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Original

Microbiological quality and antibacterial activity of honey produced by *Melipona beecheii* in Yucatan, Mexico

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ABSTRACT

Objective. To analyze the microbiological quality and antibacterial activity in 43 samples of honey produced by *Melipona beecheii*, extracted during the years 2020 and 2021 in the harvest (January to May) and post-harvest (June to October) seasons from meliponaries located in the low deciduous forest of southeast of Mexico. **Materials and methods**. Microbiological quality was determined by evaluating the content of total aerobic mesophiles, coliforms, molds, yeasts, and spore-forming anaerobes. For antibacterial activity, the agar well diffusion assay was used using 5, 10, 20, 40 and 80% honey concentrations. **Results.** The presence of aerobic mesophiles (83.7% of the samples), coliforms (4.6%), molds (20.9%) and yeasts (39.5%) was observed, with a maximum of $4.5x10^2$, 2.5x10, 9.5x10 and $3.2x10^3$ CFU/g, respectively. The presence of sporulated forms of sulfite-reducing clostridia was not observed in any sample. With respect to the antibacterial activity, the highest zones of inhibition were recorded against *Staphylococcus aureus* at a honey concentration of 80 and 40%, contrary to what was observed with *Salmonella* var. *Typhimurium*, *Pseudomonas aureginosa* and *Escherichia coli,* where the interference in bacterial growth was not so evident. **Conclusions.** However, the growth of mesophiles and yeasts in most of the samples showed antibacterial activity against the pathogens mentioned above, which can be attributed to the interactions between microbiome, plants, bees and physicochemical characteristics of honey.

Keywords: Melipona; mesophiles; coliforms; yeasts; *S. aureus* (*Source: USDA*).

RESUMEN

Objetivo. Analizar la calidad microbiológica y actividad antibacteriana en 43 muestras de miel producida por *Melipona beecheii*, extraída durante las épocas de cosecha (enero a mayo del 2020 y 2021) y poscosecha (junio a octubre 2020) de meliponarios ubicados en selva baja caducifolia del Sureste de México. **Materiales y métodos**. La calidad microbiológica se determinó evaluando el

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contenido de mesófilos aerobios totales, coliformes, hongos, levaduras y anaerobios formadores de esporas. Para la actividad antibacteriana, se utilizó el ensayo de difusión en agar con pocillos utilizando concentraciones de miel al 5, 10, 20, 40 y 80%. **Resultados.** Se observó la presencia de mesófilos aerobios (83.7% de las muestras), coliformes (4.6%), mohos (20.9%) y levaduras (39.5%), con un máximo de $4.5x10^2$, 2.5x10, 9.5x10 y $3.2x10^3$ UFC/g, respectivamente. En ninguna muestra se observó la presencia de formas esporuladas de clostridios sulfito reductores. Con respecto a la actividad antibacteriana las mayores zonas de inhibición se registraron contra *Staphylococcus aureus* a una concentración de la miel al 80 y 40%, contrario a lo observado *con Salmonella* var. *Typhimurium, Pseudomonas aureginosa* y *Escherichia coli*, en donde la interferencia en el crecimiento bacteriano no fue tan evidente. **Conclusiones**. No obstante, el crecimiento de mesófilos y levaduras en la mayoría de las muestras, éstas presentaron actividad antibacteriana contra los patógenos referidos, lo cual puede ser atribuido a las interacciones entre microbioma, plantas, abejas y características fisicoquímicas de la miel.

Palabras clave: Melipona; mesófilos; coliformes; levaduras; *S. aureus* (*Fuente: USDA*).

INTRODUCTION

Meliponiculture in Mexico has had a resurgence in the last 15 years due to the growing demand for organic products with functional properties (1). However, the introduction of honey produced by stingless bees into the market is limited by the lack of knowledge of the characteristics that define its quality, as well as the absence of regulations and technical procedures that define its quality standards (2).

Microorganisms can influence the quality or safety of the honey. In Mexico, the sanitary quality is specified in NMX-F-036-NORMEX-2006, however, this refers to *Apis mellifera.* Although it is known to be a microbiologically safe food, little is known about pathogenic microorganisms in stingless bee honey. Some studies have shown the presence of sanitary indicators and some pathogenic microorganisms in this types of honey (3,4).

In the Yucatan peninsula, the production of *Melipona beecheii* honey dates back to pre-Hispanic times, being used in religious ceremonies and as food. In traditional Maya medicine (5) it was widely used for the treatment of digestive disorders, eye diseases, respiratory infections, wound healing, and skin ulcers, as well as in post-partum recovery (6). Currently, modern medicine has considered the therapeutic use of stingless bee honey after different studies have reported its antimicrobial activity (7) against both, Gram-positive and Gram-negative bacteria (8), including antibiotic-resistant strains (9).

The main factors contributing to the antibacterial activity of honey are related to low water activity, high acidity, enzymes, hydrogen peroxide, non-peroxide compounds such as methylglyoxal (MGO $C_{15}H_{12}O_7$), antimicrobial peptides (defensin-1), and phenolic compounds (10); as well as the honey microbiome (11). The antimicrobial potential of honey depends, in addition to the hive health and conditions during harvest and storage, on its botanical origin (12).

In the state of Yucatan, the low deciduous forest is characterized by a diversity of native plant species, approximately 2,400 registered to date; 600 of them are melliferous and 30 are considered of great apicultural importance (13). Is in this ecoregion where the api-botanical cycle, which includes the pre-harvest season from October to December, harvest from January to May and post-harvest from June to September, is strongly related to the environmental conditions that affect flowering and honey production (14). The objective of this study was to evaluate the microbiological quality and in vitro antibacterial activity of honey produced by *M. beecheii* extracted during the harvest and post-harvest seasons from meliponaries located in the low deciduous forest of the state of Yucatan, Mexico.

MATERIALS AND METHODS

Sample collection. From February 2020 to May 2021, 43 samples of *M. beecheii* honey were collected from meliponaries located in 18 municipalities located in low deciduous forests as shown in Figure 1.

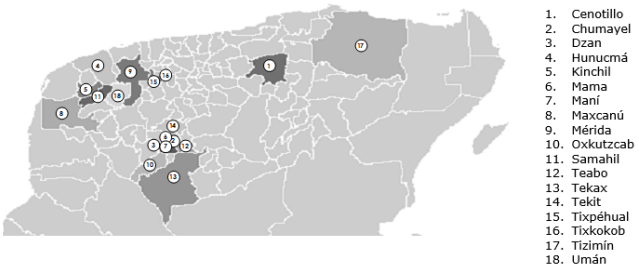

Figure 1. Municipalities in the state of Yucatan where *M. beecheii* honey was collected.

Nineteen samples were obtained in the post-harvest season (June to October 2020) and twenty-four in the harvest season (February to May 2020 and January to May 2021) according to the api-botanical calendar of the Yucatan Peninsula. The extraction was done by each meliponiculturist. Samples were collected, transported in 500 mL polyethylene containers, insulated from light in an isothermal container, and kept refrigerated at 4 °C until analysis in the UAEM, School of Medicine laboratories.

Microbiological analysis. Total aerobic mesophilic microorganisms, coliforms, molds, and yeast were counted using the technique described by Tanuğur-Samanci & Meral Kekecoğlu (15), for which 10 g of sample were diluted in 90 mL of buffered peptonized water (MCD Lab). For optimal growth and recovery of microorganisms, 1 N NaOH was used to adjust the pH of the solution to 7.0±0.2. 1 mL of the sample solution was placed on aerobic counting pads (MC-Media Pad Rapid Aerobic Count®, MC-Media Pad Coliform® and MC-Media Pad Yeast & Molds®, Merck Millipore), to be subsequently incubated at: 35±1°C for 48 hours for aerobic mesophilic microorganisms count, 35±1°C for 24 hours for coliforms, and 25±1°C for 72 hours for molds and yeasts. Finally, all colonies were tallied, regardless of the size and intensity of the color designated for each test.

For the count of spore-forming sulfite-reducing anaerobes, 10 g were diluted in 90 mL of buffered peptonized water (MCD Lab). In the inactivation of plant cells, a heat treatment of 80°C was applied for 5 minutes in a water bath, followed by rapid cooling. 1 mL of the solution was transferred to a Petri dish to which 15 mL of sulfite-iron agar (Condalab) was poured. The inoculum was homogenized with the medium by circular (right and left), horizontal, and vertical movements. After the medium solidified, approximately 10 mL was poured into the plate in order to ensure anaerobiosis. The boxes were sealed with Parafilm and incubated in an anaerobic jar at 37±1°C for 48, and then black colonies, surrounded by a black halo, were counted and identified as sulfite-reducing bacteria (16).

Antibacterial activity. A modification of the well agar diffusion assay proposed by Ng et al (9) was used to evaluate the antibacterial activity. The strains used in this study were: *Staphylococcus aureus* (ATCC 6538), *Salmonella enterica* subsp. *enterica* var. *Typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 14028), and *Escherichia coli* (ATCC 8739), which were inoculated in boxes with Müeller-Hinton (MH) agar (BD Bioxon) and incubated at 37°C, for 24 h. The strains were suspended in 5 mL of Müeller-Hinton broth solution (Difco) adjusted to 0.5 on the McFarland scale, which corresponds to an absorbance of 0.08 to 0.13 at a length of 625 nm. This suspension was spread by streaking on MH agar plates (thickness 4 mm) using a sterile swab. After drying the inoculum, 5 wells of approximately 5 mm in diameter were cut and filled with 10mL of each dilution in sterile distilled water from the samples to be tested (80, 40, 20, 20, 10, and 5 %) (v/v). The boxes were incubated at 37°C for 18 hours, and then the inhibition halos were measured using a vernier. Dicloxacillin (1 mg/10mL) was used as a positive control in the case of *S. aureus,* and gentamicin (10mg/10mL) for Gram-negatives.

Statistical analysis. Samples were taken entirely at random and all determinations in this study were performed in duplicate. Harvest and post-harvest groups were compared using the non-parametric Mann-Whitney U test. The R statistical software package was used (v. 4.0.2).

RESULTS

Microbiological analysis. Figure 2 shows some illustrative images of the results observed for the growth of aerobic mesophilic microorganisms and yeasts.

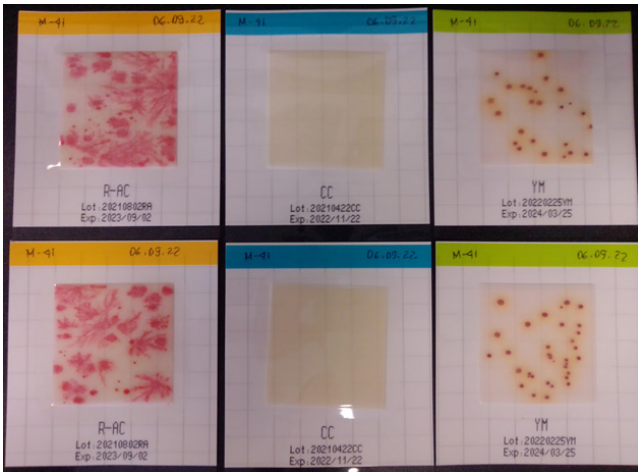

Figure 2. Representation of aerobic mesophilic microorganisms (R-AC) and yeast (YM) growth, absence of coliforms (CC) and molds (YM) in one of the honey samples of *M. beecheii* analyzed.

Based on the microbiological evaluation, 4 samples (9.30%) from the municipalities of Mama, Merida, Oxkutzcab, and Tekit showed no growth of any of the sanitary indicators evaluated. In 36 of the 43 samples (83.7%) there was an aerobic mesophilic microorganism growth, with a maximum of $4.5x10^2$ CFU/g being observed.

Regarding coliforms, only two samples (4.6%) showed counts of 5 and 25 CFU/g, respectively. Mold growth with a maximum of 9.5 x 10 CFU/g was observed in 9 samples (20.9%). Yeast growth was observed in 17 samples (39.5%), with a maximum of $3.2x10^3$ CFU/g. The presence of sporulated forms of sulfite-reducing clostridia was not observed in any sample. Only in the case of yeasts, 10 samples presented a higher count than the specification indicated for *A. mellifera* honey in NMX-F-036-1997-NORMEX, "Alimentos-Miel-Especificaciones y métodos de prueba" (Table 1).

It is worth noting that there was no statistically significant difference ($p>0.05$) between the honeys extracted at harvest and post-harvest.

Antimicrobial activity. Regarding the antimicrobial activity, due to insufficient volume required for the test in one of the samples, only 42 of the 43 samples were analyzed. Of the samples, 78.6% showed activity against *S. aureus* at a concentration of 80%, 59.5% at a concentration of 40%, and 21.4% at a concentration of 20%. However, halos >18 mm were observed in only 30.9% and 2.4% at the 80% and 40% concentrations, respectively (Figure 3).

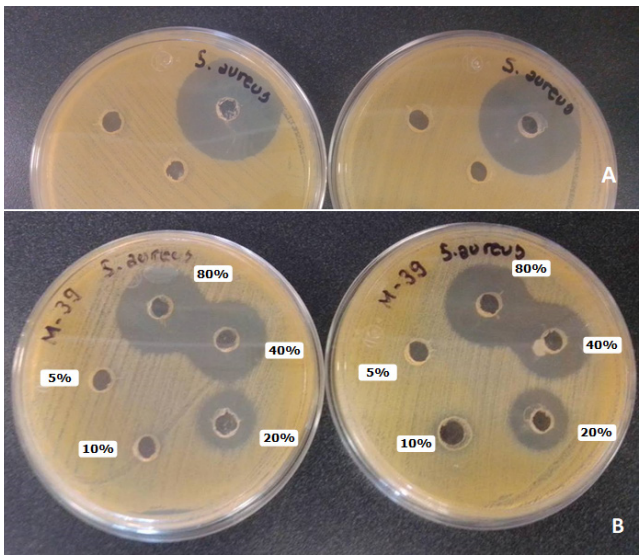

Figure 3. Agar diffusion well assay for determination of antibacterial effect against *S. aureus*: A) positive control dicloxacillin 1 mg/100 mL; B) *M. beecheii* honey at 80, 40, 20, 10n and 5 % (v/v).

Table1. Number of honey samples analyzed with sanitary indicator counts above 10^2 and 10^3 CFU/g, in accordance with the normative sanitary specifications for honey from *A. mellifera.*

Season	Mesophiles > 10^3 CFU/g	Coliforms > 10^3 CFU/g	Molds > 10^2 CFU/g	Yeasts > 10^2 CFU/g	Clostridia > 10^2 CFU/g
Harvest	0/24[a]	0/24 [a]	0/2 [a]	10/24 [a]	0/24 [a]
Post-harvest	0/19 [a]	0/19 [a]	0/19 [a]	7/19 [a]	0/19 [a]

[a] Same letter in the same column indicate that there is no significant difference ($p>0.05$).

With regard to Gram-negative bacteria, the reduction in bacterial growth occurred in 57.1%, 38.0%, and 28.6% of the samples at a concentration of 80% for *Salmonella enterica* var. *Typhimurium*, *P. aeruginosa*, and *E. coli,* respectively. While at a 40% concentration, the activity was observed only in 28. 6%, 9.5%, and 2.4% of the samples for the same strains. However, only one of the samples had an inhibition halo > 15 mm.

In this study, the largest inhibition halos were recorded against *S. aureus*. The means of the inhibition zones for honey collected at harvest and post-harvest seasons were 12.34±1.40 mm and 13.37±2.07 mm at 80% concentration, 6.73±1.28 and 8.48±1.69 mm at 40% concentration, and 0.92±0.65 and 3.38±1.11 mm at 20% concentration (Table 2). At a concentration of 20%, a significant difference ($p<0.05$) was observed between seasons.

However, in Gram-negatives, resistance was observed against all honey samples tested, with inhibition halos of 6.81±1.13 mm and 6.06±1.28 mm for *S. enterica* var. *Typhimurium*, 2.76±0.83 mm and 3.10±0.88 mm for *P. aeruginosa*, and 2.11±0.70 mm and 2.76±0.89 mm for *E. coli*, all at 80% concentrations for harvest and post-harvest honey, respectively (Table 2). No statistically significant differences ($p>0.05$) were observed between seasons.

Although reduced inhibitory activity was observed against these strains, there were lighter areas around the wells in all cases, indicating bacteriostatic activity (Figure 4).

Table 2. Mean diameter (mm) of inhibition halos for 20%, 40%, and 80% (v/v) *M. beecheii* honey against *S. aureus, Salmonella, P. aeruginosa,* and *E. coli*.

	Harvest			Post-harvest		
	20%	**40%**	**80%**	**20%**	**40%**	**80%**
S. aureus ATCC 6538	0.92 ± 0.65[b]	6.73 ± 1.28[c]	12.34 ± 1.40 [a]	3.48 ± 1.1 [b]	8.48 ± 1.69[d]	13.37 ± 2.07[c]
S. typhimurium ATCC 14028	No inhibition [a]	1.81 ± 0.8[b]	6.81 ± 1.13[c]	No inhibition[a]	3.44 ± 1.04[c]	6.06 ± 1.28[b]
P. aeruginosa ATCC 14028	No inhibition [a]	0.34 ± 0.34[a]	2.76 ± 0.83[b]	No inhibition[a]	1.08 ± 0.61[b]	3.10 ± 0.88[a]
E.coli ATCC 8739	No inhibition [a]	0.25 ± 0.25[a]	2.11 ± 0.7[b]	No inhibition[a]	No inhibition [a]	2.76 ± 0.89[a]

[a-d] Same letters in the same column indicate that there is significant difference ($p< 0.05$). The table shows mean ± standard error.

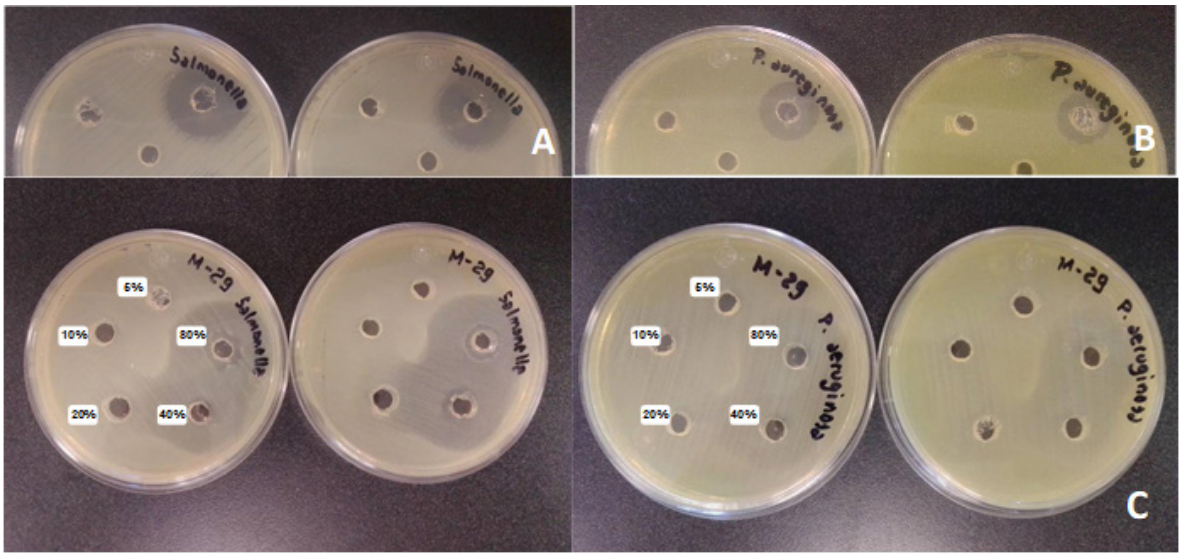

Figure 4. Agar diffusion well assay for determination of antibacterial effect against *Salmonella* and *P. aeruginosa*: A) positive control gentamicin 10 mg/100 mL; B) *M. beecheii* honey at 80, 40, 20, 10 and 5% (v/v).

DISCUSSION

Although the presence of aerobic mesophilic microorganisms was observed in 83.7% of the samples, none of them showed values higher than those established in appendix B of NMX-F-036-2006 (17), that is, the counts were $<10^3$ CFU/g, with a maximum of 4.5×10^2 CFU/g (Table 1).

The presence of molds was near the maximum limit and yeasts was exceeded as per the NMX-F-036-2006, which establishes a value $<10^2$ CFU/g for each specification. However, when compared with the ADAB regulation No. 207 for honey of the *Melipona genus*, which specifies between 10^3 and 10^4 CFU/g, the samples comply (18).

The results of this research agree with Fernandez et al (19) for honey from *Melipona fasciculata* in Brazil regarding the absence of contamination by total coliforms and sulfite-reducing clostridia but not for molds and yeasts, where it was reported that only one of the 40 samples presented a total count of molds and yeasts above 100 CFU/g. Other authors, such as Nadja et al (20), reported finding 1 to 9.7×10^2 CFU/g for aerobic mesophilic microorganisms and the presence of values higher than 100 CFU/g for molds and yeasts at 50% in their samples of stingless bee honey collected from different areas of Malaysia. The opposite case was reported by Marconi et al (2) in honey from *Melipona iotta* from Peru, which were free of bacterial contamination as well as molds and yeasts.

As there are several sources of contamination in honey that are difficult to control, such as air, water, nectar and pollen, the growth of microorganisms can occur; however, during maturation there are changes in the physicochemical conditions of honey, gradual evaporation of water, acidification, and increase in the concentration of sugars, these factors promote the selection of osmotolerant, xerotolerant, and acid tolerant microorganisms (11) whose presence is acceptable up to 10 000 CFU/g for aerobic mesophilic if they comply with the criteria for yeasts and are free of fecal contamination (21). Considering the above, the low presence of total coliforms in the samples analyzed could indicate the implementation of good hygienic practices during harvest and storage or that the antibacterial activity did not allow the survival and proliferation of these microorganisms.

The absence of sporulated forms of sulfite-reducing clostridia suggests that the contamination of the honey analyzed was low; however, since it cannot be guaranteed that all honey is free of spores, it would be advisable to apply some method, such as gamma radiation, to ensure that the honey used for the treatment of wounds and ulcers is free of spores (22) as well as to continue with the recommendation that its consumption should not be encouraged in children under one year of age (23).

Concerning the mold and yeast counts, it is necessary to consider the limit established in ADAB regulation No. 207 of up to 10^4 CFU/100 g for honey of the *Melipona genus*, since the humidity of up to 30% and acidity of up to 70 meq/100 g reported in this type of honey are factors that favor the growth of these microorganisms. Yeast genera such as *Starmerella, Candida*, and *Zygosaccharomyces* have been reported in stingless bee honey (24,25), which along with other genera, favor the honey fermentation process when the honey has a high degree of humidity (>21%) (20). This natural fermentative process could be related to its biological potential (26), such as the antibacterial activity observed in this research.

The statistical difference ($p<0.05$) in the aerobic mesophilic microorganism count between the honey extracted on the harvest and post-harvest seasons can be explained by the deficiencies of good practices during extraction, especially when the sealing of the jobon (a traditional wood hive) with mud, which causes a product with soil particles to be obtained during honey extraction. It may also be linked to the season of extraction, the high rainfall during the post-harvest season, and poor storage conditions.

In this study, the largest inhibition zones were recorded against *S. aureus*, and, despite the minimal bactericidal activity observed against these strains, in all cases there were halos with bacteriostatic activity, that is, lighter zones were observed, indicating inhibition of bacterial growth (Figure 4). This might be due to differences in the cell walls of the bacteria since in gram-negative organisms the complex cell wall with an additional outer membrane protects the bacteria from the environment by excluding toxic molecules, providing an additional stabilizing layer around the cell as has been indicated by Silhavy et al (27).

The mean of the zone of inhibition of the honey samples analyzed was similar to that reported by Chan-Rodriguez et al (28) for *M. beecheii* in Mexico, with inhibition halos of 10.5 mm for S. aureus and 4.0 mm for *E. coli* at 80% honey concentrations. This is contrary to what was described by Dória et al (29) and Domingos et al (7) with respect to the inhibition of *S. aureus*, reporting halos of up to 32.7±1.5 mm and 20.6±0.6 mm for 50% honey from *M. quadrifasciata anthidioides* and *M. seminigra,* respectively. However, it is confirmed that there is little or no sensitivity against *E. coli* and *P. aureginosa.*

As mentioned above, honey can constitute a reservoir for microorganisms from the environment. Bacteria of the genus *Bacillus* spp., *Clostridium* spp., *Enterococcus* spp, *Staphylococcus* spp, *Streptomyces* spp., as well as lactic acid bacteria have been associated with stingless bee honey (4), so the antibacterial activity of the analyzed samples could be due to other factors, such as the profile and concentration of phenolic compounds, unavoidable components in the vegetation found in the low deciduous forest where the time of the year has a notable effect, increasing their concentration during summer (30), and how antibacterial activity has been associated with a modification in the permeability of cell membranes and enzyme binding, which causes loss of integrity, inducing the consequent outflow of intracellular compounds (31).

In the case of Gram-positive bacteria, flavonoids, 3.61 CE mg/100 g reported by Ruiz-Ruiz et al (32) for honey from *M. beecheii* in Mexico and 4.19 CE mg/100 g by Alvarez et al (33) in Cuba, have been reported to modify intracellular pH and interfere with the energy generating system (ATP). They can also act by inhibiting both energy metabolism and DNA synthesis, thus affecting protein and RNA synthesis (31), which would explain the bacteriostatic effect against Gram-negative bacteria obtained in this research.

Lee et al (34) reported that 92.5% of the total bacteria isolated from various floral honey analyzed in their research manifested antimicrobial activity against at least one of the microorganisms tested, so an aspect of interest in this study was the observed antimicrobial activity of the honey analyzed in spite of the mesophilic counts, molds, and yeasts, which could be explained by the production of secondary metabolites with antagonistic interspecies interactions such as antibiotics, bacteriocins, biosurfactants, siderophores, antibiotics, toxins, quorum sensing or self-induction inhibitors, produced mainly by bacilli, lactobacilli, molds and yeasts (11). Thus, the identification of the microbiota of stingless bee honey and their antibacterial benefits could lead to their direct use in the production of probiotics in the food technology industry (35).

In conclusion, although it is known that honey has antibacterial properties, in this study bacterial growth was observed in most of the honey analyzed; however, only the yeast count exceeded that established by the Mexican regulations that apply to honey from *A. mellifera*. Due to its high moisture content, stingless bee honey was more susceptible to microbial contamination, which makes it more susceptible to fermentation and therefore has a short shelf life. Therefore, it is necessary to avoid contamination and follow good hygienic practices to maintain the quality of these types of honey, especially if they are to be used as therapeutic agents. Thus, it is important to establish microbiological specifications for recently harvested honey from stingless bees, like *M. beecheii*, in the current Mexican regulations, in order to ensure their sanitary quality. Regarding antibacterial activity, the results showed susceptibility to *S. aureus* in 78.6% of the samples, but not in the case of *S. Typhimurium*, *P. aeruginosa* y *E. coli.* Such antibacterial activity may be associated, not only with the physicochemical characteristics of honey, but also with the interactions between microbiome, plants, and bees.

Conflict of interest

The authors declare no conflicts of interest.

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