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

Carolina de Gouveia Mendes da Escóssia Pinheiro1 Maria Rociene Abrantes1
Rodrigo Otávio Silveira Silva2 Carlos Augusto Oliveira Júnior2
Francisco Carlos Faria Lobato1 Jean Berg Alves da Silva1

1Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, Brasil.
2Escola de Veterinária. Universidade Federal de Minas Gerais (UFMG), 31.270-901, Belo Horizonte, MG, Brasil. E-mail: rodrigo.otaviosilva@gmail.com.

*Corresponding author.

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.

Quality 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 ºC, 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.
(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-µL 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, 10µL 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; MERCÉS 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 9.12×10^3CFU/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 (2.00log_{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 1.0×10^3CFU/g (4.00log_{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 (jandaíra 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; BS – Bacillus subtilis.

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

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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; MERCÉS 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., 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).

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DECLARATION OF CONFLICTING 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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