honeys. This technique based on an interphase emulsion test can be performed by bee-keepers, farmers, housekeepers, professionals and consumers of honey in general. Five genuine honeys produced by* Apis mellifera, Geotrigona leucogastra, Melipona mimetica, Scaptotrigona vitorum, Tetragonisca angustula *by a fake honey from Ecuador were analyzed. The HATIE was fast and effective to detect fake honey (two phases), and genuine honeys (one and three phases). A further screening of 51 Asian, Australian, European and Latinamerican honeys was done. Additionally, the HATIE generated a new application as the Honey Biosurfactant Test (HBT) of* Scaptotrigona vitorum *'Catiana' (one phase) with a potential microbial origin, and its entomological origin in this set of honeys. 'Catiana' nests smell like Roquefort cheese, indicating a suspected fungi association with this rural stingless bee highlighted by its distribution, productivity, and the peculiarities described in this research, 80 years after the description of the new genus* Scaptotrigona *Moure, 1942. Paradoxically, this communication without microbiological analysis, infers the fungal presence in the nest of* Scaptotrigona vitorum *by sensory observations different from the classic sensory evaluation of honey.*

Introduction

Neotropical stingless bees comprise 500 of the 600 identified species in the world. They can be found from 27.03°N in North America, Alamos, Sonora, Mexico, up to 34.90°S in South America, Montevideo, Uruguay (Roubik and Vergara, 2021). *Scaptotrigona* Moure 1942 was the first new genus described by Father Jesus Santiago Moure (1912-2010), the most prolific meliponine taxonomist to date. *Scaptotrigona* is the most abundant stingless bee in Ecuador producing the most pot-honey in the country. They are kept by farmers who are familiar with them in the field. Basic knowledge of stingless bee management is transmitted within families. A market study determined that there was a supply of about 500L of honey and a demand of 2,000L, for which the feasibility of breeding *Scaptotrigona* in the high zone of El Oro province, Ecuador was proposed (Aguirre Zambrano, 2015). The ethnic name of this bee is 'Catiana' in El Oro province, and 'Catana' in the Loja province.

The oldest records of meliponiculture are by the Mayan culture with the stingless bee *Melipona beecheii* called ‘Xunan Kab’ (Weaver and Weaver, 1981), still productive and managed to preserve the Mayan ancestral traditions for the cosmovision in the Yucatan Peninsula, Mexico (Sotelo Santos and Alvarez Asomoza, 2018). In Venezuela, the Huottuja (Piaroa) from the Amazonas

KEYWORDS / Blue Cheese Odor / Honey / Honey Authenticity Test by Interphase Emulsion HATIE / Honey Biosurfactant Test HBT / Meliponini / *Scaptotrigona vitorum* /

Receipt: 09/05/2022. Modified: 10/14/2022. Accepted: 10/17/2022.

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state keep stingless bees of diverse species (Vit *et al*., 2011) of the *Melipona*, *Scaptotrigona*, and *Tetragona* genera. Ecuador has an apifauna of about 200 stingless bees, which have not reached the Galapagos. Coloma (1986) collected and identified 78 species. Dr. Silvia RM Pedro from the Universidade de São Paulo in Ribeirão Preto, Brazil, identified 54 species of stingless bees collected during a year (Vit *et al*., 2018), and 100 species of stingless bees come from a tiny 64 km2 plot of Yasuní (Roubik, 2018). There are about 250 species of meliponines in Brazil which has much larger territory (Melo, 2020).

Sustainable bee-keeping and meliponiculture allow for the preservation of bee and plant biodiversity, along with medicinal and social benefits for producers, aimed at reducing poverty (FAO, 2021). Its integration into rural development programs in Ecuador to improve well-being (Álvarez Montalvo, 2021) has versatile strategic potential in key aspects of pollination, beehive products and apitherapy. In an organized community with bee-keeping and meliponiculture professionals, there is no room for fake honey. With so many floral and entomological resources, honey biodiversity in Latin America is a treasure protected by ancestral knowledge but forgotten by the industry and agricultural development plans, the optimal terrain for the marketing of fake honey. Honey from bees is a product that occupies a place of honor in cupboards, due to its medicinal use (Vit *et al*., 2015a). Together with pollen, propolis and royal jelly, they are eye-catching products at any agricultural fair where they can be promoted. In addition to its economic impact, Paredes and Saravia (2021) highlighted the function of social and cultural meetings in the farmer fair space to communicate specialized agricultural management.

Recently, the composition of honey from meliponines was reviewed in a context where the global honey market is dominated by *Apis mellifera* (Vit, 2017). The physical-chemical composition of honey from *Apis mellifera* marketed in Quito was analyzed (Velásquez and Goetschel, 2019), and honey from Meliponini stingless bees from Ecuador (Villacrés-Granda *et al*., 2021), Costa Rica (Umaña *et al*., 2021), Australia and Malaysia (Zawawi *et al*., 2022). Nuclear magnetic resonance is a rapid method to confirm the authenticity of honey (Spiteri, 2015; Yong *et al*., 2022) and its entomological origin (Vit *et al*., 2022). Since 2014, the Honey Authenticity Test by Interphase Emulsion (HATIE) produced a novel result in honey from *Scaptotrigona vitorum*, but it's possible explanation allowed expanding the application of the authenticity test to a metabolic field of honey biosurfactant activity with suspected microbial origin. Furthermore, the nests of *Scaptotrigona vitorum* smell like Roquefort cheese, which is reported for the first time here as a particularity observed in only one of the 54 species of stingless bees collected in Ecuador.

The HATIE was tested on 51 Asian, Australian, European and Latin American honeys, to show its universality. When it was published (Vit, 1998), it had already been tested by about a thousand honey consumers (bee-keepers, farmers, scientists, housewives, students, teachers, employees and professors, other professions). The Andean bee-keeper with hives located at highest altitudes in Venezuela, remembers the "little cloud" as he memorized the emulsion formed by genuine honey more than 30 years ago (Juan Carlos Schwartzenberg, La Casita de la Miel, Escagüey, Mérida, Venezuela, personal communication, 21st April 2022).

The honey authenticity test by interphase emulsion (HATIE) was done in order to value non-traditional techniques to detect fake honey as useful in a scientific and social context for honey consumers. Thus, decision-making on authenticity was based on interphasic visual perception of genuine honey from *Apis mellifera*, and from stingless bees such as *Geotrigona leucogastra*, *Melipona mimetica*, *Scaptotrigona vitorum*, *Tetragonisca angustula*, and a false honey.

Materials and Methods

Honey sampling

Pot-honeys were harvested by suction with a syringe adapted with a plastic tube, introduced in pierced cerumen of honey pots. The six honeys analyzed were a genuine commercial *Apis mellifera* honey, four honeys from stingless bees of the most frequent genera in Ecuador *Geotrigona*, *Melipona*, *Scaptotrigona* and *Tetragonisca*, and a commercial fake honey recognized for its smell of eucalyptus oil, received from Mr. Julio Vásquez, bee-keeper in Pichincha province. These four genera of Ecuadorian stingless bees were recommended for the first Pot-Honey Standard Project in Ecuador (INEN, 2015; P. Vit, personal observation), during the revision of the Ecuadorian Technical Standard for Honey NTE INEN 1572, in Spanish. The proposal of a honey standard for each of these genera of stingless bees was justified like the first standard for *Melipona* honey, from the state of Bahia in Brazil (ADAB, 2014). During the honey sampling, the nest of *Scaptotrigona* presented the characteristic odor of Roquefort cheese, which was observed in the numerous nests of this bee. A screening with Asian, Australian, European, and Latin American honeys, was performed on a collection of 51 frozen honeys from the Apitherapy and Bioactivity Group, Food Science Department at Universidad de Los Andes.

Identification of stingless bees

Stingless bees were collected with a plastic bag, and transferred to a plastic bottle with isopropyl alcohol, dried, protected with plastic jewelry boxes, then only enveloped with tissue, and identified by: 1. Prof. Dr. C. Vergara, Department of Chemical and Biological Sciences, Universidad de Las Américas, Puebla, México, as Geotrigona leucogastra (Cockerell 1914) 'Earth bee', deposited in the entomological collection UPSE, Peninsula State University of Santa Elena, Ecuador, and 2. Dr. SRM Pedro, Department of Biology, Universidade de São Paulo, Brazil as: 1) *Melipona mimetica* Cockerell 1914 'Bermejo', and 2) *Tetragonisca angustula* (Latreille 1811) 'Angelita', deposited in the Camargo RPSP Collection, Ribeirão Preto, São Paulo, Brazil. The new species *Scaptotrigona vitorum* Engel 2022 was differentiated from *Scaptotrigona ederi* (Engel, 2022a), which was not the Schwarz *nomina nuda*, known that the Schwarz and Engel species are not conspecific with the Ecuadorian 'Catiana'. The new Ecuadorian species (Engel, 2022b) is deposited in the Snow Entomological Collection, University of Kansas Natural History Museum, Lawrence, Kansas, USA (SEM). From the 54 stingless bee species identified, 26 were reported for the first time in Ecuador (Vit *et al*., 1918).

Honey Authenticity Test by Interphase Emulsion (HATIE)

The test to evaluate the authenticity of honey by forming an emulsion as an intermediate phase between the aqueous and the ethereal phases (Vit, 1998), has a patented prototype (Vit, 1995, 1999). A glass vial with a Bakelite lid was used, 1.0g of honey was weighed, which was diluted with 1.0g of distilled water, then 2.0mL of diethyl ether (analytical grade, Sigma Aldrich, St. Louis, USA) were added and it was strongly shaken, left undisturbed for 1 min to allow separation of phases. False honeys form two phases and genuine honeys form three phases, with an emulsion as an intermediate phase, this allows visual detection of false honeys. Except for the genuine honey of *Scaptotrigona vitorum*, with a single phase, which is the novel result of the present investigation

Results

In the interphase emulsion test, the false honey presented two phases, unlike the genuine honeys of *Apis mellifera* and of stingless bees, with three phases, except *Scaptotrigona vitorum* with one phase.

Two organoleptic discoveries are reported here for the honey and nest of the Ecuadorian 'Catiana': 1. The biosurfactant activity in the authenticity test applied to honey from *Scaptotrigona* sp. by visual observation, and 2. The smell of blue Roquefort-type cheese from the nest of *Scaptotrigona vitorum* by olfactory observation.

Figure 1 shows a diagram with the results obtained for the different types of honey with the honey authenticity test by interphase emulsion. The decision making was very simple, the honey with two phases is false, the honey with three phases is genuine as indicated by Vit (1998), and the new pattern of having a unique phase is also a genuine honey of *Scaptotrigona vitorum*. The origin of this new result with one phase, has been an unsolved puzzle for a long time. Images of the test for each type of honey analyzed are also illustrated in Figure 1, where the differences between the two types of genuine honey, with a unique emulsion phase dispersed in the reaction volume (*Scaptotrigona*) or with an interphase emulsion, intermediate between the aqueous and the ethereal phases (*Apis*, *Geotrigona*, *Melipona*, and *Tetragonisca*) are easily observed. Fake honey presented only two phases, one aqueous and one ethereal, without the interphasic emulsion.

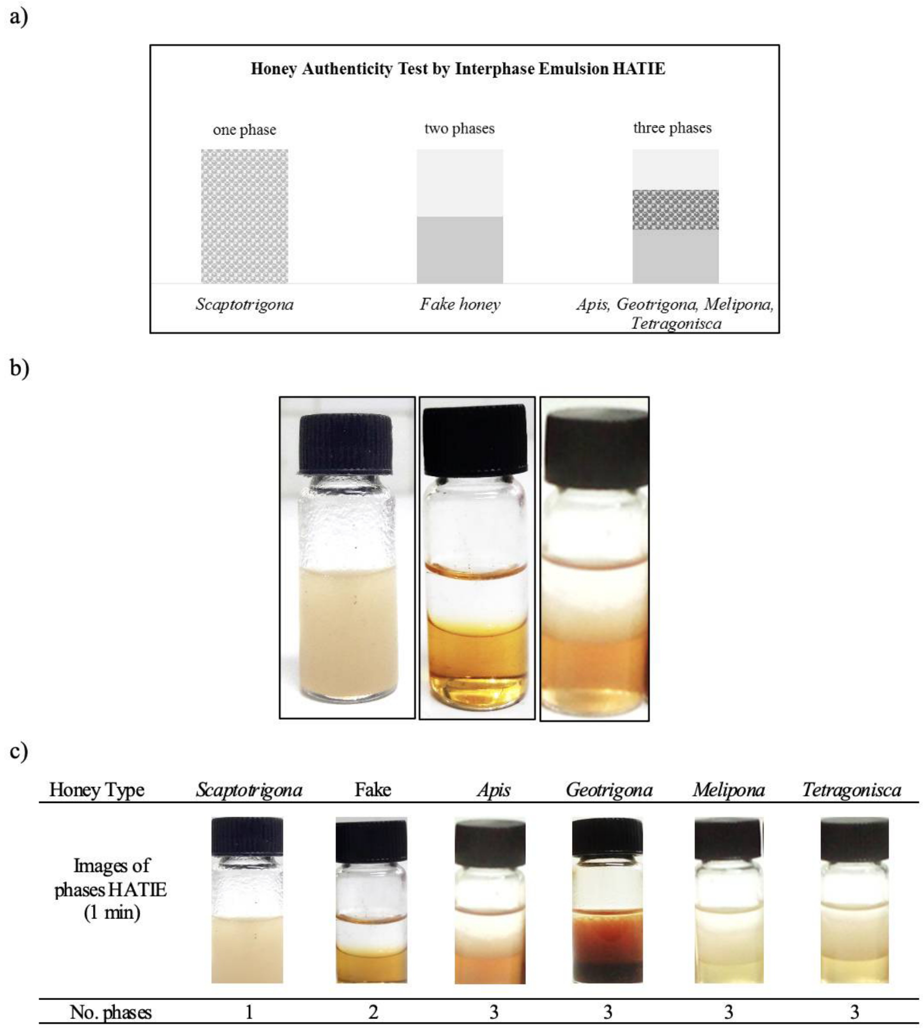

Figure 1. Number of phases formed with the HATIE. a) Diagram with number of phases formed in the Honey Authenticity Test by Interphase Emulsion. b) Genuine *Scaptotrigona vitorum* honey has a biosurfactant that causes a single phase (first vial), there are two phases in the fake honey (second vial), and three phases in the genuine *Apis mellifera* honey (third vial). c) Results in different honey types 1: *Scaptotrigona vitorum* honey (one phase), 2: Fake honey (two phases), 3: *Apis*, *Geotrigona*, *Melipona*, *Tetragonisca* honeys (three phases).

A further HATIE screening was carried out with various Asian, Australian, European and Neotropical honey types (*Apis cerana, Apis dorsata, Apis mellifera,* unifloral, *Melipona fasciculata, Melipona scutellaris, Scaptotrigona depilis, Scaptotrigona mexicana, Tetragonisca angustula* and fake) (Table 1). The genuine honey presents one or three phases and the false honey two phases. *Scaptotrigona depilis* from Argentina, Bolivia, and Brazil, a meliponine honey from Malaysia informed to be *Geotrigona thoracica* without entomological authority, and that of *Tetragonula carbonaria* from Australia also presented one phase.

Honeys of *Apis cerana* from Japan and *Apis dorsata* 'Tualang' –forest from Malaysia– *Apis mellifera* unifloral of acacia, *Acacia mangium* FABACEAE, 'belimbing' star fruit *Averrhoa carambola* OXALIDACEAE, durian *Durio ziberthinus* MALVACEAE, 'nanas' pineapple *Ananas comosus* BROMELIACEAE, 'gelam' *Melaleuca cajaputi* MYRTACEAE, 'getah' rubber tree *Hevea brasiliensis* EUPHORBIACEAE, 'kelapa' coconut *Cocus nucifera* ARECACEAE, rambutan *Naphelium lappaceum* SAPINDACEAE and 'Kelulut' –stingless bees– *Geniotrigona thoracica* of Malaysia were tasted during a Workshop on Sensory Evaluation of Asian Honeys 26 and 27 September before the *11th Asian Apicultural Association's Conference & ApiEXPO*, Kuala Terengganu, Malaysia, 28 September - 2 October 2012. *Frieseomelitta paupera* 'Guanotica' from Venezuela, *Melipona fasciculata* 'Tiúba' y *Melipona scutellaris* 'Uruçu' from Brazil three phases. One phase was observed in the honey of *Scaptotrigona depilis* 'Obobosí' from Bolivia, 'Tapezuá' from Argentina and 'Mandaguari' from Brazil. Three phases were formed in the honey of *Scaptotrigona mexicana* 'Pisil Nekmej' from Mexico, *Tetragonisca angustula* 'Jataí' from Brazil, Mariola from 'Costa Rica', 'Ramichi' from Peru and 'Angelita' from Venezuela, and *Tetragonula biroi* 'Kiwot' from Philippines. *Tetragonula carbonaria* 'Karbi' from Australia formed one phase. Unifloral *Apis mellifera* honeys of lavender *Lavandula officinalis* from France, *Astragalus* from Iran, chestnut of

Castanea sativa from Italy, manuka *Leptospermum scoparium*, pohutukawa *Metrosideros excelsa* and rewarena *Knightia excelsa* from New Zealand, acacia *Robinia pseudoacacia*, raspberry *Rubus idaeus*, sunflower *Helianthus annuus*, rape *Brassica napus*, and linden *Tilia cordata* from Czech Republic, developed three phases. Fake honeys from Brazil, Colombia, Malaysia, Singapore and Venezuela, formed two phases with the HATIE. All these botanical and entomological origins were informed, some were in the honey labels, but vouchers of botanical and entomological origins were not available.

Discussion

Honey Authenticity Test by Interphase Emulsion and suspected biosurfactants

Fake honeys were detected by this test because they do not form an intermediate emulsion as they do not have the characteristic components of genuine honey (Vit, 1998). This behavior was observed in 500 Venezuelan commercial honeys (Vit *et al*., 1994) where the idea of the interphase test arose, since genuine honeys generate three phases and false honeys only two. It is important to distinguish interface, which refers to the contact surface, from interphase, which refers to the volume of the emulsion (Geckeler *et al*., 1997). However, Table 1 shows that the honey from *Scaptotrigona vitorum* produces a unique phase. This difference is illustrated in the diagram of Figure 1a. It is a cloudy phase as can be seen in Figure 1b. With the light microscope, this phase (vial 1) consists of multiple bubbles of liquid-air interaction at 400X, which contract and collapse on the slide, like the interphase (vial 3). The interphase emulsion that separates the ethereal phase from the aqueous phase is not formed, but dispersed in the reaction volume as a result of the action of a biosurfactant, therefore a single phase is observed. It is a phenomenon of the nest of this species of *Scaptotrigona* – not observed in honeys from *Apis mellifera*, *Frieseomelitta*, *Geotrigona*, *Melipona*, *Nannotrigona*, or *Tetragonisca* from Argentina, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Mexico, Peru, Trinidad & Tobago and Venezuela. However, it was also observed in honey from *Scaptotrigona depilis* from Argentina, Bolivia, and Brazil, and that of *Tetragonula carbonaria* from Australia also presented one phase.

How to explain this differentiated interphase phenomenon? Biosurfactants produced by a yeast associated with this bee but not with the others could originate the phase observed only in *Scaptotrigona vitorum* honey. There are interesting associations of microbes with insects (Schmidt and Engel, 2021). Bees function as vectors to transport yeasts from their natural reservoirs in floral nectar to the different substrates of bee nests (Starmer and Lachance, 2011). The yeast *Candida bombicola* initially isolated from *Bombus* bumblebee honey synthesizes a sophorolipid with surfactant properties (Kim *et al*., 1997), in particular extracellular glycolipids with biosurfactant activity, known as sophorolipids $C_{34}H_{58}O_{15}$. Sophorolipids (SL) are composed of a sophoroside molecular fraction (glucose disaccharide with an unusual β-1,2 bond whose etymology derives from *Sophora japonica* pods) and a lipid tail that is the aglycone.

The nomenclature of the yeast *Candida bombicola* changed with the new genus, and since 1998 it has been known as *Starmerella bombicola* (Rosa and Lachance, 1998); however, *Candida bombicola* still appears in some publications (CA Rosa, personal communication, 7th February 2022). In other cases gender change is indicated as in *Starmerella (Candida) bombicola* JCM 9596 from the Japan Microorganism Collection at the Riken BioResource Center (Hirata *et al*., 2021). The study of the surfactants produced by *Starmerella* is of current interest due to its biotechnological applications (Van Renterghem *et al*., 2018). SLs are the surfactants of the future due to their versatility in formulations, and because they are ecologically friendly. In fact, 75% of the research on *Starmerella bombicola* focuses on its SLs. But why does *S. bombicola* invest so much energy to produce this secondary metabolite? Although SLs do not represent osmotic protection in common habitats such as nectar and honey, they protect the niche of *S. bombicola* with a double advantage to compete against other microbes: a) Extracellular storage of usable energy in starvation emergency, and b) Antimicrobial activity (De Clercq *et al*., 2021). Proteomic and genetic research on *S. bombicola* is recent, such as the study of its exoproteome (Ciesielska *et al*., 2014) and its transportome (Claus *et al*., 2022), demonstrating the potential of omics sciences for the functional design of molecular biosynthesis in industrial biotechnology. One could think of some species of *Starmerella* or the same *S. bombicola* associated with *Scaptotrigona* sp. from Ecuador, which should be isolated from their honey to demonstrate this potential microbial origin of a biosurfactant that causes the behavior observed in the honey authenticity test based on an interphase emulsion.

Starmerella bombicola was identified in the honey microbiome of *Scaptotrigona bipunctata* and *Scaptotrigona depilis* from Brazil, but was absent in *Scaptotrigona tubiba* and other 14 species of studied stingless bees (Echeverrigaray *et al*., 2021). What are the traits of these two species of *Scaptotrigona* for the yeast *Starmerella bombicola* to be associated with them? They could be related to the bee diet, secretions, or even microbiome. The result of the interphase emulsion test performed with *Scaptotrigona depilis* honey from Argentina, Bolivia and Brazil, was one phase (Table 1), which could confirm the production of extracellular sophorolipids with biosurfactant activity by a yeast type *Starmerella bombicola*, located in the same subclade. The honey produced by the Australian *Tetragonula carbonaria* and the Malaysian 'Kelulut' putatively *Geotrigona thoracica*, also had a one phase result. Biosurfactant sophorolipids are produced by multiple species of the yeast clade, subclade such as *Candida apicola*, *Candida riodocensis*, *Candida stellata* and a new species Candida sp. NRRL Y-27208 (Kurtzman *et al*., 2010).

The presence of *sophorosides* has also been associated with the botanical origin of bee and floral substrates, they are not SL but flavonoid sophorosides. For example:

a) Rosemary nectar extracted from the stomach of *Apis mellifera* contains glycosides, 93% kaempferol 3-sophoroside and 7% quercetin 3-sophoroside, but only their aglycones were found in honey (Gil *et al*., 1995).

b) Carnations contain kaempferol 3-O-sophoroside (Iwashina *et al*., 2010).

c) Bee pollen contains quercetin 3-O-sophoroside (Čeksteryte *et al*., 2016).

Natural fermentation of pot-honey and studies of traditional fermented food

Pot-honey produced by stingless bees is a fermented product. Traditionally, fermented honey from *Apis mellifera* was considered a defect caused by harvests of unripe honey with high moisture content. Fake honeys meet the <20% moisture requirement. However, the honey produced by Meliponini can ferment from the nests of stingless bees, a fermentation that can continue after harvest (Drummond, 2013), and therefore merited the inclusion of the new sensory family Fermentation, with the acetic, alcoholic and

TABLE I
HATIE SCREENING OF GLOBAL HONEY TYPES

Bee Ethnic name	Interphase emulsion (number of phases)	Botanical origin	Country
Apis cerana	3	-	Japan
Apis dorsata 'Tualang'	3	Tropical forest	Malaysia
Apis mellifera	3	Lavander *Lavandula officinalis* LAMIACEAE	France
	3	Persian manna *Astragalus* FABACEAE	Iran
	3	Chestnut *Castanea sativa* FAGACEAE	Italy
	3	Orange *Citrus* RUTACEAE	Japan
	3	Acacia *Acacia mangium* FABACEAE	Malaysia
	3	'belimbing' tamarindo chino *Averrhoa carambola* OXALIDACEAE	Malaysia
	3	Durian *Durio ziberthinus* MALVACEAE	Malaysia
	3	'gelam' *Melaleuca cajuputi* MYRTACEAE	Malaysia
	3	'getah' Caucho *Hevea brasiliensis* EUPHORBIACEAE	Malaysia
	3	'kelapa' Coco *Cocus nucifera* ARECACEAE	Malaysia
	3	'nanas' Piña *Ananas comosus* BROMELIACEAE	Malaysia
	3	Rambutan *Naphelium lappaceum* SAPINDACEAE	Malaysia
	3	Manuka *Leptospermum scoparium* MYRTACEAE	New Zealand
	3	Pohutukawa *Metrosideros excelsa* MYRTACEAE	New Zealand
	3	Rewarena *Knightia excelsa* PROTEACEAE	New Zealand
	3	Multifloral	Venezuela
	3	Acacia *Robinia pseudoacacia* FABACEAE	Czech Republic
	3	Strawberry *Rubus idaeus* ROSACEAE	Czech Republic
	3	Sunflower *Helianthus annuus* ASTERACEAE	Czech Republic
	3	Rape *Brassica napus* BRASSICACEAE	Czech Republic
	3	Linden *Tilia cordata* MALVACEAE	Czech Republic
Frieseomelitta paupera 'Guanotica'	3	'merey' cashew *Spondias mombin* ANACARDIACEAE	Venezuela
Geniotrigona thoracica 'Kelulut'	1	-	Malaysia
3 *Melipona fasciculata* 'Tiúba'	3	-	Brazil
8 *Melipona scutellaris* 'Uruçu'	3	-	Brazil
Scaptotrigona depilis 'Tapezuá'	1	-	Argentina
Scaptotrigona depilis 'Obobosí'	1	-	Bolivia
Scaptotrigona depilis 'Mandaguari'	1	-	Brazil
Scaptotrigona mexicana 'Pisil Nekmej'	3	-	Mexico
Tetragonisca angustula 'Jataí'	3	-	Brazil
Tetragonisca angustula 'Mariola'	3	-	Costa Rica
Tetragonisca angustula 'Ramichi'	3		Peru
Tetragonisca angustula 'Angelita'	3	-	Venezuela
Tetragonula biroi 'Kiwot'	3	-	Philippines
Tetragonula carbonaria 'Karbi'	1	-	Australia
Fake	2	-	Brazil
	2	-	Colombia
	2	-	Malaysia
	2	-	Singapore
	2	-	Venezuela

lactic subfamilies, and their sensory descriptors: vinegar, pot pollen, brandy, fermented fruit, brewer's yeast, liquor, must, sake, vinasse, white wine, red wine, miso, cheese, yogurt (Vit, 2008c). Fermentation is not a defect of meliponine honey but its method of food preservation. That is why there are so many yeasts in their nests (Rosa *et al*., 2003; Silva *et al*., 2019; Echeverrigaray *et al*., 2021; Gavazzoni *et al*., 2022) as in industrial fermentations. Ethanol production in *Tetragonisca angustula* honey from Venezuela was stabilized in 30 days post-harvest (Pérez-Pérez *et al*., 2007) and lactic acid bacteria were inactivated in *Heterotrigona itama* honey from Malaysia (Yaacob *et al*., 2021). In another study, the proportion of gluconic acid was optimized to improve the taste quality of kombucha, using a Synthetic Microbial Community (SMC). *Starmerella davenportii* with the highest ethanol yield and *Gluconacetobacter intermedius* with the highest acetic acid yield were selected to rebuild the microbial community that allowed obtaining a better sensory quality kombucha with 74% gluconic acid (Li *et al*., 2022).

Microbial metabolites as non-morphological traits of stingless bees

Some metabolites of microbial origin in honey corresponded to sensory descriptors for the proposed fermented subfamilies: Acetic, Alcoholic, and Lactic (Vit, 2008). These metabolites produced by the microbiota associated with stingless bees, have precise functions in the bee colony. Both the nutritional improvement of the substrate, the defense against entomopathogens, and others such as: Acetic acid and lactic acid bacteria (Leonhardt and Kaltenpoth, 2014), Yeasts (Rosa *et al*., 2003; Echeverrigaray *et al*., 2021), and Edible filamentous fungi *Monascus* [sic] *Zygosaccharomyces* grown by *Scaptotrigona depilis* in brood cells (Menezes *et al*., 2015), orchestrated with two other nest microbes –*Candida* sp. and *Monascus ruber*– that modulate metamorphosis from larva to pupa (Paludo *et al*., 2019). Sensory evaluation brings us closer to the evolution of the associations between microbes and bees, to their ecological host-host role as investigated by Schmidt and Engel (2021), in mutualisms possibly originating from the Late Cretaceous, the era of the first fossil of a bee in our planet: *Cretotrigona prisca*, a stingless bee (Michener and Grimaldi, 1988a,b; Camargo, 2013). Some aromas of honey are so harmoniously amalgamated that if we knew how to express these sensory attributes with musical notes, it could be more precise.

In his taxonomic notes on *Scaptotrigona*, Engel (2022b) is surprised that in Rasmussen and Cameron's (2010) phylogenetic study, *Scaptotrigona* was not placed close to the shy *Nannotrigona*, but in a clade with *Oxytrigona*, with a higher degree of aggressiveness It was learned during the collection of the first Ecuadorian pot-honey for the Prometeo Project, requiring water for prompt removal of those bees from the collector hair. For this reason the scaptotrigonicultors work with a veil. Engel specifies that *Nannotrigona* and *Scaptotrigona* are morphologically similar, but have other non-morphological features that separate them. Just as *Scaptotrigona* is morphologically more similar to *Trigonini* than to *Melipona*, but in the chemical composition of its honeys, it turned out to be more similar to *Melipona* (Vit *et al*., 1998). Remarkable phylogenetic demonstrations could emerge by broadening the spectrum of biological traits not limited to morphology. Metabolites of microbial origin are novel for interpreting coevolution with stingless bees and the underlying causes of evolutionary preferences among bee-microbe associations. The study of the bacterial, fungal and yeast microbiomes associated with different species of stingless bees and their substrates begins to reveal particular differences between specialized and generalist dynamic evolution.

The Roquefort blue cheese smell in the nest of Scaptotrigona vitorum

Lipolysis gives rise to carboxylic acids and other malodorous molecules that impart sensory complexity to fungus-inoculated blue cheeses, such as British Stilton, Italian Gorgonzola, or French Roquefort. For example, fatty acids form methyl ketones (alkan-2-one, heptan-2-one, nonan-2-one) with blue cheese notes, and they also react with alcohols such as ethanol to produce flavoring esters. Other decarboxylation, deamination, oxidation, and reduction transformations produce short-chain volatile organic compounds. Lactate and citrate produce molecules such as butter-flavored diacetyl, ethanal, and ethanol (Cotton, 2010). Explaining the origin of the sensory complexity of blue cheese in a dairy-free stingless bee nest is astounding, and a challenge soon to be revealed with the advancement of omics sciences in stark contrast to the growing cross-border bureaucracy, which far from cooperating, makes it difficult to transfer honey from the tropics to specialized laboratories. It can be said that the biotechnology of inoculum-type fungi in blue cheese produces this biotransformation in some nest substrate of *Scaptotrigona vitorum*. They were perceived by the sense of smell, although its functions for the bee colony are not known. It could also be an unknown secretion from the bee or a derivative from foraged materials, particularly biotransformed.

In a European patent, three aliphatic organic acids that cause unpleasant odors (acetic acid, butyric acid, and isovaleric acid) are indicated as by-products of the microbial biosynthesis of sophorolipids (European Patent EP 2 821 495 B, 2016).

On fake honey, omics sciences and honey consumer protection

Walker *et al*. (2022) reviewed multiple analytical techniques needed to detect and report sophisticated honey adulteration. A recent strategy to detect fake honey is to apply metabolomics in order to identify foreign compounds in bee honey. Honey diluted in deuterated water allowed detection of benzoic acid, citric acid, sorbic acid and vanillin in high concentrations by ^{1}H-NMR, as well as HMF and sucrose (Schievano *et al*., 2015). In Malaysia, 80% of the honeys are counterfeit adulterated, and their packaging is very luxurious (P. Vit, personal observation). ^{1}H-NMR spectra of genuine stingless bee honeys were fingerprinted for the detection of controlled adulterations of 1% C3 and C4 sugars, along with routine OPLS-DA chemometric models in metabolomics (Yong *et al*., 2022).

There are few molecular studies on the honey maturation mechanism by honey bees. Therefore, the metabolomics of the formation of mature honey is a resource to recognize false honeys (Guo, 2021). A state-of-the-art study compared immature honey with mature honey obtained during a turnip *Brassica napus* bloom by UPLC-QToF-MS-based metabolomics coupled with multivariate analysis of PCA, OPLS-DA, and VIP. Its authors found that mature operculated honey doubles the content of decenedioic acid originating from the bee (Sun *et al*., 2021).

Depending on the objective of the study of fake honey in the market, metabolomic approaches can be used to identify and quantify foreign substances to this product. Either by NMR spectroscopy, by chromatographic, or electrophoretic techniques coupled to multivariate statistics. These techniques are very effective for searching markers of botanical (Schievano *et al*., 2016; Seraglio *et al*., 2021), geographic (Spiteri *et al*., 2015) and entomological (Vit *et al*., 2015b) origins.

It is recommended to identify the valuable bee products with

Protected Geographical Indications (PGI) to value the agricultural product linked to its place of origin, and also raise its quality and the reputation of honey professionals. These actions protect the consumer of honey by offering a genuine product. The Protected Designation of Origin (PDO) could be requested for honey from four types of stingless bees (*Geotrigona*, *Melipona*, *Scaptotrigona*, *Tetragonisca*). The Protected Geographical Indication (PGI) could be proposed for particular biome, at least in its geographic stripes, coast, mountains, and rainforest, in the case of Ecuador, and corresponding biome in other countries. The Guaranteed Traditional Specialty (GTS) protects the production method, which is artisanal in the case of meliponine honey "The PDO/ PGI protects a name that identifies a product originating from a certain place, and the GTS protects the production methods and traditional recipes. In a product with PDO/PGI, the specificity is due to the origin of the product, while in one with GTS it is due to its traditional nature" (Mapa, mapa.gob.es).

A story about stingless bees, associated yeasts, and etymologies

A homage was accepted from CA Rosa from Universidade Federal de Minas Gerais in Brazil, to name a new species of yeast. An attentive and formal Email was received, with a manuscript that became comprehensive several years later. *Starmerella vitae* sp. nov. (Santos *et al*., 2018), yeast isolated from the stingless bee *Trigona fulviventris* Guérin, 1844 known as 'Jicote', from Santa Rosa National Park, Guanacaste, Costa Rica, and from Brazilian flowers of the vine *Thunbergia grandiflora* ACANTHACEAE, from Ilha Grande State Park (Atlantic Tropical Forest), Rio de Janeiro state, and *Tabebuia* spp. BIGNONIACEAE Belo Horizonte, Minas Gerais state. CA Rosa, witnessed this food honey is transforming into ecology of microbiota and its metabolites in nests of meliponines. Rosa also dedicated a new species to the memory of João MF Camargo of the Universidade de São Paulo, who identified the meliponines from Venezuelan honeys, *Starmerella camargoi* sp. nov. In the phylogram based on the D1/D2 domains of the ribosomal large subunit LSU rRNA gene, *Starmerella vitae* is in the subclade of *Starmerella bombicola* together with the bio-sophorolipid-producing species.

Conclusions and Recommendations

The interphase emulsion method formed by stirring diethyl ether with aqueous honey dilution was fast and effective in order to detect false honey, and protect the consumer. The classic analysis such as those of the NTE INEN (2016) are between this alternative approach and that of the major analytical equipments (CE, HPLC, NMR). However, they are limited, and do not allow the identification of any foreign additive in the honey. Like other national standards, they do not detect all false honeys, and group genuine fermented or heated honeys as out of standard.

For the first time, the results of a unique phase in the Honey Authenticity Test of *Scaptotrigona vitorum* honey, was suspected to be associated with the presence of a natural biosurfactant, possibly of microbial origin. The production of sophorolipids by the yeast *Starmerella bombicola* –frequent in the scientific literature– and its associations with Neotropical stingless bees, also demonstrated, the basis of this interpretation. The microbiome of *Scaptotrigona vitorum* from Ecuador has not been studied in the bee or in the different substrates of its nest, where the suspected microbial origin of the biosurfactant in this investigation could be evidenced. It will be necessary to study the nature and chemical functionality of this metabolite in another investigation, to postulate its functions for *Scaptotrigona vitorum* and for the yeast that produces it. Likewise, the distinctive Roquefort cheese odor in its nest may have a microbial origin. The new interdisciplinary rurality of Pérez (2004) is ready to promote social development from territorial revaluation needed for all stingless bees kept in Ecuador. The 'Catiana' stingless bee is embedded in the Ecuadorian geography, and, in the knowledge of those who care for their nests and harvest their honey. They are stingless bee-keepers that would deserve a complex name –scaptotrigonicultors– imitating that of meliponicultors.

In addition, another application for this test was proposed, useful for those interested in honey metabolites. When a unique phase is obtained with this procedure, this test detects the presence of biosurfactant activity, and becomes the Honey Biosurfactant Test (HBT). This use allows screening to detect stingless bees associated with biosurfactant-producing microbiota in honey. In particular, if demonstrated, it could be a Test for Sophorolipids in Honey (SST) because the yeast *Starmerella bombicola* does not produce any biosurfactant but sophorolipids. Its industrial applications in food, bioremediation, cosmetics, pharmacy, and eco-friendly home products are known. The biotechnological development of biosurfactants of microbial origin has constant parametric optimizations of cell cultures (solid phase, use of dual lipophilic substrates, standardization).

Starmerella bombicola has been widely studied in biotechnology for its massive production of extracellular sophorolipids. It was initially isolated from honey of the *Bombus* bumble bee, later from the stingless bee 'Jicote' *Trigona fulviventris* from Costa Rica. Recently, from honey produced by two species of stingless bees (*S. bipunctata* and *S. depilis*) of the genus *Scaptotrigona* associated with this yeast in Brazil (Echeverrigaray *et al*., 2021). Noteworthy, a recent discovery isolated this yeast from propolis of the Australian *Tetragonula carbonaria* (Flavia Massaro, personal communication, 14th October 2022).

The type of emulsions obtained with the test deserves further research, and also the component causing the biosurfactant activity. The physical state of the *Scaptotrigona* honey is liquid. Given the biosurfactant activity observed under the test conditions, could be there any nanoemulsion? Just to think on possible applications of this honey itself. Nanoemulsions are nano-sized emulsions with medicinal industrial uses, manufactured for improving the delivery of active pharmaceutical ingredients (Jaiswal *et al*., 2015).

Knowing the types of genuine honey, characterizing them, and promoting their consumption with cutting-edge bee keeping and meliponiculture, targeting the informed consumer, is a shared commitment. The Ecuadorian National Institute for Standardization (INEN), the Phytosanitary and Zoosanitary Regulation and Control Agency (AGROCALIDAD) and the Ministry of Agriculture and Livestock (MAGAP) could join efforts to revitalize the future development of meliponiculture in Ecuador in all its provinces except Galapagos where the unique bee is the solitary *Xylocopa darwini* Cockerell, 1926.

Paradoxically, in this communication without microbiological analysis, the fungal presence was perceived from the nest of *Scaptotrigona* sp. by sensory observations different from the classic sensory evaluation of honey, and corresponding scientific interpretations: 1) Biosurfactant activity in honey perceived visually by a unique emulsion phase formed in the honey authenticity test by interphase emulsion for genuine honeys, and 2) Typical odor of blue cheese such as Roquefort obtained after inoculated fungi develop and perceived olfactorily when opening the nest.

Ethics statement

The author declares that she has made the contributions that justify her authorship. There is no conflict of interest of any kind and the relevant ethical and legal requirements and procedures have been complied with no funding received.

ACKNOWLEDGEMENTS

To my father, Mr. Giovanni Vit, who sponsored this publication because he valued innovation. To the stingless bee keepers and bee keepers of the world, producers and defenders of genuine honey. To Carlos Augusto Rosa, Department of Microbiology, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, for his long-standing research on microbial associations with stingless bees. To my family for their constant and unconditional support, my parents Giovanna and Giovanni, my brothers Daniele, Massimo and Leo, all participated in the development of the honey authenticity test. The manufacture of the prototype was carried out in VITCA, Vanguardia de Industria Tecnológica C.A, Judibana, Falcón state, Venezuela. To Michael S Engel from Kansas University for the discovery of a new species in the specimens sent to Charles D Michener, and deposited at the Snow Entomological Museum Collection (SEMC –besides nearly 5 million pinned insect specimens–) where other collections saw dust or could not decipher. To Silvia RM Pedro, JMF Camargo, and Carlos Vergara for their friendship, meliponine taxonomic talent, and timely availability. To the Scientific, Humanistic, Technological and Art Development Council of the Universidad de Los Andes, Mérida, Venezuela, for the trust and encouragement received since 1986 to investigate the honey produced by bees and their imitations. To the Universidad Técnica de Machala, El Oro, Ecuador, and the Prometeo Program. To the Universitá Politecnica delle Marche, Ancona, Italy.

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PRUEBA DE AUTENTICIDAD DE LA MIEL POR EMULSIÓN INTERFÁSICA REVELA ACTIVIDAD BIOSURFACTANTE Y BIOTECNOLOGÍA EN NIDOS DE LA ABEJA SIN AGUIJÓN *Scaptotrigona vitorum* 'CATIANA' DE ECUADOR

Patricia Vit

RESUMEN

Las abejas son valiosas polinizadoras de cultivos frutales y otras plantas con flores. La miel es un alimento medicinal de origen vegetal y animal, con impacto social en el bienestar de los apicultores. La apifauna neotropical tiene alrededor de 500 especies de abejas sin aguijón (Meliponini). La apicultura comercial con Apis mellifera *subestima el legado cultural de la meliponicultura, y ambas son afectadas por la presencia de mieles falsas en el mercado. Se investigó la técnica integrativa Test de Autenticidad de la Miel por Emulsión Interfásica (TAMEI) para detectar mieles falsas. Esta técnica basada en una prueba de emulsión en interfase puede ser realizada por apicultores, agricultores, amas de casa, profesionales y consumidores de miel en general. Cinco mieles genuinas producidas por* Apis mellifera, Geotrigona leucogastra, Melipona mimetica, Scaptotrigona vitorum, Tetragonisca angustula *y una miel falsa de Ecuador fueron analizadas. El TAMEI fue rápido y eficaz para detectar mieles falsas (dos fases), y mieles genuinas (una y tres fases). Se analizó una selección adicional de 51 mieles asiáticas, australianas, europeas y latinoamericanas. Además, el TAMEI generó una nueva aplicación como el Test de Miel Biosurfactante (TMB) de* Scaptotrigona vitorum *'Catiana' (una fase) con un posible origen microbiano, y su origen entomológico en este conjunto de mieles. Los nidos de 'Catiana' huelen a queso Roquefort, lo que indica una posible asociación de hongos con esta especie de abeja sin aguijón rural. Esta abeja se destaca por su distribución, productividad y las peculiaridades descritas en esta investigación, 80 años después de la descripción del nuevo género* Scaptotrigona *Moure, 1942. Paradójicamente, esta comunicación sin análisis microbiológico, infiere la presencia de hongos en el nido de* Scaptotrigona vitorum *mediante observaciones sensoriales diferentes a la evaluación sensorial clásica de miel.*

UM TESTE DE AUTENTICIDADE DO MEL POR EMULSÃO INTERFÁSICA REVELA ATIVIDADE BIOSSURFACTANTE E BIOTECNOLOGIA NO NINHOS DE ABELHAS SEM FERRÃO DE *Scaptotrigona vitorum* 'CATIANA' DO EQUADOR

Patricia Vit

RESUMO

As abelhas são polinizadores valiosos de frutíferas e outras plantas com flores. O mel é um alimento medicinal de origem vegetal e animal, com impacto social no bem-estar dos apicultores. A apifauna neotropical possui cerca de 500 espécies de abelhas sem ferrão (Meliponini). A apicultura comercial com Apis mellifera subestima o legado cultural da meliponicultura, e ambas são afetadas pela presença de méis falsos no mercado. A técnica integrativa Teste de Autenticidade de Mel por Emulsão Interfásica (TAMEI) foi investigada para detectar mel falso. Esta técnica baseada em um teste de emulsão de interface pode ser realizada por apicultores, agricultores, donas de casa, profissionais e consumidores de mel em geral. Foram analisados cinco méis genuínos produzidos por Apis mellifera, Geotrigona leucogastra, Melipona mimetica, Scaptotrigona vitorum, Tetragonisca angustula e um mel falso do Equador. O TAMEI foi rápido e eficaz na detecção de méis falsos (duas fases), e méis genuínos (uma e três fases). Uma seleção adicional de 51 méis asiáticos, australianos, europeus e latino-americanos foi analisada. Além disso, o TAMEI gerou uma nova aplicação como o Teste de Biossurfactante de Mel (TMB) de Scaptotrigona vitorum 'Catiana' (uma fase) com possível origem microbiana, e sua origem entomológica neste conjunto de méis. Os ninhos de 'Catiana' cheiram a queijo Roquefort, indicando uma possível associação de fungos com esta espécie de abelha sem ferrão rural. Esta abelha se destaca por sua distribuição, produtividade e pelas peculiaridades descritas nesta pesquisa, 80 anos após a descrição do novo gênero Scaptotrigona Moure, 1942. Paradoxalmente, esta comunicação sem análise microbiológica, infere a presença de fungos no ninho de Scaptotrigona vitorum através de observações sensoriais diferentes da avaliação sensorial clássica do mel.