



ORIGINAL ARTICLE

Microbiological assessment of honey in México



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Abstract Honey is a product used as a natural sweetener and in several regions of Mexico and other countries it is also used as a therapeutic agent. Microbiological contamination of honey can occur during its extraction and handling. Due to the use and consumption of honey we highlighted here the importance of the assessment of its microbiological quality. One thousand nine hundred twenty samples obtained from 8 honey-producing states from Mexico were analyzed. From these samples, 40.5% (777/1920) did not comply with the NMX-036-NORMEX-2006 specification. Forty five percent (777/1920) of the samples did not comply with the mesophilic aerobic microorganism specification, neither did 17% (327/1920) of the samples with the specification for molds and 18.1% (348/1920) with the specification for yeasts. With regard to coliform bacteria, the samples contained less than 3 NMP/g. Two percent of the samples contained lactic acid bacteria (LAB), *Clostridium perfringens* was observed in amounts of more than 100 CFU/g. None of the samples from the different states contained more than 100 CFU/g of *Staphylococcus aureus*; *Salmonella* spp. was absent in all samples. It is important to avoid contamination sources and implement good hygienic practices in order to maintain and improve the quality of Mexican honeys since a large percentage of them are intended for export. If these honeys are intended for therapeutic use, it is necessary to ensure that they comply with all quality parameters and to apply specific treatments that guarantee the removal of any pathogen that may represent a risk to the patients's health.

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PALABRAS CLAVE

Miel;
Seguridad
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Mohos;
Levaduras

Calidad microbiológica de la miel en México

Resumen La miel es un producto que se usa como edulcorante natural y en algunas regiones de México y otros países, como agente terapéutico. La contaminación de la miel con microorganismos se puede presentar durante la extracción y el manejo. Dado el uso y consumo de la miel, se consideró importante evaluar su calidad microbiológica. Se trabajó con 1.920 muestras de miel obtenidas de 8 estados productores en México. De las muestras de miel analizadas, el 40,5% (777/1.920) estuvo fuera de las especificaciones de la NMX-036-NORMEX-2006. Todas esas muestras no cumplían con las especificaciones referidas a mesófilos aerobios, en tanto que el 17% (327/1920) no cumplían las relativas a mohos y el 18,1% (348/1920), las fijadas para levaduras. En lo que se refiere a los organismos coliformes, las muestras contenían < 3 NMP/g. Se encontró que el 2% contenía BAL; *Clostridium perfringens* se puso en evidencia en el 12%, con más de 100 UFC/g. En el caso de *Staphylococcus aureus*, ninguna muestra estuvo por arriba de 100 UFC/g. *Salmonella* spp. no se detectó en ninguna de las muestras analizadas. Es importante evitar las fuentes de contaminación y observar buenas prácticas de higiene para mantener y mejorar la calidad de las mieles mexicanas, ya que un buen porcentaje de ellas están destinadas a la exportación. Si estas van a ser destinadas a un uso terapéutico es necesario asegurarse de cumplir con los parámetros de calidad y dar tratamientos específicos que aseguren la eliminación de algún patógeno que pueda poner en riesgo la salud del paciente que utilice la miel.

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Introduction

Honey is a product used as sweetener and in some regions of Mexico and other countries it is also used as a therapeutic agent. It has a high sugar content, mainly fructose and glucose, low water activity ($a_w = 0.50-0.60$), and a high osmotic potential with a humidity lower than 18%, a high acidity and low pH (3.4-6.1)¹⁷. In our country, Apiculture has a high social and economic value. Forty five thousand producers depend on it, which accounts for 19 million of beehives and allow Mexico to be placed as the fifth producing country and as the third exporting country in the world²⁷. Several studies have shown the presence of substances with antimicrobial activity such as hydrogen peroxide and several phytochemical compounds such as flavonoids, phenols, organic acids like cinnamic acid, methyl syringate, and methylglyoxal, which limit the amount of microorganisms present in honey and allow only those that manage to survive to remain viable^{4,11,36}.

The microbial characteristics of honey are a consequence of the intrinsic biota of bees, since honey is the product of the nectar of different flowers of distinct species that is kept during a different amount of time in their honey stomach without getting contaminated since it never gets in touch with the gut of the bee³. Contamination of honey with other microorganisms can occur during extraction and handling¹⁵. The following are some of the primary sources of microbial contamination in honey: the digestive tracts of honey bees, dust, air, dirt and flowers. Secondary sources of microbes in honey are likely to be the same as for other foods³⁰.

Different types of microorganisms such as *Actinobacter* spp., *Bacillus* spp., *Clostridium* spp., *Corynebacterium* spp., *Pseudomonas* spp. are bacteria commonly

found in soil. *Saccharomyces* and *Torula* yeasts can be found in high-moisture sugars, and *Leuconostoc mesenteroides* has been found in sugar refineries. *Brochothrix* spp., *Citrobacter* spp., *Enterobacter* spp., *Erwinia* spp., *Flavobacterium* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Listeria* spp. and *Pediococcus* spp. are found in plants and plant products³⁰.

Among the sporulating bacteria, different species of *Bacillus* have been reported as well as *Clostridium botulinum* spores and in a lesser proportion those of *Clostridium perfringens*. *C. botulinum* can occasionally cause infant botulism in children less than one year of age; *C. perfringens* can cause gastrointestinal alterations or gas gangrene in deep wounds, a possibility that is considered because of the current use of honey as a healing agent in wounds²³. There is no Official Mexican Standard, however, in 2006 a non-mandatory standard was elaborated (NMX-036-NORMEX-2006)¹⁹ establishing that the presence of no more than 1000 CFU/g of nonpathogenic bacteria and up to 100 CFU/g of yeast and molds should be accepted.

Mexico is one of the main honey producers worldwide; in 2010 it was the third exporter with 56 thousand tons and in 2012 it raised to 86 thousand tons, being the state of Yucatán, the main producer, followed by Campeche and Jalisco with a total aggregate of almost 40% of the national production among these three states²⁵⁻²⁷.

As a result and due to the use and consumption of honey, we considered it important to assess the microbiological quality of honey by studying the presence of aerobic mesophilic bacteria, coliforms, molds, yeasts, lactic acid bacteria, *Staphylococcus aureus*, *Salmonella* spp. and *C. perfringens* in samples from several producing regions in Mexico.

Materials and methods

Sampling sites

A total of 1920 samples of honey obtained from 8 producing states in Mexico (Mexico City, the States of: México, Yucatán, Jalisco, Morelos, Chihuahua, Oaxaca and Michoacán) were analyzed during two years (2010–2011). Two hundred forty samples from each state were assessed, handling 80 samples a month (10 of each state).

Sample handling

One thousand grams of honey were collected from the sites of process in clean containers, which were shipped in ice boxes at approximately 10 °C by air or ground transportation to the laboratory for their analysis.

Microbiological analysis

Twenty five (25) g of each sample were added to 225 ml of 0.1% peptone water homogenized on a STOMACHER 400 Circulator (Seward®, London), and decimal dilutions were made up to 10⁻⁶.

Each dilution was plated on potato dextrose agar acidified with tartaric acid using the pour plate technique and incubated at 25 °C during 5 days for molds and at 35 °C during 48 h for yeasts²¹. Standard method agar was used for the quantification of aerobic mesophilic (MA) bacteria with an incubation period of 48 h at 35 °C²⁰. Coliform organisms (OC) were assessed by the most probable number (MPN) using 10¹ to 10³ dilution by the 3,3,3 series on lactose broth; 1.0 ml of each dilution was transferred to the broth and incubated at 35 °C. Gas formation was observed after 24 and 48 h. A sample from the positive tubes was inoculated in 2% brilliant green bile lactose, incubated at 35 °C for 24–48 h and afterwards, tables were consulted in order to establish the MPN/g on each sample of honey²². For the determination of lactic acid bacteria (LAB), samples were inoculated onto de Man, Rogosa, and Sharpe agar (MRS)⁵ containing 1% of glucose and peptonized milk agar by spreading the plate and incubated at 37 °C with low CO₂ tension (Labcon Co®), for 48 h. For species identification several tests were performed, such as Gram staining, determination of catalase and oxidase activities, carbohydrate fermentation patterns (glucose, lactose, sucrose to detect gas production), motility and ability of strains to grow at different temperatures, pH and salt concentrations^{5,28,32}.

S. aureus samples were inoculated onto Baird-Parker media by the spreading technique and incubated for 48 h at 35 °C. Plates containing between 15 and 150 typical colonies of *S. aureus* were selected for coagulase and thermonuclease tests to confirm this microorganism²². For the determination of *Salmonella* spp., 25 g of each honey sample were added to 225 ml of lactose broth, incubated for 24 h at 35 °C, and 0.1 ml was transferred to 10 ml of Rappaport-Vassiliadis media and 1 ml to tetrathionate broth, incubated at 37 and 42 °C respectively for 24 h; the obtained cultures were streaked onto MacConkey, xylose lysine deoxycholate and brilliant green agar plates, incubated at 35 °C for

24 h and the biochemical and serological identification was performed²².

For the isolation of *C. perfringens* we used the methodology outlined in the BAM-FDA²⁴. *C. perfringens* ATCC 10239 was used as a positive control. Using an aseptic technique, 25 g of honey sample were placed into a sterile blender jar, 225 ml peptone dilution fluid (1:10 dilution) were added, and the mixture was homogenized for 1–2 min at low speed, avoiding aeration to obtain a uniform homogenate. Using the 1:10 dilution described above, serial dilutions were made from 10¹ to 10⁶ by spreading 0.1 ml of each dilution using a sterile glass rod spreader over previously poured plates of TSC agar containing egg yolk emulsion. Plates were overlaid with 10 ml of TSC agar without egg yolk emulsion. Anaerobic conditions were established, and the jars were placed in a 35 °C incubator for 20–24 h. Plates containing 20–200 black colonies were selected for counting (*C. perfringens* colonies in egg yolk medium are black with a 2–4 mm opaque white zone surrounding the colony, as a result of lecithinase activity). Black colonies were counted and the number of *Clostridium* cells per gram of food was calculated. Plates were saved for identification tests. All was done in triplicate. Ten typical *C. perfringens* colonies were selected and each of them was inoculated into a tube of freshly deaerated and cooled thioglycollate broth. Tubes were incubated in a standard incubator for 18–24 h at 35 °C. Each culture was examined by Gram staining and checked for purity. A 1-ml sample of each actively growing fluid thioglycollate culture was inoculated into modified iron-milk medium, and then incubated at 46 °C in a water bath. After 2 h, the cultures were checked every 60 min for stormy fermentation²⁴. When this test was positive, a complete test was carried out. Afterwards, biochemical identification was performed²⁴.

Results

From the 1920 honey samples analyzed, 40.5% (777/1920) did not meet the specification for NMX-036-NORMEX-2006. With regard to the indicator groups for mesophilic aerobic bacteria, 40.5% (777/1920) of the samples did not comply with meet the normative standards. Table 1 shows the number of analyzed samples that did not meet the specification established by the standard, which corresponds to 56.7% of the honey samples from Oaxaca, 53.3% from Jalisco, 50% from the State of Mexico, 48.8% from Michoacán, 36.3% from Chihuahua, 33.3% from Morelos, 25.8% from Mexico city and 19.6% from Yucatán. The bacterial content ranged from 1200 to 9400 CFU/g.

With regard to molds, 17% (327/1920) of the analyzed samples were outside the limits established by the standard while in yeasts, 18.1% (348/1920) of the samples showed the same behavior. CFU for molds ranged from 100 to 160 CFU/g and for yeasts from 120 to 990 CFU/g. Molds were found in honeys samples from the following states: 27.01% from Oaxaca, 25% from Michoacán, 21.3% from Jalisco, 20.8% from the State of Mexico, 15% from Morelos, 12.5% from Chihuahua, 8.3% from Mexico City and 6.3% from Yucatán. Yeasts were found in 29.2% of the honeys from Michoacán, 25% from the State of Jalisco, 23.3% from Oaxaca, 22.5% from the State of Mexico, 14.6% from Morelos, 11.7% from Mexico City, 11.3% from Chihuahua and 7.5% from Yucatán (Table 1).

Table 1 Number of analyzed honey samples with pathogen or microbial group presence above 100 and 1000 CFU/g

Sampling place	MA >1000 CFU/g	Molds >100 CFU/g	Yeasts >100 CFU/g	LAB >100 CFU/g	<i>C. perfringens</i> >100 CFU/g
Chihuahua	87/240	30/240	27/240	0/240	18/240
Ciudad de México	62/240	20/240	28/240	5/240	22/240
Estado de México	120/240	50/240	54/240	8/240	37/240
Jalisco	128/240	51/240	60/240	12/240	41/240
Michoacán	117/240	60/240	70/240	6/240	51/240
Morelos	80/240	36/240	35/240	2/240	15/240
Oaxaca	136/240	65/240	56/240	6/240	47/240
Yucatán	47/240	15/240	18/240	0/240	16/240
Totales	777/1920	327/1920	348/1920	39/1920	247/1920

MA – mesophilic aerobic bacteria, LAB – lactic acid bacteria.

Table 2 CFU intervals in samples above 100 and 1000 CFU/g of honey per indicator group

State	Mesophilic aerobic bacteria (CFU/g)	Molds (CFU/g)	Yeasts (CFU/g)	LAB (CFU/g)	<i>C. perfringens</i> (CFU/g)
Chihuahua	1200–9400	160–920	230–920	–	110–890
Cd. México	1100–9900	150–950	270–970	170–810	32–940
Edo. México	1100–9900	110–990	120–990	26–83	42–950
Jalisco	1200–9900	120–990	120–980	12–99	18–930
Michoacán	1000–9800	110–1600	210–980	110–670	12–99
Morelos	1000–9900	150–8900	230–990	560–980	17–990
Oaxaca	1000–9900	100–7000	290–990	140–700	11–950
Yucatán	1100–9700	240–970	180–860	–	150–820

With respect to coliforms, samples contained less than 3 NMP/g. For the other bacterial groups not considered in the standard for honey, we found that 2% contained LAB (39/1920), and 12% (247/1920) showed the presence of *C. perfringens* with more than 100 CFU/g (Table 1). None of the samples from the different states exhibited more than 10 CFU/g of *S. aureus*. *Salmonella* spp. was not detected in any of the samples.

Tables 1 and 2 show the presence of *C. perfringens* in the samples from each state: 21.3% for Michoacán, 19.6% for Oaxaca, 17.1% for Jalisco, 15.4% for the State of Mexico, 9.2% for Mexico City, 7.5% of Chihuahua and 6.7% for Yucatán.

Discussion

Forty point five (40.5%) percent from the 1920 analyzed honey samples exceeded the limits permitted by the regulation (NMX-036-NORMEX-200619), with bacterial counts higher than 1000 CFU/g but lower than 10 000 for the aerobic mesophilic count (Table 2). Similar studies in Argentina⁹, Brazil³¹, Colombia³⁶, Costa Rica³⁸, Croatia¹², Portugal¹¹ and Romania³⁷ have determined that none of the samples analyzed exceeds 1000 CFU/g of aerobic mesophilic bacteria and that they are below 100 CFU/g of this indicator group as in Argentinian samples⁹. Moreover, 75% of Turkish samples showed counts ranging from 100 to 1000; however, the

remaining 25% were between 1000 and 10 000 CFU/g. Nevertheless, none of these studies mention whether they comply with the normative standards of the country, although the study from Turkey mentions that the analyzed honeys have good microbiological quality⁷. Snowden and Cliver³⁰ mention that honeys can have an acceptable quality with a bacterial count up to 10 000 CFU/g of aerobic mesophilic bacteria if they comply with the criteria for yeasts and are free of fecal contamination.

For the counts of yeasts and molds we observed that 18.1% and 17% of the samples showed more than 100 CFU/g respectively; in the studies conducted by Gomes et al.¹⁰; Iglesias et al.¹⁴; Gradvol et al.¹²; Vica et al.³⁷; Zamora and Arias³⁸, the counts for the group of molds were lower than 100 CFU/g, however, in Turkey, 5% of the samples showed counts higher than 1000 CFU/g and 10% higher than 100 CFU/g for molds whereas only 20% of the samples were above 100 CFU/g for molds³⁵. In Brazil, 50% of the samples analyzed showed counts above 100 CFU/g for yeasts and molds³¹.

Although honey has high osmolarity and low water activity, two factors that prevent the development of microorganisms, it may contain microorganisms that are found in several sources of contamination that are hard to control such as dust, air, soil and nectar. However, microbial contamination can also be caused by food handlers, equipment and crossed contamination. The latter can be controlled by standard sanitation and good manufacturing

practices during honey harvesting and processing, otherwise, the honey would have as a consequence high levels of vegetative cells. Among the molds and yeasts involved in contamination, some genera such as *Penicillium*, *Mucor*, *Saccharomyces*, *Schizosaccharomyces* and *Torula* have been reported, which are responsible for the fermentation of honey when it shows a high degree of humidity (>21%)³⁰.

All samples contained <3 CFU/g of coliforms; for *S. aureus* no sample was above 100 CFU/g, *Salmonella* spp. was not detected in the 25 g of all the analyzed samples. Several authors mention that all the honey samples analyzed have shown absence of *Salmonella* spp. on the 25 g analyzed of each sample and not more than 1 or 3 CFU/g of coliforms, and none of these authors analyzed the count for *S. aureus*^{10,12,14,30,31,35,37,38}. In 80 honey samples from Istanbul, positive samples for coliform counts, *Escherichia coli* and *S. aureus* were 16, 3.6 and 13.4% respectively, with a positive correlation of their presence among these microorganisms⁷.

Contamination by *S. aureus* is feasible due to an inadequate handling of honey. The authors mention that the presence of coliforms and *Salmonella* spp. can probably be attributed to fecal or environmental contamination⁷. Other authors mention that the presence of these bacteria can come from inappropriate premises, insects, material with a deficient process of de-contamination as well as extraction, packaging and storage^{9,15}.

In situ we observed that in all sampled sites the handling of honey complied with the hygienic conditions for its processing and consumption no matter what production region of the Mexican Republic did the sample come from.

With regard to *C. perfringens* counts, we observed that 12% of the contaminated samples with this sporulating bacteria contained more than 100 CFU/g of honey (Tables 1 and 2); the presence of this microorganism may be due to contamination by bees, nectar or external sources. There have been observations that the bee gut can contain up to 27% of gram-positive bacteria, some of which include several species of *Clostridium*, including *C. perfringens*.

It has been reported that spores of *C. perfringens* can survive and be maintained in a latent form for long periods of time at low temperatures when honey is inoculated with this microorganism^{3,16}, therefore, the samples containing *C. perfringens* may have been contaminated in the apiary as a primary source of contamination through bees containing a sporulating microorganism in their guts. In honeys from Argentina, a low incidence of sulphite-reducing clostridia was reported¹⁵.

Furthermore, if honey is used for therapeutic purposes to heal wounds, spores of *C. perfringens* can germinate and proliferate causing problems of gangrene in deep wounds, although it has been observed that there are differences in bacterial survival. It has been reported that the use of honey prevents the wound from getting infected and can heal without risk of infection for up to 6 days although other authors report that bacteria can remain viable for 2, 3 and even 5 weeks²³.

By the analysis and study of the therapeutic properties of honey, the medical significance of honey has been highlighted in the healing of wounds, ulcers, eye infections and burns^{13,18,33}; currently, it has been used by some doctors for

cancer care⁶. Its antioxidant and antimicrobial properties have also been considered^{1,10,29,34}.

It is clear that the honey to be used for this latter purpose should be picked from areas where no sources of contamination exist⁸. Moreover, it is recommended that when honey is used, a previous microbial analysis should be carried out to avoid the contamination of wounds. It is also necessary to continue carrying out studies to establish factors affecting the standardization of honey, including source (monofloral or multifloral), type of honey (processed or raw), origin (natural or commercial honey), type and size of wounds to treat or the intended treatment^{2,13}.

With regard to lactic acid bacteria, only 15.79% of the samples contained more than 100 CFU/g (Tables 1 and 2). These kind of bacteria are considered to be safe (GRAS) and can play an important role in the preservation of the product since many of them have the ability to produce antimicrobial agents such as organic acid and bacteriocins that can inhibit or destroy pathogenic gram-negative and gram-positive bacteria, although these characteristics may vary depending on the type of LAB. For the reasons already exposed, it may be an extra benefit to the properties of honey if it were to contain these acid lactic bacteria¹³.

The state that showed the highest percentage of samples with a high microbial count for aerobic mesophilic and molds was Oaxaca, and the one with less count was Yucatán. Samples from Michoacán were the ones with the highest count for *C. perfringens*. It is important to point out that Yucatán exports most of the honey in our country; according to these results, samples from this state are the ones with the best sanitary quality.

It is important to mention that over 40% of honey samples did not comply with the specification related to the presence of aerobic mesophilic bacteria that implies an emphasis on the handling and sources of contamination by local producers so that honeys can fit the standard. With regard to other microorganisms, we can conclude that the percentage of samples with more than 100 CFU/g is less than 20% throughout the country, which can be the result of good sanitary handling of this food. It is important to avoid the sources of contamination and to follow good hygienic practices to maintain or improve the quality of the Mexican honey since a great percentage is exported. If honey is going to be used as a therapeutic agent, it should fulfill quality parameters and specific treatments to remove pathogens that can be threatening to the patients' health should be implemented.

Ethical responsibilities

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 they have followed the protocols of their work center on the publication of patient data.

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

Conflict of interest

There is no conflict of interest between the authors

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