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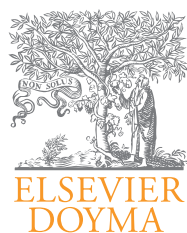
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ORIGINAL ARTICLE

Microbiological and physicochemical analysis of yateí (*Tetragonisca angustula*) honey for assessing quality standards and commercialization

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Yateí honey;
Microbiological
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Quality standards

Abstract

Due to the interest in the production and trading of yateí (*Tetragonisca angustula*) honey in the province of Misiones, Argentina, in this work we assessed microbiological and physicochemical parameters in order to contribute to the elaboration of standards for quality control and promote commercialization. Results showed that yateí honey samples had significantly different microbiological and physicochemical characteristics in comparison to established quality standards for *Apis mellifera* honey. Thus, we observed that values for pH (3.72), glucose (19.01 g/100 g) and fructose (23.74 g/100 g) were lower than *A. mellifera* quality standards, while acidity (79.42 meq/kg), moisture (24%), and mould and yeast count (MY) (3.02 log CFU/g) were higher. The acid content was correlated with glucose ($R^2=0.75$) and fructose ($R^2=0.68$) content, and also with mould and yeast counts ($R^2=0.45$) to a lesser extent. The incidence of microorganisms in yateí honey samples reached 42.85% and 39% for *Clostridium* sulfite-reducers and *Bacillus* spp., respectively. No *C. botulinum* or *B. cereus* cells were detected. *Enterococcus* spp. and *Staphylococcus* spp. incidence was similar (ca. 7.14%), whereas *Escherichia coli* and *Salmonella* spp. were not detected. We conclude that the microbiological and physicochemical properties of yateí honey are different from those of *A. mellifera* honey; hence, different quality standards could be implemented to promote its commercialization. © 2014 Asociación Argentina de Microbiología. Published by Elsevier España, S.L. All rights reserved.

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PALABRAS CLAVE

Miel de yateí;
Parámetros
microbiológicos;
Parámetros
físico-químicos;
Estándares de calidad

Análisis microbiológico y fisicoquímico de la miel de yateí (*Tetragonisca angustula*) para la evaluación de estándares de calidad y comercialización

Resumen

Debido al interés en la producción y comercialización de la miel de yateí (*Tetragonisca angustula*) en la provincia de Misiones, Argentina, en este trabajo se evaluaron parámetros microbiológicos y fisicoquímicos con el fin de contribuir con la elaboración de normas para el control de calidad y promover su comercialización. Los resultados demostraron que los parámetros analizados en esta miel ($n = 28$) diferían significativamente de los valores aceptables establecidos para la miel de *Apis mellifera*. En comparación, se observó que los valores de pH (3,72) y de concentración de glucosa (19,01 g/100 g) y fructosa (23,74 g/100 g) eran más bajos, mientras que los valores de acidez (79,42 meq/kg) y humedad (24 %), al igual que el recuento de hongos y levaduras (HyL) (3,02 log UFC/g), eran más altos. La acidez mostró una correlación inversamente proporcional con el contenido de glucosa ($R^2 = 0,75$) y fructosa ($R^2 = 0,68$), y directamente proporcional con el recuento de HyL, aunque en este caso la correlación fue menor ($R^2 = 0,45$). En lo que respecta a los parámetros microbiológicos, se observó 42,85 % de *Clostridium* sulfito-reductores y 39 % de *Bacillus* spp., y no se detectó presencia de *C. botulinum* ni de *B. cereus*. *Enterococcus* spp. y *Staphylococcus* spp. se encontraron en una proporción similar (ca. 7,14 %), mientras que *Escherichia coli* y *Salmonella* spp. no fueron detectados. Concluimos que las propiedades microbiológicas y fisicoquímicas de la miel de yateí difieren de las de la miel de *A. mellifera*, por lo cual sería oportuno establecer normas de calidad diferentes para facilitar su comercialización.

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Introduction

The most common stingless bees in the Province of Misiones, Argentina, are the yateí bees (*Tetragonisca angustula*), from the *Meliponini* subfamily. Their habitat covers the tropical and subtropical regions of the American continent from Argentina up to Panamá and Mexico²¹. There are two subspecies of *T. angustula* identified as *T. angustula* Latreille and *T. angustula* Fiebrigi having a black and yellow mesepisternum, respectively. The latter is restricted to southern Brazil, Paraguay and Northeast provinces of Argentina including Misiones, Chaco, Formosa and Corrientes⁸. They are smaller than the *Apis mellifera* bees, with shorter flights to a wide variety of floral resources; they construct small nests in tree cavities or branches, and also underground²⁷.

On the whole, the physicochemical and organoleptic characteristics of yateí honey depend on the sugar content, maturity (ripe) and presence of active compounds such as aliphatic and aromatic alcohols, aldehydes, acids and their esters. These active compounds give special properties and define the honey flavour^{4,7}.

Although honey have high osmolarity and low water activity and nutrients, it can hold microorganisms present in pollen, dust, air, soil and nectar which are very difficult sources to control. However, microbial contamination can also be originated from food handlers, equipment and cross-contamination which can be easily controlled by standard sanitation and good manufacturing practices during harvest and honey processing²⁴; otherwise, honey would have high counts of vegetative bacteria¹¹. The microorganisms of concern in honey are some fungi and

yeast genera, such as *Penicillium*, *Mucor*, *Saccharomyces*, *Schizosaccharomyces* and *Torula*¹⁹; which are responsible for fermentation of honey with high moisture content (>21%). Spore-forming bacteria such as *Bacillus cereus* and *Clostridium* spp. are also regularly found in honey, which under certain conditions could cause illness in humans, specially bacteria such as *Clostridium botulinum* which are a high risk in children foods²⁹. Coliforms and yeasts are indicative of sanitary or commercial quality concern²⁴. In this sense, we found no reports in the literature about bacterial contamination, such as *Escherichia coli*, *Staphylococcus*, *Enterococcus* and *Salmonella* in yateí honey.

Meliponi culture is steadily growing with commercial purposes; for this reason it is very important to study the characteristics and quality of local honey³⁴. The aim of this work was to assess the microbiological and physicochemical properties of yateí honey in order to contribute to the elaboration of standards for quality control for this type of honey, which is not ruled by Mercado Común del Sur (MERCOSUR) Legislation and Código Alimentario Argentino (CAA).

Materials and methods**Honey samples**

Twenty eight samples of yateí honey were collected from bee hives located within a variety of handmade boxes designed by producers from different regions of the Province of Misiones, Argentina. All samples were aseptically collected

from the shaped combs using sterile syringes. Sampling was carried out during the months of November, December, March and April on sunny days at room temperature (25-35 °C). Honey samples in syringes were stored at 5 °C and processed within 24-48 h after harvest time.

Physicochemical analysis

The moisture content in homogenized undiluted honey samples was determined by the Official Method of Analysis³ (AOAC International). The refractive index of transparent and translucent liquid samples was determined using an Abbe refractometer (Lambda Scientific Systems, Inc., USA) at 20 °C and the values recorded were converted to percent moisture using the conversion table⁹ modified by Wedmore³⁵. Values are expressed as moisture percentages (%).

The pH of honey samples was measured using an Orion 230A pH meter (Orion Research, Inc., USA).

Acidity was determined in a solution containing 2 g of honey and 15 ml of previously heated and cooled water. This solution was titrated with 0.1 N NaOH until the pH reached 8.5. Values are expressed as milliequivalents of total acid per kilogram of honey (meq/kg).

Sugar (glucose, fructose and sucrose) content was measured in a 10 µl aliquot by high pressure liquid chromatography (HPLC) using a chromatographer (Waters & Associates, Inc., USA) connected to an amino column, 250 × 4.6 mm, 5µ (Grace, Inc., USA). For the HPLC analysis of sugar content, samples were prepared as follows: 0.25 g of honey were diluted in 25 ml of the mobile phase (70:30, acetonitrile:water). The mix was centrifuged for 2000 g and filtered with a 0.45 µm filter in a Minisart high flow Syringe Filter (Sartorius AG, Germany). Chromatographic separation was performed under isocratic conditions using the above mobile phase with a flow rate of 1.1 ml/min. A refractive index detector, Model 410 (Waters & Associates, Inc., USA), was used for data analysis. The concentration of sugar is expressed as grams per 100 grams of honey (g/100 g).

Microbiological analysis

Counts of microorganisms were carried out by plating appropriate dilutions of the honey samples. For this procedure, a 10 g sample was homogenized in 90 ml of 0.1% peptone water (Britania, Argentina) in an Erlenmeyer flask. Subsequent decimal dilutions were prepared in sterile peptone water and analyzed by duplicate by plate count or by the most probable number (MPN) method, depending on the kind of microorganism. The following microbial analyses were carried out:

- Total aerobic mesophilic bacteria (TAMB): 1 ml aliquots from each decimal dilution were plated onto count plate agar (PCA; Britania) and incubated at 30 °C for 48 h¹⁶. Microbial counts are expressed as logarithm of colony-forming units per gram of honey (log CFU/g).

- Moulds and yeasts (MY): 0.1 ml aliquots from each decimal dilution were spread on a mould and yeast medium (MY; Britania) and incubated at 25 °C for 5 days¹⁶. Microbial counts are expressed as logarithm of colony-forming units per gram of honey (log CFU/g).

- Total coliforms: a series of 3 sequential MPN tubes were inoculated from 10⁻¹, 10⁻² and 10⁻³ dilutions. Lauryl sulphate broth (LSB; Britania) was used as enrichment medium and brilliant green bile (2%) broth (Brilla; Britania) for confirmation. In both cases, samples were incubated at 35 °C for 48 h, positive samples (growth and gas production) in Brilla medium were used to calculate total coliforms⁶. Results are expressed as log MPN/g.

- *Clostridium* spp.: 5 ml aliquots from 10⁻¹ dilution were treated at 80 °C for 5 min. Then, 1 ml aliquot was added to a tube (RCM; Britania) molten and cooled at 46 °C, and the top covered with Vaseline for anaerobic conditions. The tubes were incubated at 45 °C for 5 days for growth and gas production. Cell morphology was observed by Gram stain and malaquita green stain using a CX optical microscope (Olympus, Inc., Japan). Further traditional assays including catalase, gelatine, lecithinase, nitrate and lactose assimilation were carried out to discard *Clostridium botulinum* presence. Results are expressed as presence or absence of *Clostridium* spp.

- *Bacillus* spp.: 0.1 ml aliquots from appropriate dilution were spread on *Bacillus cereus* selective agar (BCS; Britania) and incubated at 35 °C for 24 h. Suspected colonies were identified by Gram stain and biochemical reactions: catalase, modified Voges-Proskauer (VP), gelatine and growth in brain-heart infusion broth (BHI; Britania) plus 6.5% NaCl⁶. Results are expressed as presence or absence of *Bacillus* spp.

- *Escherichia coli*: positive tubes from Brilla broth were tested by growth in EC broth (Britania) and EMB agar (Britania). Typical colony growth on EMB agar was confirmed by traditional assays⁶ including indole, methyl red, VP and citrate. Results are expressed as presence or absence of *E. coli*.

- *Salmonella* spp.: these bacteria were investigated according to a modification of the standard method suggested by the FDA-BAM⁶. For the pre-enrichment, 1 g of honey was added to 9 ml of lactose broth (LB, Merck, Germany) and cultures were incubated at 35 °C for 24 h. The enrichment step was performed onto selenite cystine broth (SCB; Merck) incubated at 35 °C for 24 h. Isolations were examined onto bismuth sulfite agar (BSA; Merck) and Hektoen enteric agar (HEA; Britania), after incubation at 35 °C for 48 and 24 h, respectively. Suspected colonies of *Salmonella* were tested into triple sugar iron (TSI, Britania) and lysine iron (LIA, Britania) agar. Colonies exhibiting typical reactions on TSI and LIA were purified and further characterized by traditional assays: urease, oxidase, phenylalanine decarboxylase, VP, indole, citrate and gelatine. Results are expressed as presence or absence of *Salmonella* spp.

- *Enterococcus* spp.: a series of 3 sequential MPN tubes with glucose-azide broth (Merck) were inoculated from 10⁻¹, 10⁻² and 10⁻³ dilutions and incubated at 35 °C for 48 h. The positive tubes were inoculated onto KF agar (Merck) and typical colonies were analyzed by Gram stain, catalase, growth on BHI broth plus 6.5% NaCl, at 45 °C for 48 h and 10 °C for 5 days¹⁶. Results are expressed as log MPN/g.

- *Staphylococcus* spp.: 0.1 ml aliquots from 10⁻¹ dilution were spread on Baird Parker agar (BPA, Britania) and incubated at 35 °C for 48 h. The presence of coagulase

positive staphylococci (CPS) was determined by picking colonies from BPA and testing them by Gram stain, TSI agar reactions and coagulation of rabbit plasma⁶ (BD, USA). Results are expressed as presence or absence of *Staphylococcus* spp. and CPS.

Data analysis

Mean values and significant differences between parameters were evaluated by one way Analysis of Variance and by the Fisher-test in Minitab-15 software (Minitab Inc., USA). Histogram analyses were also performed by Minitab-15, whereas correlation analyses were performed by using Sigma Plot 10 (Systat Software, Inc., USA).

Results and discussion

Physicochemical properties

The pH value of honey samples from *T. angustula* (Yatei) showed a mean of 3.72 ± 0.02 . This value is similar to that obtained by Anacleto *et al.*² and Vit *et al.*³³ for yatei honey, but lower than that obtained by Iurlina and Fritz¹⁷ for *A. mellifera* honey (pH means value 4.6).

The acidity of yatei honey ranged from 25 to 160 meq/kg with a mean value of 79 ± 43 (Fig. 1A). A similar range (7.7–109 meq/kg) was previously indicated by Souza *et al.*²⁵ for yatei honey from Brazil, Mexico and Venezuela. Sgariglia *et al.*²³ also reported a wide range of acidity (45.4–98.4 meq/kg) for yatei honey from the northwest of Argentina. However, no previous reports showed acidity values as high as those obtained in this work (130–160 meq/kg). This fact may be related to the harvest time, i.e. the maturity of honey, and/or climatic factors, which would favor chemical, enzymatic and microbiological reactions able to release acidic compounds in honey (see below). In contrast, acidity values of *A. mellifera* honey are significantly lower, between 15 and 38 meq/kg. Therefore, the CAA establishes that the acidity value should not exceed 40 meq/kg for edible honey.

The amount of water contained in yatei honey was $24 \pm 1.5\%$, similar to values previously reported by several authors for honey from different stingless bees^{1,17,28,32}. The water content in yatei honey is higher than the average reported for *A. mellifera* honey^{10,13,25} (ca. 18%).

The concentration of glucose and fructose varied from 11.5 to 31.6 g/100 g but had significantly different means with a confidence interval of 95% for Fisher test. In Figure 1B we observed that data have a log normal distribution, being the average of 19 ± 6 and 24 ± 4 g/100 g for glucose and fructose, respectively. These mean values are lower than those reported by other authors for yatei honey, where values reached 22–27 and 31–40 g/100 g for glucose and fructose, respectively^{23,27}. These differences may be associated with the high levels of acidity that were observed in this work (see above). In comparison, *A. mellifera* honey has higher concentration of glucose (32 g/100 g) and fructose (39 g/100 g) than the yatei honey analyzed in this work¹⁰.

We also observed that the sum of water content plus monosaccharides measured in yatei honey represented only

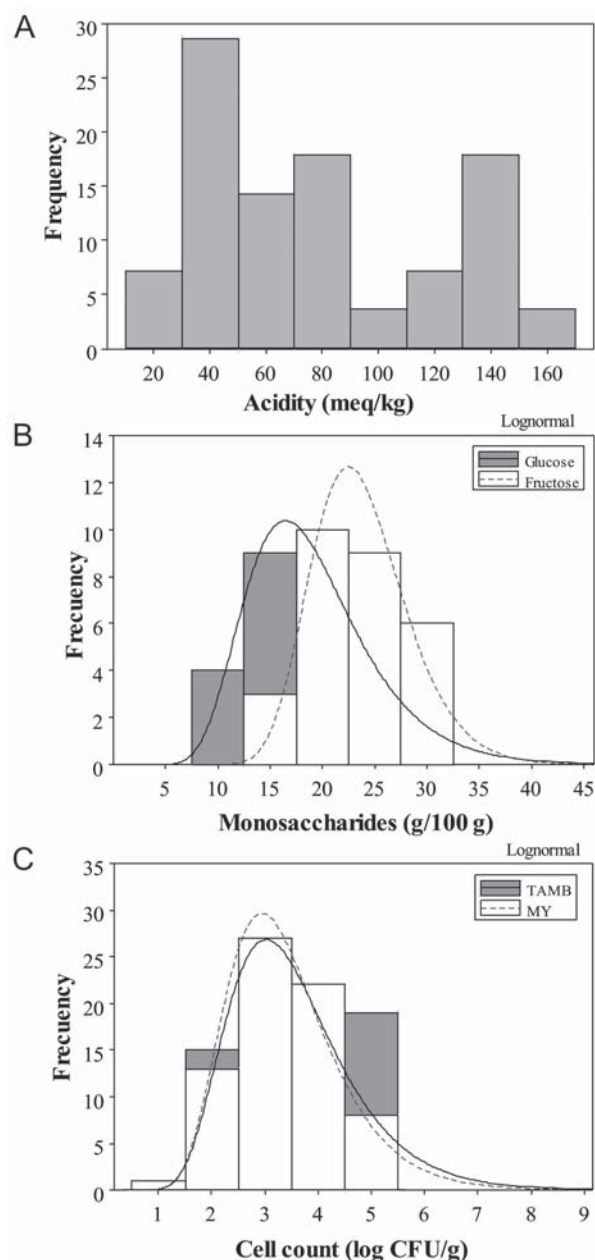


Figure 1 Frequency distribution of acidity (A), glucose and fructose (B) and total aerobic mesophilic bacteria (TAMB) and mould and yeast (MY) (C) in honey samples from *T. angustula* (n=28).

65.5 g/100 g of the total honey mass, which suggests that other substances, including polysaccharides, would be present in high concentration in this honey. The findings reported by Vit *et al.*³¹ support this hypothesis since they showed that the genus *Trigona*, such as *T. angustula*, produces honey with high maltose content (24–56 g/100 g), whereas the genus *Melipona*, such as *Melipona paraensis*, produces honey with low maltose content (1–1.23 g/100 g). The HPLC methodology we used in this study was suitable to detect sucrose but not maltose. As sucrose was not found in the analyzed samples, we suspect that maltose could be another main compound in yatei honey composition.

Absence of sucrose in yateí honey was previously reported by Torres *et al.*²⁷, who related this fact to the activity of invertase enzymes present in this honey. In contrast, other authors reported the presence of sucrose in yateí honey but in low concentrations^{2,25} (0.13 and 6.00 g/100 g).

Microbial counts

Honey microorganisms can originate from primary sources (at harvest) such as pollen, the digestive tracts of honey bees, dust, nectar, and others, which are very difficult to control. Other sources (after-harvest) include air, food handlers, cross-contamination, equipment and buildings, which can be controlled by good manufacturing practices²⁴. In this work, we try to avoid these secondary sources of contamination by using sterile syringes and gloves for honey sampling. However, yateí honey showed a wide range of MY counts (1.2–4.7 log CFU/g), with an average of 3.02 ± 0.99 log CFU/g (Fig. 1C), which came from a primary source of contamination. The subsistence of MY in honey is due to the ability of fungi, mainly yeasts, to grow under high sugar concentrations even with limited available water²⁴. However, it is worth highlighting that the mean values for MY in yateí honey were higher than those reported for *A. mellifera* honey¹⁷. This fact was related to the higher water content observed in the former.

About 90% out of MY in yateí honey were yeast strains. Although we found no reports about the total “mould and yeast” count in this honey, we found that a yeast count of ca. 4 Log CFU/g was reported by Texeira *et al.*²⁶. This author indicated that this count is due to the species *Starmerella meliponinorum*, which would be metabolically active and able to grow at the expense of sugars present in this food.

The number of TAMB was similar to MY, with an average of 3.13 ± 1.05 Log CFU/g. This value is similar to data reported by several authors for *A. mellifera* honey^{17,22,28} and falls within the legislative limits ruled by MERCOSUR and CAA. Only in 10% of samples counts were higher than the established maximum limit (1×10^4 CFU/g).

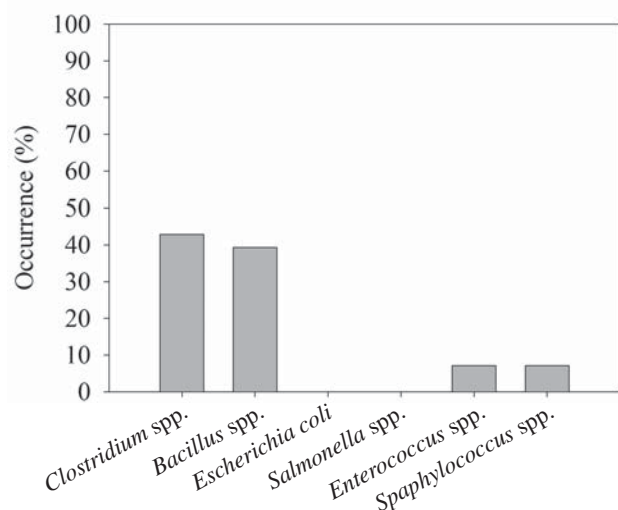


Figure 2 Occurrence (%) of different bacteria in honey samples from *T. angustula* (n=28).

Bacterial characterization

On the whole, honey is a hostile environment for the growth of food-borne pathogenic bacteria. However, spore and vegetative latent forms may be present due to primary and/or secondary pollution. In this work, we evaluated six bacterial genera that can be present in honey and can produce food-borne diseases. These genera included: *Bacillus*, *Clostridium*, *Staphylococcus*, *Enterococcus*, *E. coli* and *Salmonella*.

Clostridium spp. (64%) and *Bacillus* spp. (39%) were the most prevalent genera in analyzed honey samples (Fig. 2). These spore-forming bacteria are common in air, soil and dust becoming a primary source of contamination; thus, they are commonly found in honey²⁴. The detection of *Clostridium* spp. in honey is very important to determine its quality mainly taking into account the severity of *C. botulinum* foodborne disease. Although we detected a high percentage of *Clostridium* spores in honey from *T. angustula*, we did not characterize any of the isolates as *C. botulinum* by the biochemical test performed. The presence of *Clostridium* spp. in *A. mellifera* honey is also high. Finola *et al.*¹¹ found sulfite-reducing spores in about 70% of samples from Córdoba, Argentina. Kokubo *et al.*¹⁸ showed that 79% of the samples analyzed contained sulfite-reducing spores, with six samples containing *C. perfringens* but none *C. botulinum*. In stingless bee honey, a few reports indicated absence of *C. botulinum* in honey samples^{12,37}.

The presence of *Bacillus* spp. would be expected in honey, since a symbiotic relationship between this microorganism with insects, including honeybees, solitary bees and stingless bees had been reported²⁰. Specifically, *Bacillus* species were found in abdominal tissues, larval food and honey of several species of tropical stingless bees¹⁴.

Since the native habitat of *E. coli* is the enteric tract of animals, its presence in foods generally indicates direct or indirect contamination of fecal origin²². Only one out of twenty eight samples had total coliforms, in which the count was 1.45 log NMP/g. Fecal coliforms, *E. coli*, *Salmonella* spp. were not detected (Fig. 2). These results reflect good manufacturing practices during harvest and laboratory store analysis.

Two samples were positive for *Enterococcus* spp., with counts of 1.63 and 3.04 log MPN/g (Fig. 2). Cultures were characterized as lactic acid bacteria (LAB), which can originate from bee microbiota, since associations between LAB and invertebrates have been shown. Symbiotic LAB has been found inside the honey crop in *A. mellifera*, from where it can be transferred to the honey comb³⁰; a similar event could happen in *T. angustula* honey.

Two samples of *T. angustula* honey showed the presence of *Staphylococcus* spp. but none of the isolated strains produced coagulase. We have not found any reports of *Staphylococcus* in honey from stingless bees. In *A. mellifera* honey this genus is rarely found, it appears to be unable to survive during processing and is not likely to grow in honey. In that respect, some authors²⁴ have emphasized the need to improve the procedures of honey harvesting and processing to reduce the introduction of microbes like *Staphylococcus*, and have also indicated that the

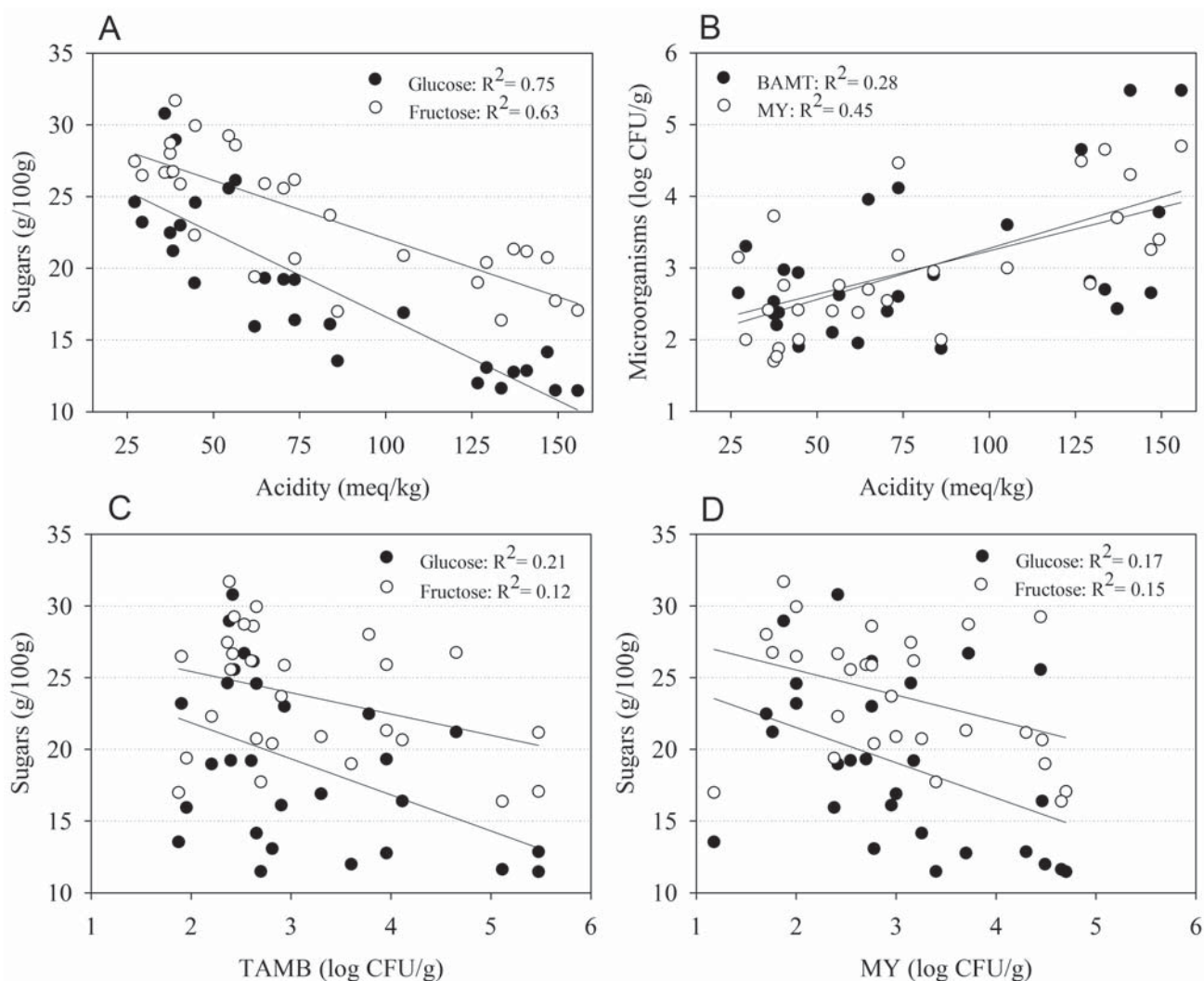


Figure 3 Correlation analysis between physicochemical and microbiological parameters: Acidity and sugars (A), acidity and microorganisms (B), total aerobic mesophilic bacteria (TAMB) and sugars (C), and mould and yeast (MY) and sugars (D).

antibacterial properties of honey should be carefully considered²⁴.

Correlation between acidity, sugars and microorganisms

The high acidity of yateí honey is linked to its high water content, which favors the development of some chemical and enzymatic reactions able to release acidic compounds. As examples of these reactions, we can quote the decomposition of fructose into levulinic and/or methanoic acids^{5,15} and the conversion of glucose into gluconic acid by the enzyme glucose oxidase³⁶. On the other hand, the high water content in yateí honey can also decrease its osmotic pressure and allow the development of some fermentative microorganisms able to release acidic compounds³⁴. In order to know which of these reactions would be the main cause for acidity in yateí honey, we evaluated the correlation between the acid content, the sugar concentration (glucose and fructose), and the microorganism count (TAMB and MY).

We observed that acid content was associated with the sugar concentration with a correlation coefficient (R^2) of 0.75 for glucose and 0.68 for fructose (Fig. 3A). On the other hand, the correlation between the acid content and the MY count was significantly lower ($R^2=0.45$) (Fig. 3B). No correlation ($R^2 \leq 0.28$) was observed between the acid content and the TAMB count (Fig. 3B), neither between the sugar concentration and the microorganism count (Figs. 3C and 3D). These results indicate that the acidity of yateí honey is related to chemical and enzymatic reactions of honey itself rather than with fermentative processes carried out by microorganisms.

Conclusion

The results obtained for yateí honey were quite different compared to the quality standards established in the CAA for *A. mellifera* honey; therefore, we recommend that some quality parameters, such as acidity, moisture content

and mould and yeast counts, should be adapted to yateí honey in order to promote its commercialization.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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