

Physicochemical composition and antimicrobial potential of stingless honey: a food of differentiated quality

Composição físico-química e potencial antimicrobiano do mel de abelhas sem ferrão: um alimento de qualidade diferenciada

Composición físico-química y potencial antimicrobiano de la miel sin aguijón: un alimento de calidad diferenciada

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Abstract

This study aimed to assess the antimicrobial activity of various honeys against strains of gram-negative and -positive bacteria, as well as to determine the physicochemical parameters of these honeys. Seven honeys from various species of stingless bees were evaluated. The

physical-chemical parameters evaluated were pH, moisture, water activity, acidity, ash, electrical conductivity and color. Antimicrobial activity was determined using disc diffusion agar tests and minimum inhibitory concentrations. We found that there was a relationship between the physical-chemical parameters and the antimicrobial activity. The minimum inhibitory concentration of 25% honey was able to inhibit the growth of both gram-positive and -negative bacteria; the greatest efficacy was verified for the species of bees *Melipona mondury*, *M. quadrifasciata*, *Scaptotrigona bipunctata* and *Tetragona clavipes*. Regarding synergism, *Escherichia coli* maintained its sensitivity profile in relation to all studied honeys combined with antimicrobials. An important factor to consider is the concentration of honey capable of sensitizing the microorganism, as it has been shown to be dependent on the species of the stingless bee. Nevertheless, all honeys showed antimicrobial activity in various methods of analysis. These data suggest that honey is a promising alternative to sensitize resistant microorganisms, for the health of humans and animals alike.

Keywords: Antibiogram; Quality; Stingless bees; Resistance; Antibiotics; Foods.

Resumo

Este estudo teve como objetivo avaliar a atividade antimicrobiana de mel de abelhas frente a cepas de bactérias gram-negativas e positivas, bem como determinar os parâmetros físico-químicos desse mel. Foram avaliadas sete amostras mel de espécies de abelhas sem ferrão. Os parâmetros físico-químicos avaliados foram pH, umidade, atividade de água, acidez, cinzas, condutividade elétrica e cor. A atividade antimicrobiana foi determinada por meio de testes de ágar de difusão em disco e concentrações inibitórias mínimas. Verificamos que existe uma relação entre os parâmetros físico-químicos e a atividade antimicrobiana. A concentração inibitória mínima de 25% de mel foi capaz de inibir o crescimento de bactérias gram-positivas e-negativas; a maior eficácia foi verificada para as espécies de abelhas *Melipona mondury*, *M. quadrifasciata*, *Scaptotrigona bipunctata* e *Tetragona clavipes*. Em relação ao sinergismo, *Escherichia coli* manteve seu perfil de sensibilidade em relação a todos os méis estudados associados a antimicrobianos. Um fator importante a se considerar é a concentração de mel capaz de sensibilizar o microrganismo, pois tem se mostrado dependente da espécie de abelha sem ferrão. No entanto, todas as amostras de mel apresentaram atividade antimicrobiana em diferentes métodos de análise. Esses dados sugerem que o mel é uma alternativa promissora para sensibilizar microrganismos resistentes, para a saúde de humanos e animais.

Palavras-chave: Antibiograma; Qualidade; Abelhas sem ferrão; Resistência; Antibióticos; Alimentos.

Resumen

Este estudio tuvo como objetivo evaluar la actividad antimicrobiana de diversas mieles frente a cepas de bacterias gram negativas y positivas, así como determinar los parámetros físico-químicos de estas mieles. Se evaluaron siete mieles de varias especies de abejas sin aguijón. Los parámetros físico-químicos evaluados fueron pH, humedad, actividad del agua, acidez, ceniza, conductividad eléctrica y color. La actividad antimicrobiana se determinó mediante pruebas de agar de difusión en disco y concentraciones inhibitorias mínimas. Encontramos que existe una relación entre los parámetros físico-químicos y la actividad antimicrobiana. La concentración mínima inhibitoria del 25% de miel fue capaz de inhibir el crecimiento de bacterias tanto grampositivas como negativas; la mayor eficacia se verificó para las especies de abejas *Melipona mondury*, *M. quadrifasciata*, *Scaptotrigona bipunctata* y *Tetragona clavipes*. En cuanto al sinergismo, *Escherichia coli* mantuvo su perfil de sensibilidad en relación a todas las mieles estudiadas combinadas con antimicrobianos. Un factor importante a considerar es la concentración de miel capaz de sensibilizar al microorganismo, ya que se ha demostrado que depende de la especie de abeja sin aguijón. Sin embargo, todas las mieles mostraron actividad antimicrobiana en varios métodos de análisis. Estos datos sugieren que la miel es una alternativa prometedora para sensibilizar a los microorganismos resistentes, tanto para la salud de los seres humanos como de los animales.

Palabras clave: Antibiograma; Calidad; Abejas sin aguijón; Resistencia; Antibióticos; Alimentos.

1. Introduction

Stingless bees are social insects, belong to the order Hymenoptera and the family Apidae (Meliponini), as well as species that are closely linked to tropical and subtropical regions for honey production (Crane 1990; Michener et al. 2013; Chuttong et al. 2016). Honey is a natural product recognized in traditional world medicine for having medicinal properties, including antimicrobial, antifungal, antioxidant, antiviral, antiparasitic, anti-inflammatory, anticancer, immunosuppressive, and healing activities (Irish et al. 2006; Bogdanov et al. 2008; Alvarez-Suares et al. 2010; Mcloone et al. 2016).

Its antimicrobial potential and the physicochemical profiles of honey depend on their geographical location, the climatic conditions, and the variation of the nutritional source of the hive during the seasons (Mavric et al. 2008; Sherlock et al. 2010; Kwakman et al. 2011; Hussain et al. 2015; Laallam et al. 2015; Nishio et al. 2016; Ekhtelat et al. 2016; Wasfi et al.

2016). In addition, the processing, handling and storage of honey can influence its composition (Sherlock et al. 2010; Alvarez-Suarez et al. 2010; Nishio et al. 2016). The honey of stingless bees is stored in jars made of wax and propolis. Some of the phytochemical constituents of propolis can diffuse into the honey during storage, giving it antimicrobial properties (Temaru et al. 2007; Ballivián 2008; Suntiparapop et al. 2012).

In Brazil, it is very common to use herbal medicines and homemade syrups with the use of honey in popular therapies, mainly by indigenous people and rural areas, because of the belief that this type of honey has healing properties (Posey 1987; Cortopassi-Laurino and Gelli 1991; Madaleno, 2015). Currently, due to the widespread concern about the growing emergence of resistant pathogens, honey has been promoted as an alternative for sensitizing microorganism's resistant to antibiotics and for being a natural product (Boorn et al. 2010; Pimentel et al., 2013; Campeau and Patel, 2014). Nevertheless, data on the antimicrobial activity of Brazilian honeys remains limited (Nishio et al. 2014; Bueno-Costa et al. 2016). Therefore, the present study aims to evaluate the antimicrobial activity of various stingless honeys against strains of gram-negative and positive bacteria, as well as to determine the physicochemical parameters of these honeys and their relationship with antibacterial action.

2. Methodology

2.1 Samples and material

The stingless honey samples (SHS) used in this study were collected in various cities in the state of Santa Catarina, totaling 20 honey samples from seven bee species (Table 1). The samples were collected and stored under refrigeration and were protected from light in sterile plastic bottles with airtight closures.

Table 1. Honey samples from meliponines, highlighting the species and municipality of origin in the state of Santa Catarina (Brazil).

| Stingless bee species | Popular name | Origin/Sample* |
|---|--------------|---|
| <i>Melipona rufiventris mondory</i> LEPELETIER, 1836 | Bugia | Urubici (14) |
| <i>Melipona bicolor</i> LEPELETIER, 1836 | Guaraipo | Chapecó (6, 7), Maravilha (16), Urubici (13) |
| <i>Tetragonisca angustula</i> LATREILLE, 1811 | Jataí | Chapecó (9, 19), Xanxerê (1, 3), Maravilha (15), Seara (20), |
| <i>Melipona quadrifasciata</i> LEPELETIER, 1836 | Mandaçaia | Chapecó (8, 10, 18), Xanxerê (2), Vidal ramos (17), Urubici (11) |
| <i>Melipona marginata</i> LEPELETIER, 1836 | Manduri | Chapecó (5) |
| <i>Scaptotrigona bipunctata</i> LEPELETIER, 1836 | Tubuna | Vidal ramos (12) |
| <i>Tetragona clavipes</i> FABRICIUS, 1804 | Borá | Chapecó (4) |

* Sample, corresponding sample number for MIC.
 Source: Elaborated by the authors.

The analyses of the antimicrobial activity were performed against strains of bacteria from the LABMIM library. The gram-negative bacteria isolates used in the tests were *Salmonella* Heidelberg, *S. Enteritidis*, *S. Typhimurim* (ATCC-14028) and *Escherichia coli* (ATCC 25922); the gram-positive bacterium was *Staphylococcus aureus* (ATCC 25923). The antimicrobial discs used in the in vitro tests as positive controls were: amoxicillin/clavulanic acid (AMC 0 µg + 10 µg), ceftazidime (CAZ 30 µg), gentamicin (GEN 10 µg), tetracycline (TET 30 µg) and ciprofloxacin (CIP 5 µg).

2.2. Physicochemical analyses of honey

The moisture content of the samples was determined by the refractometer (Atago Co, 1988), with values expressed in ° Brix, from which the moisture value (%) was calculated (Alves et al., 2005). The water activity (A_w) was determined according to the methodology

proposed by Kuroish et al. (2012) using the AquaLab Series 3 TE model, which uses the dew point determination technique in an encapsulated mirror (DECAGON, 2003). To determine the ash content, 5 grams of honey were weighed in a previously calcined crucible, and a muffle oven was heated to 550 °C for three hours (IAL, 2008). The pH, acidity and electrical conductivity of the samples were analyzed according to the recommendation Bogdanov et al. (1997). Color determination was performed with the aid of a spectrophotometer (Metrolab 1700 uvvis, JP) by the measurement of absorbance in the visible region at 635 nm in diluted solution of honey and distilled water (50:50) (m / v) being used the glycerin as white (Bogdanov et al., 1997). The determined values were compared to those in the Pfund table, according to the methodology described by Bianchi (1981).

2.3. Antibacterial activity

To determine the antimicrobial activity of antibiotic discs, the diffusion method described by CLSI (2015) and Brasil (2003) was used. To verify the synergism between the antibiotic discs and the different types of stingless bees honeys, the agar dilution technique described by CLSI (2003) was modified, using the different honeys in the dilution of the agar in the plate to evaluate the diffusibility and its antimicrobial activity. The technique used with honey for well agar diffusion was performed according to Perez et al. (1990). The minimum inhibitory concentration was determined according to the methodology of CLSI (2012).

2.4. Inoculum preparation

To assess the antimicrobial activity of the different techniques employed, three to five bacterial colonies isolated from each of the samples were selected from three to five bacterial colonies isolated from each of the samples, touching the top of each colony with a platinum handle and transferring them to tubes containing Brain Heart Infusion Broth (BHI). To evaluate the minimum inhibitory concentration (MIC), the same procedure was used, however the broth used was Muller–Hinton. The samples were incubated at 36 °C for 18 hours. A turbidity adjustment was performed to obtain an optical turbidity comparable to that of the 0.5 McFarland standard solution resulting in a suspension containing approximately 1×10^8 CFU/mL of *E. coli* (ATCC® 25922). For visual adjustment, a white background card with contrasting black lines was used.

2.5. Agar disc diffusion

Using a sterile swab moistened in the standardized bacterial suspension, the sample was wiped gently in all directions on petri dishes containing Muller–Hinton agar. The placement of antimicrobial discs in the petri dishes was carried out using forceps that had been flame-sterilized and then cooled. The plates were incubated in a bacteriological oven at 37 °C for 24 hours. Antimicrobial activity was measured by the diameter of the halos in millimeters, from the zone of inhibition formed around the discs according to CLSI (2015).

2.6. Agar dilution and synergism

The agar dilution technique described by CLSI (2003) was modified, each honey was diluted with agar and poured in a petri plate to assess diffusibility and synergism for the effect of antimicrobial activity. Muller–Hinton agar was autoclaved and cooled in a 45–50 °C water bath. The volume of 5% honey of each species of bee was added in relation to the total volume, obtaining a concentration of 124.54 mg/ml of honey. The mixtures were placed in Petri dishes until a 3–4 mm thick layer was obtained. Importantly, the plates were produced on the day of the test. The procedure for spreading the inoculum, placing the antimicrobial discs, and for reading and classifying the bacterial isolates was carried out in a manner similar to that described for disc diffusion (CLSI, 2003).

2.7. Agar diffusion using the well technique

The culture medium used was Muller–Hinton agar; microorganisms were inoculated on the surface using a swab. Then, the wells were drilled using a sterile mold with a diameter of 6 mm. The honeys were tested in separate plates, dispensing the volume of 100 µL/well in the concentrations of 417.3 mg/mL (25%), 550.84 mg/mL (37.5%), 924, 61 mg/mL (50%), 1312.84 mg/ml (75%) (w/v) and 1525.09 mg/ml (100%). As a control (without adding honey), one well per plate was filled with 100 µL of autoclaved distilled water. The plates were incubated at 37 °C for 24 hours, for later reading of the halos (Perez et al., 1990).

2.8. Minimal inhibitory concentration

The susceptibility to various stingless bee honeys was determined using the microdilution method in MH broth with a 96-well sterile plates, according to the CLSI

standards (2012). The honeys were tested at concentrations of 25%, 37.5%, 50%, 75% (w/v) and pure honey in total volumes per well of 200 μ L (MH broth and honey). The added volume of each standardized inoculum was 10 μ L for each well. The plates were incubated at 37 °C for 24 hours. Subsequently, 20 μ L of 2,3,5 - 1% triphenyltetrazolium chloride was added per well. If the color red appeared after 3 hours of incubation, it was considered to indicate microbial growth. The minimum inhibitory concentration was defined as the lowest concentration of honey with no visible growth after incubation (CLSI, 201; Mercês et al., 2013).

2.9. Statistical analysis

The triplicate averages of the data of the physical-chemical analyzes were subjected to the Kolmogorov–Smirnov standard testing, indicating a normal distribution. The studied variables were grouped by bee species and compared using analysis of variance; if there was a significant difference ($P < 0.05$), the Tukey test at 5% significance was used. For the analyses of honey diffusibility x antimicrobial activity, the averages of the inhibition halos were grouped by species and presented with their respective standard deviations. All statistical analyses were performed using the OriginPro 8 software (Northampton, Massachusetts, USA).

3. Results

3.1. Physico-chemical characteristics of honey

The pH of the honey samples ranged from 3.37 to 3.93, with no significant difference between the species *Tetragonisca angustula* and *Tetragona clavipes* (Table 2). There was a difference ($P < 0.05$) of these two species in relation to *Melipona* spp. and *Scaptotrigona bipunctata*. *Melipona marginata* did not differ between the species evaluated.

Table 2. Results of the physicochemical parameters of the different stingless bee honeys.

| Species | pH | Moisture (%) | Water activity | Acidity | Ash (%) | Electrical conductivity | Cor |
|---------------------------------|-------------------------------|-------------------------------|-------------------------------|---------------------------------|-------------------------------|---------------------------------|-----------------------------------|
| <i>Melipona mondury</i> | 3,69 (±0,25) ^a | 29,6 (±0,10) ^a | 0,72 (±0,02) ^{ab} | 21,18 (±1,26) ^a | 0,38 (±0,32) ^a | 108,07 (±0,25) ^a | Light Amber ^a |
| <i>Melipona bicolor</i> | 3,38 (±0,20) ^a | 30,08 (±1,58) ^a | 0,74 (±0,04) ^a | 46,58 (±17,58) ^{ab} | 1,36 (±1,50) ^a | 198,56 (±96,85) ^a | Extra-light Amber ^a |
| <i>Melipona marginata</i> | 3,61 (±0,03) ^{ab} | 30,30 (±0,36) ^a | 0,72 (±0,0) ^a | 29,96 (±2,99) ^a | 3,98 (±2,18) ^{ab} | 190,03 (±0,06) ^a | Light Amber ^a |
| <i>Melipona quadrifasciata</i> | 3,37 (±0,21) ^a | 31,23 (±0,63) ^a | 0,74 (±0,02) ^a | 44,63 (±21,95) ^a | 1,42 (±1,21) ^a | 194,01 (±96,90) ^a | Light Amber ^a |
| <i>Scaptotrigona bipunctata</i> | 3,45 (±0,0) ^a | 29,77 (±0,06) ^a | 0,78 (±0,02) ^a | 91,57 (±2,65) ^{bc} | 3,60 (±1,49) ^{ab} | 274,33 (±0,58) ^a | Dark Amber ^b |
| <i>Tetragona clavipes</i> | 3,93 (±0,02) ^b | 30,37 (±0,55) ^a | 0,75 (±0,01) ^a | 112,87 (±19,17) ^c | 0,90 (±0,64) ^a | 557,00 (±1,00) ^b | Dark Amber ^c |
| <i>Tetragonisca angustula</i> | 3,89 (±0,28) ^b | 26,98 (±1,66) ^b | 0,67 (±0,05) ^b | 45,56 (±16,74) ^a | 4,88 (±1,60) ^b | 228,34 (±67,95) ^a | Amber ^b |

Note: Different letters in the same column show differences in honey for the different species of bees evaluated here ($p \leq 0.05$). Source: Authors.

The moisture content for all samples was greater than 20%. Only *T. angustula* honey differed from the other species, with the lowest moisture content (26.98%). The water activity (a_w) was highest for *T. angustula* in relation to the others, with the exception of *Melipona mondury*, which was similar. For some honey samples, the acid values were higher than 50 mEq Kg⁻¹, varying between 21.18 to 112.87 mEq.Kg⁻¹. *T. clavipes* honey did not differ from that of *Scaptotrigona bipunctata* honey with respect to water activity, which, in turn, did not differ from *Melipona bicolor* honey, which was similar to the other species. The ash contents varied between 0.38% to 4.88%; *T. angustula* honey differed from *M. mondury*, *M. bicolor*, *M. quadrifasciata* and *T. clavipes* honey. Only the honey of *T. clavipes* differed from the others with respect to electrical conductivity (557 $\mu\text{S}\cdot\text{cm}^{-1}$) and color (dark amber).

3.2. Antimicrobial activity

3.2.1. Agar disc diffusion

S. aureus was sensitive to all antimicrobials according to Table 3. Gram-negative bacteria were classified as 60% sensitive, 25% intermediate and 15% resistant to the antimicrobials used in our study.

Only the *S. Heidelberg* showed a resistance pattern against the antimicrobials amoxicillin/clavulanic acid (7.0 mm measured halo <13 mm reference halo for resistance), ceftazidime (11 mm measured halo <14 mm reference halo for resistance) and tetracycline (0,0 mm measured <14 mm halo reference for resistance).

3.2.2. Agar dilution and synergism

E. coli showed no change in the susceptibility profile, remaining sensitive against antimicrobials and when evaluating the synergism of stingless honey versus antimicrobials (Table 3). *S. aureus* was sensitive to all antimicrobials tested in isolation; however, there was a change in their susceptibility profile compared to the stingless honey versus antimicrobial test. When evaluated the honey of *M. quadrifasciata*, *S. bipunctata* and *M. mondory* versus ciprofloxacin, for *S. aureus* it changed its profile to intermediate.

Table 3. Results of the classification of the sensitivity of the strains according to CLSI (2005) and the evaluation of the synergistic effect of antimicrobial activity between antibiotics and the different stingless honeys against the strains of *E. coli* ATCC 259, *S. aureus* ATCC 25923, *S. Typhimurium* ATCC 14028, *S. Enteritidis* and *S. Heidelberg*, by measuring the diameter of the halos (mm).

| Antibiotic* | CLSI. 2005 | Halo of inhibition (mm) ** | | | | | | |
|-------------------------------|------------|----------------------------|-------------------------|-------------------------------|---------------------------|--------------------------------|---------------------------------|---------------------------|
| | | <i>Melipo mondory</i> | <i>Melipona bicolor</i> | <i>Tetragonisca angustula</i> | <i>Melipona marginata</i> | <i>Melipona quadrifasciata</i> | <i>Scaptotrigona bipunctata</i> | <i>Tetragona clavipes</i> |
| <i>Escherichia coli</i> | | | | | | | | |
| AMC (30µg) | 25 S | 22 S | 26.5 (± 5.7) S | 23 (± 2.1) S | 21 S | 21.83 (± 1.8) S | 20 S | 23 S |
| CAZ (30 µg) | 30 S | 28 S | 24.5 (± 1.0) S | 25.5 (± 0.8) S | 26 S | 24.83 (± 1.2) S | 25 S | 25 S |
| GEN (10 µg) | 23 S | 19 S | 19.5 (± 2.6) S | 20.5 (± 2.0) S | 20 S | 20.16 (± 1.6) S | 20 S | 18 S |
| TET (30 µg) | 25 S | 27 S | 30.75 (± 4.3) S | 26.5 (± 2.0) S | 30 S | 26.16 (± 1.3) S | 25 S | 30 S |
| CIP (5 µg) | 35 S | 30 S | 30.75 (± 1.5) S | 30.66 (± 1.4) S | 30 S | 30 (± 1.4) S | 28 S | 29 S |
| <i>Staphylococcus aureus</i> | | | | | | | | |
| AMC (30µg) | 21 S | 19 S | 19.75 (± 1.2) S | 19.42 (± 1.4) I | 20 S | 20.25 (± 4.2) S | 22 S | 23 S |
| CAZ (30 µg) | 21 S | 22 S | 19.87 (± 0.6) S | 20.75 (± 2.0) S | 21 S | 21.33 (± 2.7) S | 22 S | 22 S |
| GEN (10 µg) | 20 S | 20 S | 21.87 (± 1.1) S | 21.83 (± 1.9) S | 22 S | 21.58 (± 2.0) S | 23 S | 24 S |
| TET (30 µg) | 20 S | 16 I | 22.25 (± 1.2) S | 22 (± 1.3) S | 21 S | 23.10 (± 2.9) S | 25 S | 25 S |
| CIP (5 µg) | 25 S | 20 I | 15.62 (± 1.4) R | 15.42 (± 1.6) R | 13 R | 15.33 (± 0.8) I | 17 I | 15 R |
| <i>Salmonella Typhimurium</i> | | | | | | | | |
| AMC (30µg) | 17 I | 23 S | 23.25 (± 1.7) S | 23.83 (± 0.8) S | 23 S | 22.83 (± 1.6) S | 24 S | 24 S |
| CAZ (30 µg) | 15 I | 20 S | 21.25 (± 1.5) S | 21.16 (± 1.0) S | 19 S | 21.16 (± 1.6) S | 23 S | 19 S |
| GEN (10 µg) | 11 I | 16 S | 15.75 (± 1.0) S | 17.83 (± 1.2) S | 15 S | 16.33 (± 1.2) S | 17 S | 18 S |
| TET (30 µg) | 16 I | 26 S | 25 (± 0.0) S | 24 (± 1.1) S | 23 S | 24 (± 1.5) S | 25 S | 23 S |

| | | | | | | | | |
|-------------------------------|------|------|-----------------|-----------------|------|-----------------|------|------|
| CIP (5 µg) | 17 I | 19 I | 19.75 (± 0.5) I | 19.33 (± 2.4) I | 18 I | 18.16 (± 1.9) I | 20 S | 15 R |
| <i>Salmonella</i> Enteritidis | | | | | | | | |
| AMC (30µg) | 20 S | 22 S | 22.25 (± 1.0) S | 22.16 (± 2.2) S | 23 S | 23.33 (± 1.9) S | 23 S | 20 S |
| CAZ (30 µg) | 20 S | 20 S | 19.5 (± 1.0) S | 19.66 (± 0.8) S | 19 S | 21.33 (± 2.7) S | 20 S | 17 I |
| GEN (10 µg) | 20 S | 12 R | 17.25 (± 2.4) S | 15.83 (± 2.0) S | 18 S | 17 (± 1.3) S | 18 S | 15 S |
| TET (30 µg) | 20 S | 20 S | 21 (± 1.5) S | 21.66 (± 1.0) S | 22 S | 22.66 (± 2.0) S | 24 S | 20 S |
| CIP (5 µg) | 22 S | 20 S | 18.5 (± 1.9) I | 18 (± 2.3) I | 19 I | 17.83 (± 4.0) I | 20 I | 16 I |
| <i>Salmonella</i> Heidelberg | | | | | | | | |
| AMC (30µg) | 7 R | 7 R | 9.5 (± 0.6) R | 10.5 (± 1.8) R | 18 S | 11.83 (± 3.8) R | 6 R | 27 S |
| CAZ (30 µg) | 11 R | 18 S | 11.75 (± 0.5) R | 12 (± 0.9) R | 12 R | 12.5 (± 0.5) R | 16 I | 12 R |
| GEN (10 µg) | 19 S | 19 S | 17.75 (± 0.5) S | 18.16 (± 1.0) S | 13 I | 16 (± 1.9) S | 17 S | 17 S |
| TET (30 µg) | 0 R | 7 R | 9 (± 0.8) R | 9 (± 2.0) R | 8 R | 9.83 (± 1.9) R | 10 R | 10 R |
| CIP (5 µg) | 22 S | 29 S | 19 (± 2.0) I | 20.33 (± 1.9) I | 18 I | 19 (± 1.8) I | 29 S | 18 I |

*AMC - Amoxicillin/clavulanic acid (AMC 20 µg + 10 µg); CAZ - Ceftazidime (CAZ 30 µg); GEN - Gentamicin (GEN 10 µg); TET – Tetracycline (TET 30 µg); CIP - ciprofloxacin (CIP 5 µg). ** R – Resistant; I – Intermediate; S – Sensitive.

Source: Authors

Furthermore, when testing the honey of *M. bicolor*, *T. angustula*, *M. marginata* and *T. clavipes* versus ciprofloxacin, the profile changed to resistant. Finally, tetracycline versus *M. mondury* changed the profile of *S. aureus* from sensitive to intermediate.

The susceptibility profile of *S. Typhimurium* was classified as intermediate when only antibiotics were used. In general, the presence of honeys increased the inhibition halos, with the exception of the honeys of *M. mondury*, *M. bicolor*, *T. angustula*, *M. marginata*, *M. quadrifasciata* versus ciprofloxacin that remained intermediate; and for *T. clavipes* versus ciprofloxacin, which changed the profile to resistant.

S. Enteritidis was sensitive to all antimicrobials. In the presence of *T. clavipes* honey, it changed its profile to intermediate versus ceftazidime. The same was true for ciprofloxacin in the presence of all honeys. Gentamicin with honey from *M. mondury* made the strain resistant. *S. Heidelberg* was considered sensitive to the antimicrobials ciprofloxacin and gentamicin, and resistant to the other antimicrobials evaluated here. In the presence of *M. marginata* honey versus gentamicin, *S. Heidelberg* presented an intermediate profile and remained sensitive in the presence of the antimicrobial ciprofloxacin versus *S. bipunctata* and *M. mondury* honeys. The resistance profile of *S. heidelberg* changed to sensitive to the antibiotic amoxicillin/clavulanate in the presence of *M. marginata* and *T. clavipes* honeys. When tetracycline was tested, there was no change in the profile that remained resistant even when associated with honeys, and the halos increased their diameter.

3.2.3. Agar diffusion using the well technique

Using this technique, we observed that the strains of gram-positive and -negative bacteria showed different behaviors in relation to honey samples, ranging from highly sensitive to totally resistant (Table 4).

We observed that there was no halo formation in the plates with *S. aureus* in the presence of *T. clavipes* honey in all concentrations. For *S. Typhimurium*, a halo was observed when only the concentration of 100% honey of the species *T. clavipes* was tested, while the addition of *M. marginata* honey presented an inhibition halo in concentrations above 50%.

S. Typhimurium presented the largest halos in relation to all concentrations of honey of *M. mondury*. *S. Heidelberg* presented a halo only when *T. clavipes* honey was added pure (100%); however, a halo was formed at 25% honey for the species *M. mondury* and *S. bipunctata*. When *S. Enteritidis* was tested, the smallest halos were observed when *T. clavipes*

honey was used in comparison to the other types of honey evaluated here. Finally, honey from *T. clavipes* was the most efficient for *E. coli* compared to honey from other bee species.

3.2.4. Minimum inhibitory concentration

Table 5 shows the minimum concentration of growth inhibition of gram-positive and gram-negative strains. The highest prevalence among the concentrations was the minimum inhibitory concentration equal to 417.3 mg mL⁻¹.

For *E. coli* the results show that for the honeys of *M. bicolor* and *M. marginata* they required a higher concentration to inhibit bacterial growth (550.8 mg mL⁻¹). In relation to *S. aureus*, the honey of *T. angustula* showed the greatest variability among the tested samples, in relation to the minimum inhibition concentrations; *M. marginata* honey inhibited its growth of *S. aureus* from 550.8 mg mL⁻¹. The honeys of *T. angustula*, *M. marginata* and *M. bicolor* inhibited the growth of *S. Typhimurium* in the concentrations of honey 924.6 mg mL⁻¹ (50%), 550.8 mg mL⁻¹ (37.5%) and 550.8 mg mL⁻¹ (37.5%). *S. Enteritidis* showed less susceptibility to the honeys of *T. angustula*, *M. marginata* and *M. bicolor*; similarly, *S. Heidelberg* required high concentrations of honeys from *T. angustula*, *M. marginata* and *M. quadrifasciata*, to prove susceptible.

Table 4. Results of diffusion tests, using the well technique, by measuring the diameter of the inhibition halos (mm) of stingless honeys against strains of *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *S. Typhimurium* ATCC 14028, *S. Enteritidis* and *S. Heidelberg*.

| % of honey in the well | <i>Melipona mondory</i> | <i>Melipona bicolor</i> | <i>Melipona marginata</i> | <i>Melipona quadrifasciata</i> | <i>Scaptotrigona bipunctata</i> | <i>Tetragonisca angustula</i> | <i>Tetragona clavipes</i> |
|-------------------------------|-------------------------|-------------------------|---------------------------|--------------------------------|---------------------------------|-------------------------------|---------------------------|
| <i>Escherichia coli</i> | | | | | | | |
| 100 | 25 | 28.75 (± 2.5) | 30 | 28.7 (± 9.1) | 30 | 30 (± 0) | 25 |
| 75 | 25 | 26.25 (± 2.5) | 27 | 27 (± 7.4) | 30 | 29.8 (± 3.3) | 30 |
| 50 | 20 | 25 (± 4.1) | 25 | 27 (± 7.4) | 27 | 27.8 (± 4.2) | 30 |
| 37.5 | 20 | 22.5 (± 2.9) | 0 | 23.5 (± 6.4) | 27 | 25 (± 3.5) | 25 |
| 25 | 20 | 22.9 (± 2.9) | 0 | 20.8 (± 10.2) | 25 | 24 (± 2.2) | 25 |
| <i>Staphylococcus aureus</i> | | | | | | | |
| 100 | 29 | 35.6 (± 6.7) | 30 | 31 (± 6.0) | 29 | 27.2 (± 2.3) | 0 |
| 75 | 27.5 | 32.6 (± 3.3) | 33.5 | 26.4 (± 13.5) | 26.5 | 26.9 (± 1.4) | 0 |
| 50 | 27.5 | 21.6 (± 8.2) | 30 | 29.8 (± 4.7) | 25 | 25.6 (± 3.0) | 0 |
| 37.5 | 25 | 16.3 (± 6.0) | 25 | 17.3 (± 10.3) | 24 | 16.7 (± 13.2) | 0 |
| 25 | 22.5 | 12.3 (± 9.8) | 12.5 | 14.4 (± 11.9) | 24 | 10.3 (± 11.5) | 0 |
| <i>Salmonella Typhimurium</i> | | | | | | | |
| 100 | 47 | 38.8 (± 7.5) | 40 | 39 (± 7.6) | 40 | 33.8 (± 3.8) | 18 |
| 75 | 47 | 32.5 (± 2.9) | 40 | 28 (± 13.9) | 30 | 28.3 (± 14) | 0 |
| 50 | 35 | 30 (± 4.1) | 35 | 30 (± 0) | 20 | 28.2 (± 5.3) | 0 |
| 37.5 | 30 | 27.5 (± 2.9) | 0 | 27.2 (± 4.0) | 18 | 25.3 (± 5.3) | 0 |
| 25 | 25 | 16.3 (± 11.1) | 0 | 20.2 (± 9.8) | 17 | 15.5 (± 8.2) | 0 |
| <i>Salmonella Enteritidis</i> | | | | | | | |
| 100 | 30 | 27.75 (± 2.5) | 32 | 26 (± 1.3) | 30 | 25.6 (± 1.6) | 20 |
| 75 | 30 | 25.5 (± 3.3) | 30 | 25 (± 2.8) | 32 | 25.8 (± 3.7) | 15 |
| 50 | 17 | 23.5 (± 1.9) | 28 | 23.3 (± 2.1) | 24 | 22.8 (± 2.8) | 10 |
| 37.5 | 17 | 19.3 (± 3.9) | 23 | 21.5 (± 3.1) | 24 | 21.8 (± 3.4) | 7 |
| 25 | 15 | 18.3 (± 3.0) | 20 | 16.3 (± 3.3) | 23 | 17.2 (± 9.3) | 7 |
| <i>Salmonella Heidelberg</i> | | | | | | | |

Heidelberg

| | | | | | | | |
|------|----|-------------------|----|--------------------|----|--------------------|----|
| 100 | 27 | 24 (\pm 3.8) | 30 | 26.7 (\pm 6.0) | 30 | 25.7 (\pm 1.0) | 20 |
| 75 | 20 | 24.3 (\pm 2.9) | 30 | 26.7 (\pm 6.9) | 23 | 23.2 (\pm 1.6) | 0 |
| 50 | 25 | 20 (\pm 3.6) | 30 | 22.7 (\pm 4.1) | 20 | 21.7 (\pm 2.6) | 0 |
| 37.5 | 15 | 15.8 (\pm 1.5) | 30 | 13.3 (\pm 11.7) | 17 | 14.5 (\pm 11.4) | 0 |
| 25 | 0 | 3.8 (\pm 7.5) | 25 | 9.2 (\pm 10.7) | 0 | 9.2 (\pm 10.2) | 0 |

Source: Authors

4. Discussion

Stingless honey has the intrinsic characteristic of being more hygroscopic, even in environments with lower humidity (Nascimento et al. 2015). The moisture content of the samples was above 20%, a parameter considered ideal by Brazilian legislation (Brasil, 2000). Nevertheless, this difference did not negatively influence the growth inhibitory capacity of microorganisms. *M. quadrifasciata* honey had the highest moisture content found, corroborating results of the study by Alves et al. (2005), who found that this species, even in a dry climate, produces honey with high levels of moisture. Bogdakov and Blumer (2001) explain that, because water is essential for the oxidation process, hydrogen peroxide is produced significantly even in immature honeys that have high water content. In cases where moisture content is low in the mature honey, the oxidation process is limited by glucose oxidase being practically inactive. Honeys with low moisture content will have a small amount of hydrogen peroxide, which is known to prevent microbial growth (Laallam et al., 2015).

Peralta (2010) found pH values for honey melon nectar between 3.2 and 3.9; Nascimento et al. (2015), obtained pH values between 2.93 to 4.08, values very close to those presented in this work. Campos et al. (2010) commented that pH is a parameter that assists in the assessment of total acidity and its variation may be related to the nutritional composition of the bee's diet, depending on the pH of the nectar and the mandibular substances. Lage et al. (2012) found no relationship between the low pH value found in relation to the high acid content. The low pH value and the high acidity detected in *Meliponas* honey give it a longer useful life, as it does not favor microbial development. By contrast, the high content of water activity would favor the growth of microorganisms (Lage et al. 2012). Water activity below 0.6 ensures low microbial proliferation (Hoffmann 2001). Lage et al. (2012) found water activity ranging from 0.59 to 0.79 in meliponids; Almeida-Muradian (2007) found values ranging from 0.74 to 0.76, values similar to those determined in this study. Bogdanov (1997) states that the antimicrobial activity of honey correlates significantly with acidity, recognizing that the acid fraction positively influences biological activity, because pH acts as an antimicrobial factor.

Table 5. Results of the evaluation of antimicrobial activity using the Minimum Inhibitory Concentration (mg mL⁻¹) of different stingless honey from the strains of *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *S. Typhimurium* ATCC 14028, *S. Enteritidis* and *S. Heidelberg*

| Species /Sample* | <i>E. coli</i> ATCC 25922 | <i>S. aureus</i> ATCC 25923 | <i>S.Typhimurium</i> ATCC 14028 | <i>S. Enteritidis</i> | <i>S. Heidelberg</i> |
|--------------------------------|------------------------------|--------------------------------|------------------------------------|-----------------------|----------------------|
| <i>Melipona mondury</i> | | | | | |
| 14 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| <i>Melipona bicolor</i> | | | | | |
| 7 | 550.8 (37.5%) | 417.3 (25%) | 550.8 (37.5%) | 550.8 (37.5%) | 417.3 (25%) |
| 13 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 16 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 6 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| <i>Tetragonisca angustula</i> | | | | | |
| 1 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 3 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 9 | 417.3 (25%) | 550.8 (37.5%) | 417.3 (25%) | 550.8 (37.5%) | 550.8 (37.5%) |
| 15 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 19 | 417.3 (25%) | 924.6 (50%) | 924.6 (50%) | 1312.8 (75%) | 1312.8 (75%) |
| 20 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| <i>Melipona marginata</i> | | | | | |
| 5 | 550.8 (37.5%) | 550.8 (37.5%) | 550.8 (37.5%) | 550.8 (37.5%) | 417.3 (25%) |
| <i>Melipona quadrifasciata</i> | | | | | |

| | | | | | |
|---------------------------------|-------------|-------------|-------------|-------------|---------------|
| 2 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 11 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 17 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 550.8 (37.5%) |
| 8 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 18 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| 10 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| <i>Scaptotrigona bipunctata</i> | | | | | |
| 12 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |
| <i>Tetragona clavipes</i> | | | | | |
| 4 | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) | 417.3 (25%) |

* Sample, corresponding sample number for MIC.
 Source: Authors

Based on our conductivity data, we classify the honey of the studied species as being derived from floral sources. Conductivity is closely related to the concentration of minerals, organic acids and proteins, with great variability depending on the floral honey source (Alves 2005; Suntiparapop et al. 2012; Nascimento et al. 2015). Nascimento et al. (2015) found values between $586.20 \mu\text{S}\cdot\text{cm}^{-1}$ and $539.60 \mu\text{S}\cdot\text{cm}^{-1}$ for various species of meliponids in Brazil. Chuttong et al. (2016), found a variation between $0.325 \mu\text{S}\cdot\text{cm}^{-1}$ to $2.8 \mu\text{S}\cdot\text{cm}^{-1}$ for various species of stingless bees from Thailand. Bogodanov et al. (1999) stated that conductivity should be used as a criterion for the botanical determination of honey, replacing the analysis of ash content, because the former would be proportional to the ash content. The data from the present study suggest that electrical conductivity positive correlates with acidity, corroborating the statements of Bogdanov et al. (1999) and Alves et al. (2005); however, the same was not found with respect to ash content.

Peralta (2010) found different values of acidity for stingless bees, ranging from 17.8 to $116.7 \text{ meq Kg}^{-1}$, similar to the data we obtained. The same author showed that there was a net negative correlation between acidity content and minimum inhibitory concentration for *E. coli*, assuming that there was slight action of acidity on the variation of biological activity. The present study also verified this relationship; the honey of *T. clavipes* and *S. bipunctata* had the highest acidity levels and the lowest minimum inhibitory concentrations, by contrast with the honey of *M. bicolor* that presented the highest value of minimum inhibitory concentration and the lowest acidity value.

The ash content indicates the amount of minerals found in honey (Alves et al., 2005); in the present work, we observed that the ash values varied between 0.38% to 4.88%. National legislation recommends up to 0.6% of ash in honey samples from *Apis mellifera* (Brasil, 2000). A factor that is correlated with the color of honey is the mineral content; according to the constitution of honey, a variety of various spectra and colors can be found. This property may be related to pollen and phenolic compounds present in honey that in turn varies with geographical origin and botanical varieties visited by bees (Moo-Huchin et al., 2015). Bueno Costa et al. (2016) studied honey from the southern region of Brazil and found that the lighter the honey, the lower the content of total phenolic compounds. This finding may explain the fact that clinical and standard strains were more sensitive to antimicrobials when we tested *S. bipunctata* honey.

We observed that the various honeys of stingless bees had an inhibitory effect on the growth of gram-positive and -negative strains. The effect was related to the concentration and type of honey. This is different from findings reported by Basualdo et al. (2007), who found

that the bactericidal effect of honey depended on the concentration of honey used and the nature of the bacteria.

With the diffusion test using the well technique, Fikselová et al. (2014) reported the highest antimicrobial activity in honey samples with 50% concentration against *E. coli*. In the present study, *E. coli* strain was sensitive only in the presence of 50% *M. marginata* honey, corroborating the results found by Ahmed et al. (2015), who reported that different honeys influenced the susceptibility of *E. coli*, and the largest halos were found at 100% concentration. *S. Heidelberg*, *S. Typhimurium* and *S. aureus* demonstrated a resistance profile against *T. clavipes* honey. *S. Heidelberg* and *S. Typhimurium* only presented halo formation when 100% concentration was used. In this study, the zone of inhibition was directly proportional to the concentration of honey, suggesting that the antimicrobial effect of honey was much greater in undiluted honey.

In the present study, *E. coli* demonstrated sensitivity to antimicrobials; when testing honey versus antimicrobial to assess the synergistic effect, there was no change in its susceptibility profile. This was contrary to the evaluation of *S. aureus* and *S. Enteritidis*, which altered their susceptibility profile against antimicrobials, depending on the type of honey evaluated, ranging from intermediate to resistant. The profile of *S. Typhimurium* was intermediate in relation to antimicrobials and, when interacting with various honeys, it was sensitive in most cases, especially in the presence of *S. bipunctata* honey. *S. heidelberg* showed a resistance profile against amoxicillin/clavulanic acid, ceftazidime and tetracycline. When assessing amoxicillin/clavulanic acid versus the honeys of *M. marginata* and *T. clavipes*, there was a change in the susceptibility profile where previously resistant strains became sensitive. The same occurred with the bacteria in the presence of *M. mondury* honey with ceftazidime, changing from resistant to sensitive, and *S. bipunctata* honey, changing the profile of this bacterium against antimicrobial from resistant to intermediate. Regarding tetracycline, although there was no change in the resistance profile; the increase in the halos suggest a possible synergy, and it is necessary to evaluate higher concentrations of diluted honey in the agar. For ciprofloxacin, the profile remained the same in the presence of *M. mondury* and *S. bipunctata* honeys.

The results of the minimum inhibitory concentration showed that the *M. mondury*, *S. bipunctata* and *T. clavipes* honeys obtained the lowest concentrations necessary to inhibit the growth of *S. Enteritidis* and *S. Heidelberg*. *M. quadrifasciata* honey had the same effect on these strains, with the exception of *S. Heidelberg*. Masoud et al. (2015) demonstrated that standard strains and clinical isolates vary in susceptibility depending on the type of honey

used. In another study carried out by Jenkins and Chapagain (2014), the clinical isolate was more susceptible to manuka honey compared to the standard strain.

Our study was conducted only *in vitro*; however, we bacteria that are highly pathogenic to humans and animals. The results were positive; however, the mechanisms involved need to be clarified. Further studies using honey *in vivo* are needed to understand how the components of honey act against various microorganisms; the goal is to increase the efficacy of antibiotics toward previously resistant strains.

5. Final Considerations

All our tested honeys have antimicrobial activity, regardless of the stingless bee species. The effect of this activity is related to the concentration of honey used capable of sensitizing gram-positive and -negative bacteria, in addition to the technique used in the antimicrobial test. In relation to synergism, there are indications that confirm the influence of the type of stingless honey and tested antibiotic, changing the susceptibility profile of the bacteria. Other factors that influence antimicrobial activity are physical-chemical parameters, because the interaction between the constituents that make up honey contributes to the inhibitory capacity and changes the susceptibility profile of bacteria of both clinical origin and reference strains.

This food of animal origin is interesting and has antimicrobial potential. The development of new research must seek to know which biochemical mechanisms are related to the capacity of antimicrobial and make it a product with wide use, as for example in nutraceutical food in human and animal health.

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Declaration of Interest

The authors declare no conflicts of interest in connection with this article.

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