









## Microbiological quality of honey from stingless bee, jandaíra (*Melipona subnitida*), from the semiarid region of Brazil

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**ABSTRACT:** The aim of this study was to evaluate the presence of microorganisms in honey produced by the stingless bee (SB) jandaíra (*Melipona subnitida*) from the semiarid region of Brazil. Thirty-five samples were analyzed and all of them were positive for mesophilic bacteria, coliforms at 45°C, fungi, and yeast. *Staphylococcus* spp. were identified in 85.7%, while *Bacillus* spp. were detected in 34.3% of honey samples. DNAs of *Clostridium perfringens* and *C. botulinum* were detected in 40% and 2.8% of the samples, respectively. *Salmonella* spp. and *C. difficile* were not detected. The present research revealed a great diversity of microorganisms in honey produced by jandaíra.

**Key words:** *Clostridium botulinum*; *Clostridium perfringens*; stingless bee.

### Qualidade microbiológica do mel da abelha sem ferrão jandaíra (*Melipona subnitida*) da região semi-árida do Brasil

**RESUMO:** O objetivo deste trabalho foi avaliar a presença de microrganismos no mel produzido pela abelha sem ferrão jandaíra (*Melipona subnitida*). Trinta e cinco amostras foram avaliadas e todas foram positivas para bactérias mesofílicas, coliformes a 45 °C, fungos e leveduras. *Staphylococcus* spp. foi identificado em 85,7% enquanto *Bacillus* foi detectado em 34,3% das amostras de mel. *Clostridium perfringens* e *C. botulinum* foram detectados em 40% e 2,8% das amostras respectivamente. *Salmonella* spp. e *C. difficile* não foram detectados. O presente trabalho revelou uma grande diversidade de microrganismos no mel produzido por jandaíra.

**Palavras-chave:** *Clostridium botulinum*; *Clostridium perfringens*; abelhas sem ferrão.

Jandaíra (*Melipona subnitida*) is a stingless bee (SB) very common in northeastern Brazil that has been increasingly used for honey production (MAIA et al., 2015). Recently, the *Polo de Mel Jandaíra* (Honey Pole Jandaíra) was implemented by the *Ministério da Integração Nacional* (Ministry of National Integration) to organize and encourage the production chain of honey from jandaíra (BRASIL, 2014). The growing demand for this honey in Brazil has encouraged studies on it and it has been already known that honey from *M. subnitida* has some remarkable physicochemical and palynological characteristics (ALMEIDA-MURADIAN et al., 2013). Despite this, reports about the microbiological quality of honey from SB are rare and there is no specific legislation that deals with it, which hinders the characterization and determination of food safety for this product

(MONTE et al., 2013). In this study, we evaluated the microbiological quality of the honey produced by SB jandaíra in the semiarid region of Brazil.

Thirty-five beekeeping farms from 12 cities of the semiarid region of Brazil were visited, with all located in a radius of 177km from the geographical location 4°56'35.9"S 37°18'31.5"W. In each farm, a 500-mL sample was collected directly from the honeycomb with a sterile syringe, packaged in transparent vials of sterile plastics, and kept protected from light with an average temperature of 25°C until microbiological analysis. Detection of mesophilic bacteria, coliforms at 45°C, *Salmonella* spp., *Staphylococcus* spp., fungi, and yeast were performed according to the Brazilian legislation (BRASIL, 2003). For detection of *Clostridium perfringens*, *C. difficile*, and *C. botulinum* DNA, each sample was inoculated into Cooked Meat Medium

(CMM, Difco, USA) broth and Reinforced Clostridial Medium (RCM, Difco, USA) Broth. After incubation at 37°C in an anaerobic atmosphere for 48 hours, a 100- $\mu$ L aliquot of the broth culture was subjected to thermal DNA extraction and DNA amplification via PCR to identify *C. difficile* (SILVA et al., 2011), *C. perfringens* (VIEIRA et al., 2008), and *C. botulinum* (PRÉVOT et al., 2007). In addition, 10 $\mu$ L of the broth was inoculated on Muller-Hinton agar (Difco, USA), which was supplemented with 5% of sheep blood, and incubated at 37°C in an anaerobic atmosphere for 48 hours. Thus, colonies not confirmed as clostridia in previous PCR analyses were subjected to further PCR assessment of 16S rRNA using universal primers (FOX et al., 1995). The amplicons were sequenced and the species were confirmed using BLAST with a similarity of 98% (MIAN et al., 2009).

Results of the present research are summarized in Table 1. Brazilian legislation categorizes the honey from SB as a product made by SB or native bees, but no official requirement related to the microbiological quality of the honey from SB is available (BRASIL, 2004; BRASIL, 2017). Beyond that, the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) requires the presentation of scientific literature to ensure the quality and innocuous of the honey from SB (CAMARGO et al., 2017). Considering this, the results of this research will be compared to the microbiological data published in literature for the honey from SB.

All samples were positive for mesophilic bacteria. Most of them (74.3%) showed counts lower than 25 colony-forming units per gram (CFU/g), 11.4% showed between 25 and 2500CFU/g, and the remaining 14.3% showed more than 2500CFU/g. The presence of mesophilic bacteria is related to the deterioration of food. However, some characteristics of honey, such as pH acidity, low water activity, low protein content, and high sugar, can diminish or stop bacterial activity, contributing to a longer shelf life of the product (EUROPEAN COMMISSION, 2002; MONTE et al., 2013). However, some samples showed a high count of mesophilic bacteria, which some authors attributed to factors associated with the location of the beekeeping farms that can result in contamination. These sources of contamination include soil, water, air, pollen, and nectar (BÁRBARA et al., 2015; OLIVEIRA et al., 2017).

Regarding coliforms at 45°C, all samples showed results lower than 3.0 most probable number per gram (MPN/g), confirming results of previous studies with different SB honeys, including that from jandaíra bees (SOUZA et al., 2009; MONTE et

al., 2013). According to SILVA et al. (2008), values under 3.0MPN/g indicated high quality of the honey. However, in a proposal of regulation for the honey from SB, CAMARGO et al. (2017) suggested a three-class plan for coliforms at 45°C, with acceptable samples between 0 and 10MPN/g and intermediate samples until 100MPN/g. These values are based on the habit of SB to use a fine mix of propolis and clay to seal the honeycombs (NOGUEIRA-NETO, 1997). Some species even land on feces from vertebrates, which can increase the number of coliforms (SANTOS & ANTONINI, 2008).

All samples tested were negative for *Salmonella* spp., in agreement with other studies evaluating the honey from SB (MONTE et al., 2013; PUCCIARELLI et al., 2014). Some studies have shown the antibacterial effect of honey, and the honey from SB in particular, against a wide range of bacteria, including *Salmonella* (PIMENTEL et al., 2013; NISHIO et al., 2016). As *Salmonella* is a relevant zoonotic pathogen, CAMARGO et al. (2017) suggested it should be absent in 25g of the honey from SB.

Only one sample (2.8%) showed the presence of *Staphylococcus* spp. higher than 20CFU/g, while 97.3% of the samples had 0 to 20CFU/g. The low number of staphylococci can be explained by the aseptic collection of honey, since the presence of *Staphylococcus* spp. in the honey from SB is rare. Equipment and physical honey handling are considered the main sources of contamination by this microorganism (DÜMEN et al., 2013; PUCCIARELLI et al., 2014). In addition, the antimicrobial activities of honey against gram-positive bacteria, specifically *Staphylococcus* spp., were also evaluated. Some studies attributed this effect to hydrogen peroxide, methyl-glyoxal (MGO), hydroxymethylfurfural (HMF), flavonoids, and bee-defensins (KWAKMAN et al., 2010; MERCÊS et al., 2013; PIMENTEL et al., 2013; NISHIO et al., 2016).

The average count of fungi and yeast reported in the samples of this study was  $9.12 \times 10^3$ CFU/g ( $3.96 \log_{10}$ CFU/g). In Brazil, some authors working with the honey from SB considered the maximum threshold to be 100CFU/g ( $2.00 \log_{10}$ CFU/g) based on national requirements for honey of *Apis mellifera* (BRASIL, 2000; SOUZA et al., 2009; MONTE et al., 2013). Despite this, CAMARGO et al. (2017) suggested a limit of  $1.0 \times 10^4$ CFU/g ( $4.00 \log_{10}$ CFU/g) for the honey from SB, as the elevated humidity of honey associated with the rich fungi microbiota of SB result in a product with higher counts, as previously described (FERRAZ et al., 2008; MONTE et al., 2013). To date,

Table 1 - Microorganisms identified in 35 samples of stingless honey from *Melipona subnitida* (jandaira bees) collected from the semiarid region of Brazil. MB – Mesophilic bacteria; CFU – Colony-forming units; Col. – Coliforms; MPN – Most probable number; FY – fungi and yeast; CPA – *Clostridium perfringens* type A; CBC – *Clostridium botulinum* type C; BC – *Bacillus cereus*; BL – *Bacillus licheniformis*; BC – *Bacillus cereus*.

Sample	MB (CFU/g)	<i>Staphylococcus</i> spp. (CFU/g)	FY (CFU/g)	<i>Bacillus</i> spp.	CPA	CBC
1	<25	<20	<15	BC	+	-
2	<25	<20	$5.4 \times 10^2$	-	-	-
3	<25	<20	$7.7 \times 10^2$	-	-	-
4	$2.5 \times 10^2$	<20	$1.0 \times 10^3$	-	-	-
5	$9.7 \times 10^2$	<20	$1.6 \times 10^3$	-	-	-
6	<25	<20	$7.1 \times 10^3$	-	-	-
7	<25	<20	$7.6 \times 10^3$	-	-	-
8	<25	<20	<15	-	-	-
9	<25	<20	<15	-	-	-
10	$>2.5 \times 10^3$	<20	<15	BL	+	-
11	$>2.5 \times 10^3$	<20	<15	-	-	-
12	$8.8 \times 10^2$	<20	$1.6 \times 10^3$	BS	+	-
13	$>2.5 \times 10^3$	<20	<15	-	-	-
14	$>2.5 \times 10^3$	<20	<15	-	-	-
15	<25	<20	<15	BC	+	-
16	$>2.5 \times 10^3$	<20	<15	-	-	-
17	<25	<20	<15	-	-	-
18	<25	<20	<15	BC	+	-
19	<25	<20	<15	BC	+	-
20	<25	<20	<15	BC	+	-
21	<25	<20	$9.3 \times 10^4$	BS	+	-
22	<25	<20	<15	-	-	-
23	$3.1 \times 10^2$	<20	$1.3 \times 10^4$	-	-	-
24	<25	$2.6 \times 10^3$	$2.5 \times 10^2$	-	-	-
25	<25	<20	$5.0 \times 10^2$	BC	+	-
26	<25	0	$2.1 \times 10^3$	-	-	-
27	<25	0	$1.0 \times 10^4$	-	-	-
28	<25	<20	$3.1 \times 10^3$	-	-	-
29	<25	<20	$1.4 \times 10^4$	BL	-	-
30	<25	<20	$1.2 \times 10^4$	-	-	-
31	<25	<20	$1.7 \times 10^4$	-	-	+
32	<25	0	$8.8 \times 10^4$	-	-	-
33	<25	<20	$5.7 \times 10^4$	-	-	-
34	<25	0	$2.2 \times 10^2$	BL	-	-
35	<25	0	$2.0 \times 10^2$	BL	-	-

\*All samples were negative for *Salmonella* spp. and *Clostridium difficile*. and showed lower than 3.0 most probable number per gram (MPN/g) of coliforms at 45°C.

there is no description of diseases caused by the fungi and yeast from the honey from SB. Thus, the main problem caused by their presence is the fermentation of honey, which can reduce the shelf life of the product (GRABOWSKY & KLEIN, 2017).

*Bacillus* species were reported in 34.3% of samples, including *B. cereus* (17.1%), *B. licheniformis* (11.4%), and *B. subtilis* (5.7%). *Bacillus* are common in SB's honey once these microorganisms have a symbiotic relationship to the bees and a strong antibacterial activity against several pathogens, contributing to the stability of the product (GILLIAM, 1997; TORRES et al., 2015). Although,

toxigenic strains of *B. cereus* in honey have been reported, there is no evidence of honey as a vehicle for this pathogen to enter humans (LÓPEZ & ALIPPI, 2010; SILVA et al., 2017).

*C. botulinum* type C and *C. perfringens* type A were detected through PCR in 2.8% and 40.0% of samples, respectively. PUCCIARELLI et al. (2014) reported that 64% of the samples tested positive for *Clostridium* spp. in the honey from SB. Studies investigating the presence of *C. botulinum* in honey from *A. mellifera* bees are very common but, to the best of our knowledge, this is the first study to confirm the presence of *C. botulinum* and



*C. perfringens* in samples of the honey from SB (NAKANO et al., 1990; NEVAS et al., 2005).

The presence of *C. perfringens* in the honey from SB is relevant once this product is being used for therapeutic purposes to heal wounds as this pathogen is commonly associated with gas gangrene in deep wounds (OLAITAN et al., 2007; MERCÊS et al., 2013). In addition, the detection of *C. botulinum* is of great significance in the present study even though the number of positive samples was lower than those in honey from *A. mellifera* bees (NAKANO et al., 1990; RAGAZANI et al., 2008). *C. botulinum* is responsible for infant botulism, which is commonly associated with honey consumption (CAGAN et al., 2010; DABRITZ et al., 2014). Interestingly, most cases of botulism are caused by *C. botulinum* type A and B. However, one case of infant botulism was caused by *C. botulinum* type C, the same type detected in the present study that had already been described previously (OGUMA et al., 1990; BROWN & DESAI, 2013). Thus, the detection of *C. botulinum* type C suggested that honey produced by jandaíra bees might pose a risk to infants under one year of age and consumers should be clearly warned about this risk on the product label (EUROPEAN COMMISSION, 2002; BRASIL, 2017).

The present study revealed a broader microbiological profile of the honey from SB compared to that of the honey from *A. mellifera*, which draws attention to the need for a specific legislation for this product and the adoption of measures to reduce the number of contaminants (SILVA et al., 2008). Good beekeeping practices associated with conservation techniques are important in guaranteeing a product of high quality. However, more studies are necessary to evaluate the effects of such practices on the physical and chemical characteristics of the honey from SB, specifically jandaíra honey (SOUZA et al., 2009; MOURA et al., 2014).

## ACKNOWLEDGMENTS

This research was supported by funds from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Pró-Reitoria de Pesquisa Universidade Federal de Minas Gerais (PRPq-UFMG).

## DECLARATION OF CONFLICTING OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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