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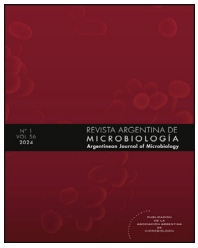

ORIGINAL ARTICLE

Antibacterial activity and effect on the outer membrane permeability of *Apis mellifera* honey from Brazil

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Antibacterial activity;
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Enterococcus faecalis;
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Abstract This study evaluates the antibacterial activity of *Apis mellifera* honey (in fresh form and in extracted fractions) and its effects on the outer membrane permeability of *Enterococcus faecalis* and *Staphylococcus epidermidis*. Five honey samples from four municipalities in the Midwest of Brazil (Mato Grosso state) were evaluated in this study. Samples of fresh honey and its hexane, ethyl acetate, and n-butanol fractions were prepared by the fractional extraction method in a separating funnel using each fraction's solvent. Antibacterial activities were assessed using the broth microdilution method with serial dilution of the samples ranging from 1000 to 1.95 μg/ml. Outer membrane permeability was determined by the broth microdilution method with a combination of honey or solvent extract fraction (80–0.15 mg/ml) and clarithromycin and erythromycin (20–0.04 μg/ml) prepared in a checkerboard. Time-kill assays were performed at 2, 4, 8 and 24 h. At a concentration of 1000 μg/ml, no antibacterial activity was observed, with inhibition below 24% for two bacteria. Honey 1 (80 mg/ml) and its n-butanol fraction increased the outer membrane permeability of *E. faecalis* when combined with clarithromycin (20 μg/ml), but no effect on membrane permeability of *E. faecalis* was observed for the remaining *r* four honey samples and their fractions. None of the samples affected the membrane permeability of *S. epidermidis*. We concluded that honey 1 increase the antibacterial activity of clarithromycin and erythromycin against *E. faecalis*, possibly due to the action of flavonoids.

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PALABRAS CLAVE
Actividad antibacteriana;
Permeabilidad de la membrana externa;
Enterococcus faecalis;
Staphylococcus epidermidis

Actividad antibacteriana de la miel de *Apis mellifera* de Brasil y su efecto sobre la permeabilidad de la membrana externa

Resumen Este estudio evaluó la actividad antibacteriana de la miel de *Apis mellifera* (fresca y fracciones extraídas) y sus efectos sobre la permeabilidad de la membrana externa de *Enterococcus faecalis* y *Staphylococcus epidermidis*. Se examinaron cinco muestras de miel de cuatro municipios centrooccidentales de Brasil (estado de Mato Grosso). Se prepararon muestras de miel fresca y se obtuvieron fracciones en hexano, acetato de etilo y N-butanol. Las actividades antibacterianas se evaluaron mediante microdilución en caldo con muestras en diluciones seriadas de 1000 a 1,95 μg/ml. La permeabilidad de la membrana externa se investigó mediante el método de microdilución en caldo con una combinación de miel o fracción de extracto con solvente (80 a 0,15 mg/ml) y claritromicina y eritromicina (20 a 0,04 μg/mL), usando el método de tablero de ajedrez. Los ensayos de tiempo-muerte se realizaron a las 2, 4, 8 y 24 h. A una concentración de 1000 μg/ml, no se observó actividad antibacteriana, con una inhibición inferior al 24% para las dos bacterias probadas. La miel 1 (80 mg/ml) y su fracción de n-butanol aumentaron la permeabilidad de la membrana externa de *E. faecalis* cuando se combinaron con claritromicina (20 μg/ml). No se observó ningún efecto sobre la permeabilidad de la membrana de *E. faecalis* con las otras cuatro muestras de miel y sus fracciones. Ninguna de las muestras afectó la permeabilidad de la membrana de *S. epidermidis*. Concluimos que la miel 1 aumenta la actividad antibacteriana de la claritromicina y la eritromicina contra *E. faecalis*, posiblemente debido a la acción de los flavonoides.

Introduction

Bee honey is the result of a mixture of nectar, pollen, and plant exudates and bee substances that are deposited in the honeycombs. Bee behavior influences honey's chemical composition, which varies in accordance with the botanical source of the nectar[22]. Honey contains volatile compounds, phenolics, and flavonoids that confer antimicrobial, anti-inflammatory and antioxidant properties[13]. These properties depend on the botanical origin of the nectar and regional flora and the chemical profile of the honey[2,28].

Antibacterial activity is the most researched aspect of honey, showing varying effects on Gram-positive and Gram-negative bacteria from different honey sources[16]. However, the *Apis mellifera* honey's activity for inhibition of *Staphylococcus epidermidis* and *Enterococcus faecalis* is limited. It has been reported in the literature, for example, that the Chilean polyfloral honey inhibits *S. epidermidis* in a concentration of 18.7% v/v, while the honey from Alegria and Amazonas had minimum inhibitory concentrations of 2.5–10% for *E. faecalis*[6,10,31].

The antibacterial effects of honey have been attributed to polyphenols such as phenolic acids and flavonoids, which disrupt bacterial functions such as inhibition of peptidoglycan and ribosome synthesis, oxidative stress, effect on cell growth and proliferation, and yet induces cell lysis and disruption of bacterial membrane permeability[2,4,19]. Some honey samples can enhance antibiotic efficacy by increasing outer membrane permeability[39], improving antibiotic efficacy[35]. The synergistic combination of Manuka honey with the antibiotic linezolid demonstrated enhanced effects against *Staphylococcus aureus* due to methylglyoxal[21]. Antibiotic-resistant strains of *S. aureus* became sensitive when combined with Manuka honey[24]. The synergistic antimicrobial interaction between quercetin and curcumin, two polyphenolic compounds commonly found in honey with antibacterial property, increased their efficacy against *E. faecalis*[8,20,28].

The combined effect of human antibiotic therapy using honey and antibiotics can reduce treatment costs, minimize antibiotic use, speed recovery, and reduce hospitalizations[2]. Combining Chinese herbal medicine with pharmacological medications such as ginseng and metformin has been shown to be a promising synergistic combination for both the prevention and treatment of diabetes[37].

Brazilian honeys, such as those from the Amazon and Mato Grosso, have demonstrated effectiveness against *Enterococcus*[10]; however, antibacterial mechanisms are not yet fully understood. The activity of honey against *Escherichia coli* may involve interference with membrane permeability, as observed with ''Yemeni Sidr honey''[3]. Other mechanisms of action of honey are protein inhibition and induction of bacterial DNA damage, as demonstrated by Manuka honey[3], highlighting the need for further studies on the antibacterial activity of honey samples from different geographical and botanical origins.

Therfore, the aim of this study was to evaluate the antibacterial activity of five samples of *A. mellifera* honey and its hexane, ethyl acetate and n-butanol fractions, as well as their effects on the permeability of the outer membrane of *E. faecalis* and *S. epidermidis*. All the honey samples were from the Midwest of Brazil (Mato Grosso state).

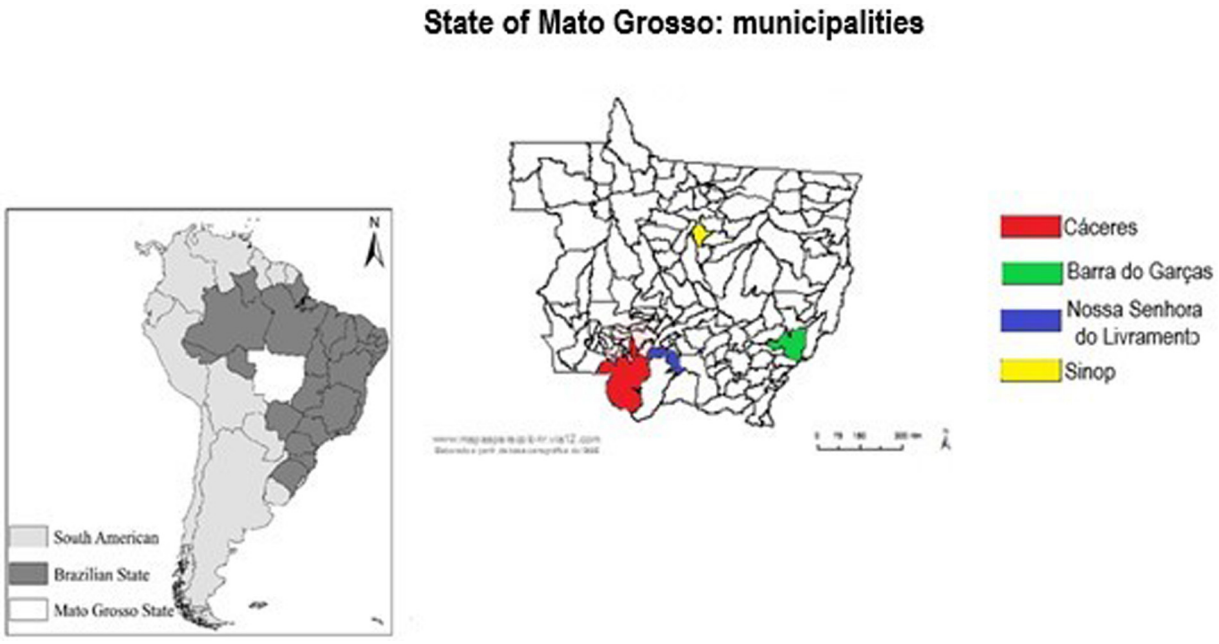

Figure 1 Geographical location of the municipalities of the Midwestern region of Brazil selected for the study.

Materials and methods

Study area

Four municipalities in the state of Mato Grosso, located in the Midwestern region of Brazil were selected for the study: Cáceres, Barra do Garças, Nossa Senhora do Livramento and Sinop (Fig. 1). These municipalities were selected due to their honey production, as they are among the 15 largest honey-producing municipalities in the state of Mato Grosso. The average production of bee honey between 2015 and 2020 in Cáceres, Barra do Garças, Nossa Senhora do Livramento and Sinop was 7083 kg, 55 061 kg, 4394 kg and 22 709 kg, respectively[7].

The municipality of Cáceres covers 24 538 591 km^2 and consists of the Amazon (6%), Cerrado (8%) and Pantanal (85%) biomes. Barra do Garças y covers 8 713 673 km^2 and contains the Cerrado biome (100%). Nossa Senhora do Livramento covers 5 537 413 km^2 and consists of the Cerrado (65%) and Pantanal (35%) biomes. Sinop covers 3 990 870 km^2 and comprises only the Amazon biome (100%). Thus, the samples cover three different biomes.

Origin of the samples

In this study, five honey samples from the state of Mato Grosso, in the Central-Western region of Brazil, were selected. Two honey samples came from Cáceres (honey 1 and honey 2) and the remaining ones from Barra do Garças (honey 3), Nossa Senhora do Livramento (honey 4), and Sinop (honey 5). The honey samples were selected from the database of the Laboratory of the Center for Apiculture Studies (CETApis)[1]. The selected samples exhibited a higher content of phenolics and flavonoids, antioxidant and antibacterial activity than the average of other honeys from Mato Grosso, verified in a preliminary screening of 32 samples (Mendonça et al., unpublished results)[1,17].

The pollen profile in the honey samples selected for this study had been previously identified[1]. *Astronium urundeuva* (*M. Allemão*) Engl. was the predominant pollen in honeys 1 and 2 and secondary pollen in honeys 3, 4, and 5, present in all samples. The species *Mimosa pudica* L. was present in all honey samples, as secondary pollen or important minor pollen, except honey 2. Honey 4 was characterized as polyfloral, with no predominant pollen. In honey 5, the predominant pollen belonged to the Moraceae family. Several plant species were classified as important minor pollen types in some honey samples, as follows: *Senna rugosa* (G. Don) H.S. Irwin & Barneby was present exclusively in honeys 1 and 2. *Miconia ferruginata* DC. and *Mimosa tenuiflora* (Willd.) Poir. were detected exclusively in honey 3. Cucurbitaceae pollen was found in honey 4. *Praxelis diffusa* (Rich.) Pruski and Borreria verticillata (L.) G. Mey. were identified in honey 5[27].

Sample preparation

This study evaluated not only the activity of fresh honey samples but also their extracted fractions. Each fraction contained some of the chemical compounds present in honey, extracted according to their polarity. Thus, comparing the activities of the fresh honey with their respective solvent extracted fractions at the same concentrations can provide an indication of possible ''chemical targets'' responsible for antibacterial action, which could be investigated in future studies. From each of the five fresh honeys, extractions were performed using hexane, ethyl acetate, and n-butanol solvents, yielding three fractions of each honey sample. A total of 20 samples were evaluated, including 5 fresh honeys and their 15 fractions. The dry extract of the honey fractions was prepared by the

fractional extraction method in a separating funnel using the solvents hexane, ethyl acetate, and n-butanol in sequence, from lowest to highest polarity (Appendix A. Supplementary Data)[26]. For extraction, 100 g of fresh honey was diluted in 150 ml of sterile distilled water, placed in a separatory funnel, and extracted with 150 ml of each solvent, with three successive extractions with 50 ml of each solvent, one every 15 min. The entire extraction process was carried out in a maximum of 24 h to avoid contamination. The three fractions collected from each honey sample were concentrated in a rotary evaporator (40 °C for hexane and 60 °C for ethyl acetate and n-butanol), and the residual solvent was evaporated in ceramic capsules for three to six days in an oven at 33 °C until reaching a constant weight, consistency and characteristic honey smell to obtain the dry extract.

Microorganisms

The following bacteria were used: *E. faecalis* (ATCC 29212, São Paulo, BR) and *S. epidermidis* (ATCC 12228, São Paulo, BR) from the collection at the Centro de Estudo em Apicultura (CETApis) Laboratory of Universidade do Estado de Mato Grosso (Brazil). These bacteria were selected based on results obtained in previous studies by CETApis Laboratory.

The bacteria were kept frozen at −80 °C in a culture medium and were reactivated just before the tests. *S. epidermidis* was reactivated from the stock culture in Mueller-Hinton broth (MBH) and grown on agar for 24 h at 37 °C. *E. faecalis* was reactivated from the stock culture in Brain Heart Infusion (BHI) broth and grown on agar for 24 h at 37 °C; the broth and agar were supplemented with vancomycin (6 μg/ml) at lethal concentrations[32].

The antibiotics used as positive controls were clarithromycin for *S. epidermidis*, and erythromycin and clarithromycin for *E. faecalis* (Sigma Aldrich®).

Antibacterial activity

The minimum inhibitory concentration (MIC) was verified by the broth microdilution method in 96-well microplates (MIC determination by the CLSI methods, 2013)[25]. A 4000 mg/ml solution was prepared to obtain serial two-fold dilutions: 100 μl of this solution were added to each well containing 100 μl of MHB or BHI, resulting in sample concentrations ranging from 2000 to 7.8 mg/ml. Then, 100 μl of the bacterial suspension (*S. epidermidis* or *E. faecalis*) prepared in its respective culture medium (McFarland scale 1.0; approximately 3.0×10^8 CFU/ml) were added to each well, resulting in a final volume of 200 μl per well, with concentrations ranging from 1000 to 1.95 μg/ml and bacterial concentration of McFarland scale 0.5.

Clarithromycin (final concentration of 20–0.04 μg/ml) was used as a positive control for *S. epidermidis* and erythromycin (final concentration of 20–0.04 μg/ml) for *E. faecalis*[9,25]. Wells containing only medium were used as sterility control. A replicate plate with the microdilution of honey or fraction samples was prepared for color control. The plates were incubated for 24 h at 35 °C[9,25]. Microplate readings were performed by a spectrophotometer (Elisa) at 450 nm. The tests were carried out in triplicate.

Effect of honey on outer membrane permeability

Time-kill

The time-kill test was performed using the same protocol described previously[4]. Samples (80–0.156 mg/ml) and antibiotics (20–0.04 μg/ml) were spiked into columns and serial dilutions were prepared. The readings were taken using a spectrophotometer (ELISA) at 450 nm at 0, 2, 4, 8, and 24 h. The assays were performed in triplicate. The samples that showed the highest inhibition of bacterial growth (≥27% in the MIC assay) for each bacterium were selected to evaluate the effect on outer membrane permeability.

Checkboard method

The effect of the samples on the outer membrane permeability of *S. epidermidis* and *E. faecalis* was evaluated by the checkerboard method, based on the broth microdilution protocol described previously in 2.4, using 96-well plates[4]. Fresh honey or its fractions were used in serial dilution (80–0.156 μg/ml), while the respective antibiotics were placed in serial dilution (20–0.04 μg/ml). Subsequently, 100 μl of bacterial suspension (McFarland scale 1 or 3×10^8 CFU/ml) prepared in sterile 0.85% NaCl solution was added to each well. The microplates were incubated in an oven at 37 °C and, after 24 h. The reading was taken using a microplate reader at 450 nm. All assays were performed in triplicate.

Data analysis

The percentage of inhibition growth was calculated by comparing the absorbance of the sample (honey and fraction) with the bacterial growth control (negative control).

The antibacterial activity of honey was analyzed using an arithmetic mean. The comparison between the honey samples was conducted using descriptive statistics, specifically the mean and the 95% confidence interval. The effect on increased bacterial membrane permeability was considered when the mean inhibition of the combined honey showed a different response pattern from that of the antibiotic alone, considering the 95% confidence interval.

Results

Antibacterial activity

It was not possible to determine the MIC of any of the honey samples or its fractions up to 1 mg. Overall, bacterial growth inhibition by honey and its fractions ranged from 2 to 37% as follow: *E. faecalis* showed inhibition from 17 to 37% and *S. epidermidis* inhibition from 2 to 37%. The positive controls inhibited 77% of the growth of *E. faecalis* and 88% of the growth of *S. epidermidis* (Table 1).

The honey or fractions that inhibited Gram-positive bacterial growth by ≥27% were selected for the membrane permeability effect assay (Table 1). For *E. faecalis*, honeys 1 and 4 (fresh and n-butanol fraction) and honey 5 (hexane fraction) were selected. For *S. epidermidis*, honey 3 (fresh, hexane fraction, ethyl acetate fraction and n-butanol frac-

Table 1 Inhibition of the growth of Gram-positive bacteria *Enterococcus faecalis* (*E. faecalis*) and *Staphylococcus epidermidis* (*S. epidermidis*) by honey samples or their fractions at a concentration of 1mg/ml.

		Bacteria (% inhibition of the growth)	
Honey sample	Fraction	*E. faecalis*	*S. epidermidis*
Honey 1	Fresh	27±5.28	24±2.93
	Hexane	23±2.99	2±0.67
	Ethyl acetate	23±1.13	20±9.31
	N-butanol	27±3.15	11±8.23
Honey 2	Fresh	25±8.22	16±4.99
	Hexane	17±5.76	24±7.71
	Ethyl acetate	17±5.36	19±7.06
	N-butanol	25±8.22	24±7.79
Honey 3	Fresh	17±5.29	36±6.61
	Hexane	26±6.68	28±7.08
	Ethyl acetate	20±8.66	32±4.34
	N-butanol	21±7.61	37±8.51
Honey 4	Fresh	37±22.40	26±1.95
	Hexane	23±6.35	30±7.62
	Ethyl acetate	21±9.91	24±3.59
	N-butanol	30±6.03	30±6.78
Honey 5	Fresh	25±10.45	28±5.96
	Hexane	29±7.75	22±10.85
	Ethyl acetate	26±7.05	16±2.76
	N-butanol	21±9.26	22±8.76
	Mean	24.00	23.55
Positive control	Erythromycin	77±11.89	
	Clarithromycin		88±3.87

tion), honey 4 (hexane fraction and n-butanol fraction) and honey 5 (fresh) were selected.

Effect of honey on outer membrane permeability

Time-kill test

Honey 1 (fresh and n-butanol fraction) combined with erythromycin increased *E. faecalis* membrane permeability after 4 h of incubation. The same effect was observed when the same samples were combined with clarithromycin. This effect persisted for up to 24 h (Fig. 2A and B).

Honey 4 (fresh and n-butanol fraction; Fig. 3A and B), as well as honey 5 (hexane fraction; Fig. 3C), also combined with erythromycin and clarithromycin, increased *E. faecalis* permeability between 4 and 8 h; however, this effect was not maintained at 24 h.

None of the honey samples or their fractions increased *S. epidermidis* outer membrane permeability at the different time points evaluated. The reduction in bacterial growth of honey or its fractions combined with antibiotics was equal to or less than the effect of the antibiotic alone.

Honey 3 (fresh; Fig. 4A) and honey 4 (n-butanol fraction; Fig. 4D), combined with clarithromycin, resulted in increased bacterial growth after 8 h; this was clearly confirmed at 24 h. These samples, evaluated alone, tended to increase *S. epidermidis* growth after 2 h. The same pattern was observed for honey 3 (ethyl acetate fraction and n-butanol fraction) and honey 5 (hexane fraction).

Honeys 3 and 4 (hexane fraction; Fig. 4B and C), combined with clarithromycin for 24 h, reduced *S. epidermidis* growth by 85% and 71%, respectively, compared to the negative control, although they had no effect on outer membrane permeability.

Checkboard method

Honey 1 (fresh and n-butanol fraction), combined with antibiotics (erythromycin and clarithromycin), increased the permeability of the *E. faecalis* membrane (Table 2). The mean inhibition of the bacterial growth of honey 1 (fresh) combined with erythromycin and clarithromycin was 92% and 91%, respectively. Honey 1 (n-butanol fraction) combined with erythromycin and clarithromycin, had a mean inhibition of 94–95%, respectively, with a mean response pattern that differed from the isolated positive control and was higher than the confidence interval.

Honey 4 (fresh and n-butanol fraction) and honey 5 (hexane fraction) had no effect on the permeability increase of the *E. faecalis* membrane. At first glance, these samples combined with the antibioticappear to have a greater effect than the antibiotic alone; however, the confidence interval excludes this hypothesis, showing no difference.

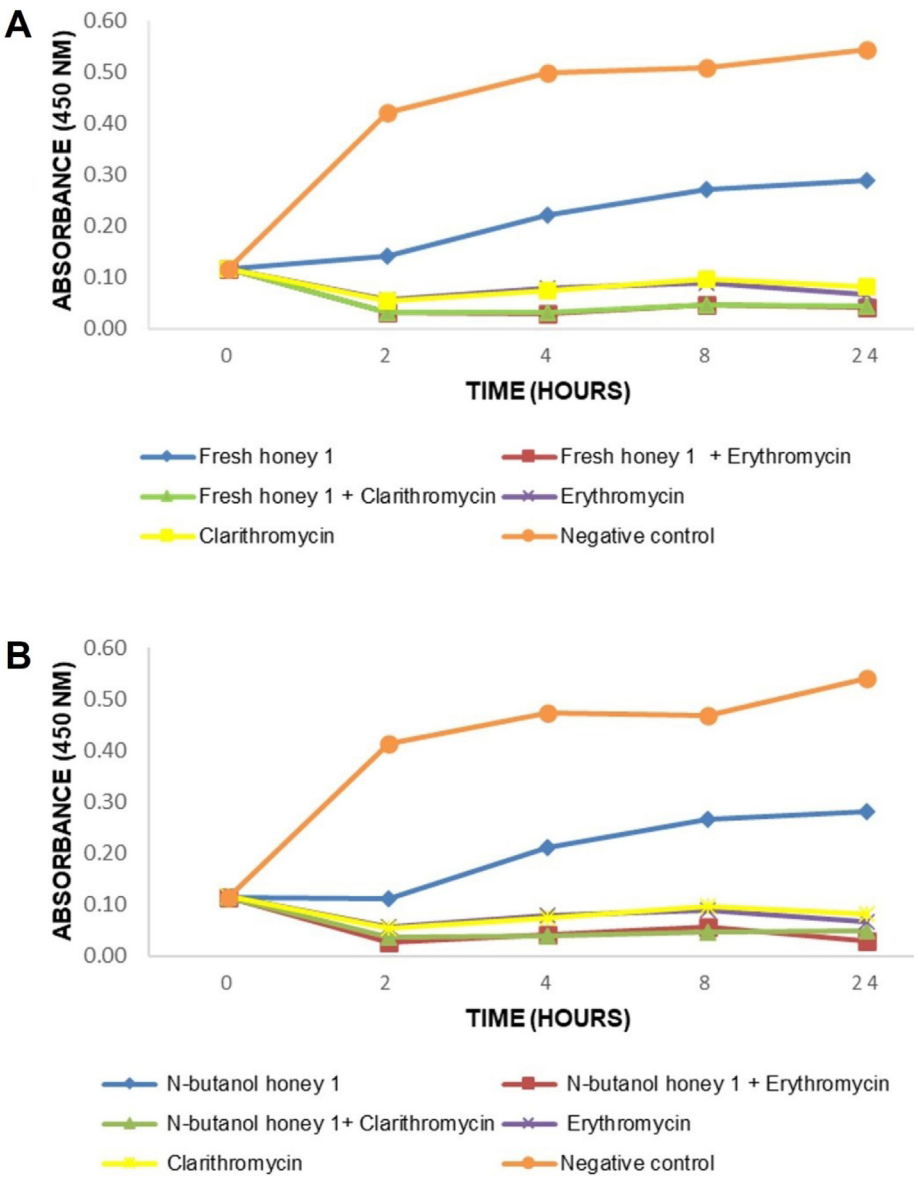

Figure 2 Time-kill curve for *Enterococcus faecalis* exposed to fresh honey 1 (A) and the n-butanol fraction of honey 1 (B) at concentrations of 80 mg/ml and the positive control at a concentration of 20 μg/ml.

Honeys 3 and 4 (hexane fraction) combined with clarithromycin did not increase the permeability of the membrane of *S. epidermidis* (Table 2). The mean pattern of inhibition of growth of these honeys combined with antibiotics was equal to that of the antibiotic alone.

Honey 3 (fresh, ethyl acetate fraction and n-butanol fraction), honey 4 (n-butanol fraction) and honey 5 (fresh), combined with clarithromycin, did not increase membrane permeability in *S. epidermidis*. These measurements were not higher than the confidence interval and still decreased the action of the antibiotic when combined.

The inhibition of Gram-positive bacterial growth at the concentration used in the checkboard assay (80 mg/ml) increased in relation to the concentration evaluated in the antibacterial activity assay (1 μg/ml). This was particularly the case for honeys 3 and 4 (hexane fraction) on *S. epidermidis*, which showed inhibition of 85% and 71%, respectively, at the concentration of 80 mg/ml, while presenting 28% and 30% at 1 mg/ml, respectively (Table 2).

Discussion

Honey's antimicrobial activity is already known, and its ability to enhance the effects of antibiotics has also been studied[36]. This effect may have a positive impact on antibiotic treatments for infections[2]. Since honey composition can vary depending on several factors, such as bee species, collection location, and flower species, each honey must be evaluated[12].

The five honey samples evaluated in this study came from regions with diverse biomes. Their botanical origin was determined[1]. Although pollen from *A. urundeuva* and *M. pudica* was present in all samples (appearing as a dominant species in some and as accessory species in others), each

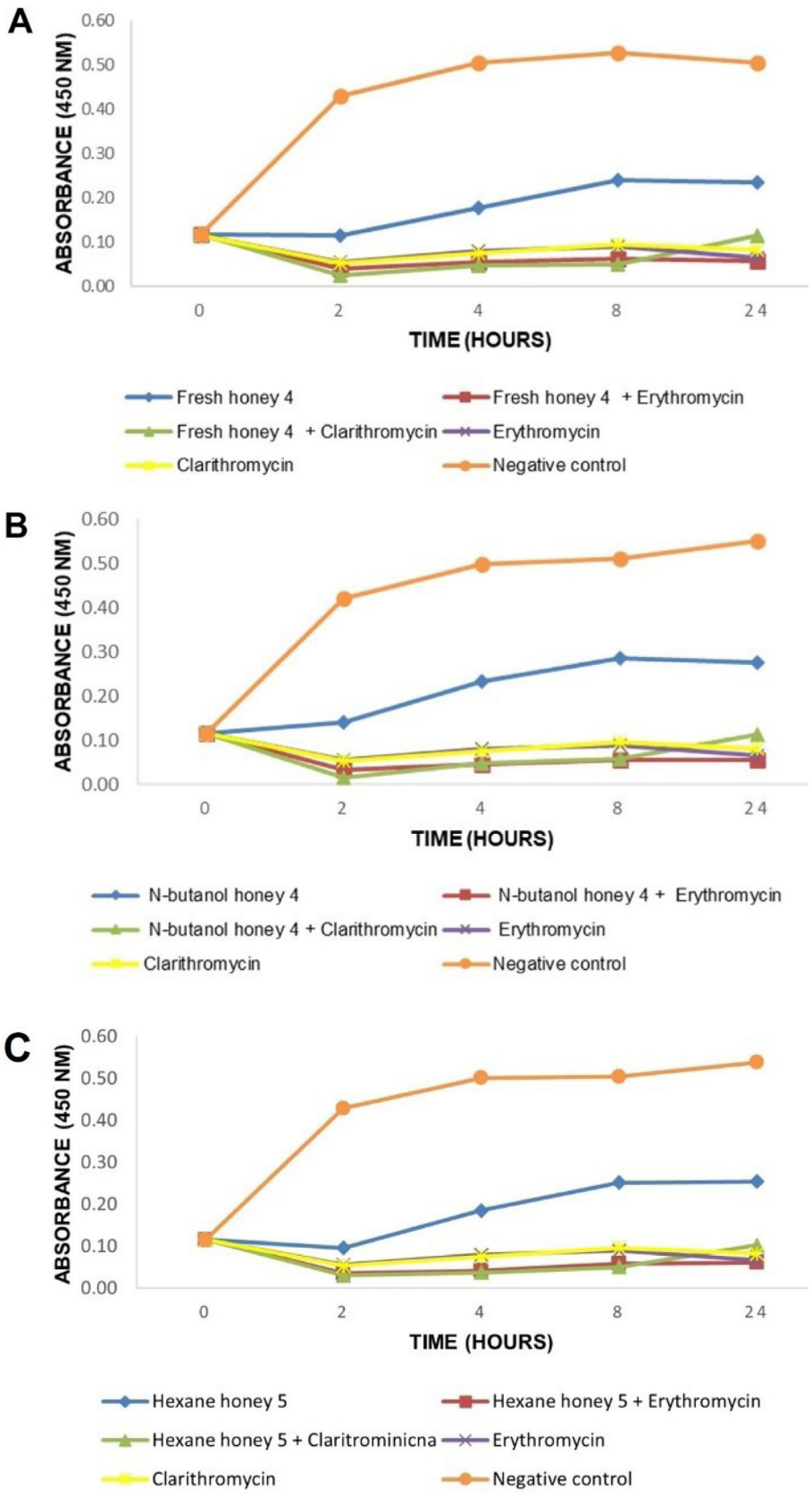

Figure 3 Time-kill curve for *Enterococcus faecalis* exposed to fresh honey 4 (A); the n-butanol fraction of honey 4 (B) at concentrations of 80 mg/ml and the positive control at a concentration of 20 μg/ml and the hexane fraction of honey 5 (C) at concentrations of 80 mg and the positive control at a concentration of 20 μg/ml.

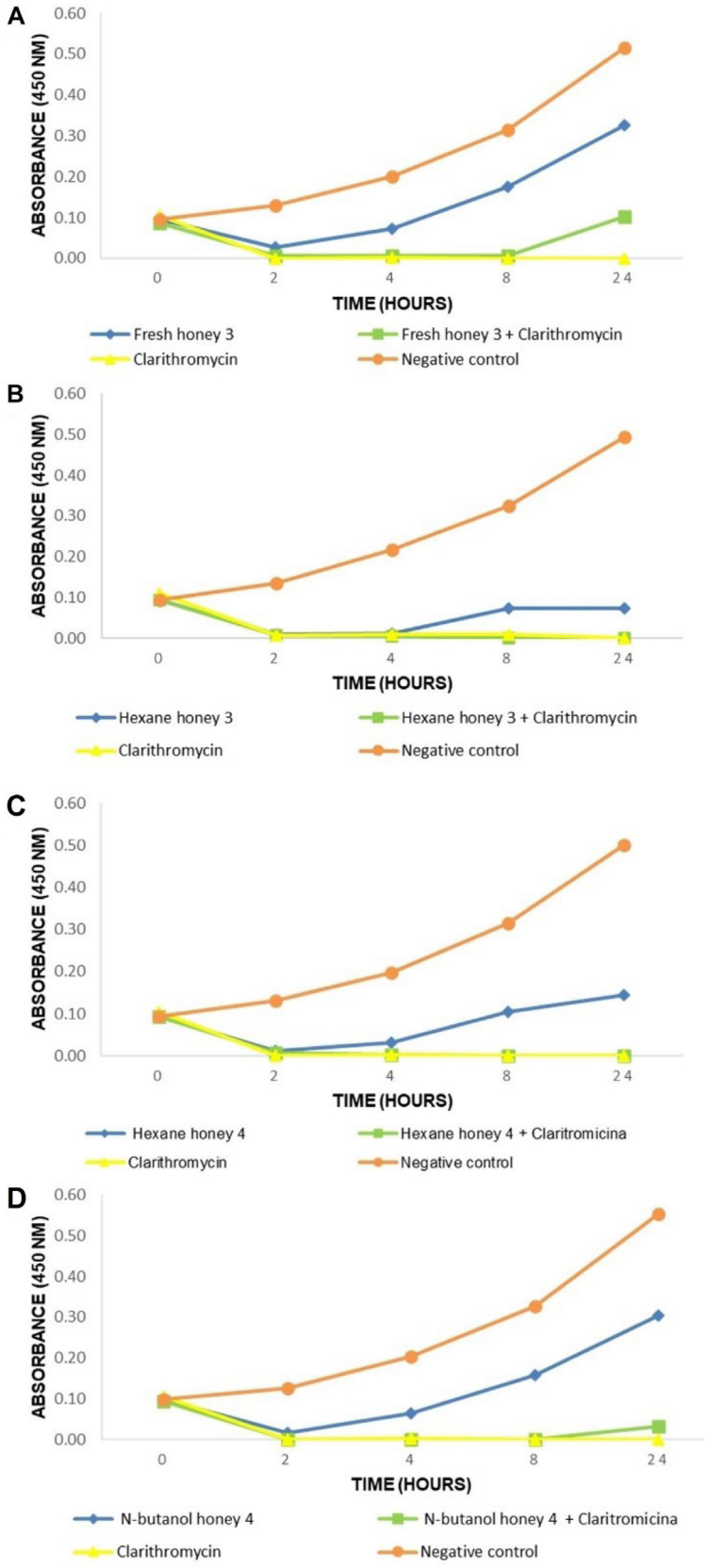

Figure 4 Time-kill curve for *Staphylococcus epidermidis* exposed to fresh honey 3 (A), hexane fraction (B), hexane fraction of honey 4 (C) and n-butanol of honey 4 (D) at concentrations of 80 mg and the positive control at a concentration of 20 μg/ml.

Table 2 Antibacterial activity expressed by inhibition of growth (%) of *Enterococcus faecalis* and *Staphylococcus epidermidis* by honeys, fractions and positive control isolated and combined (results expressed as the mean ± standard deviation) after 24 h of incubation.

Enterococcus faecalis	Isolated			Combined	
	Honey	Eri[a]	Clari[b]	Honey and Ery[a]	Honey and Clari[b]
	(80 mg/ml)	(20 μg/ml)			
Fresh honey 1	43 ± 5.49			92 ± 2.45*	91 ± 1.09*
N-butanol honey 1	47 ± 4.59			94 ± 2.04*	95 ± 2.44*
Fresh honey 4	53 ± 7.37	87 ± 2.20	85 ± 2.45	88 ± 3.54	88 ± 4.39
N-butanol honey 4	49 ± 7.67			89 ± 1.75	89 ± 2.52
Hexane honey 5	52 ± 3.46			88 ± 0.89	89 ± 0.95

Staphylococcus epidermidis	Isolated		Combined
	Honey (80 mg/ml)	Clari[b] (20 μg/ml)	Honey and Clari[b]
Fresh honey 3	36 ± 2.42		80 ± 18.31
Hexane honey 3	85 ± 4.18		100 ± 0
Ethyl acetate honey 3	39 ± 7.83		89 ± 18.30
N-butanol honey 3	49 ± 12.02	100 ± 1.19	90 ± 12.49
Hexane honey 4	71 ± 23.16		100 ± 0
N-butanol honey 4	44 ± 11.54		93 ± 16.64
Fresh honey 5	51 ± 33.88		85 ± 18.97

[a] Erythromycin.
[b] Clarithromycin.
* Different response pattern from the antibiotic alone, greater than the 95% confidence interval.

sample contained other species that characterize them as unique.

To concentrate the active compounds in the samples, such as flavonoids and phenolics, three different solvent extracted fractions of each honey were obtained and analyzed. No MIC value could be found in any of the fresh honey samples or their fractions, up to the maximum tested concentration of 1 mg/ml. At this concentration, inhibition percentages ranged from 2 to 73%.

Fresh honey 1 and its n-butanol fraction resulted in increased permeability of the outer membrane of *E. faecalis*, when combined with clarithromycin and erythromycin, an effect that may be due to the presence of flavonoids present in greater proportions in the n-butanol fraction[14]. It is known that the action of flavonoids on bacteria is based on membrane alterations, triggering damage to cell membrane structures or affecting their permeability[34]. Flavonoids can also inhibit the enzyme DNA gyrase, via peptidoglycan, and ribosome synthesis, resulting in cell lysis and inhibition of DNA helicases[2].

Previous data from the honey samples evaluated in this study show that the polyphenol content varied from 17.4 GAE/100 g (honey 3) to the highest content of 72.4 GAE/100 g for honey 2. The flavonoid content varied from 3.5 QE/100 g (honey 1) to 13.0 QE/100 g (honey 3)[1]. The total flavonoid content of these honey samples from Mato Grosso is still lower than that of other honeys, whose antibacterial activity has been evaluated, such as honey from regions of Malaysia, which showed a lower total phenolic content (TPC) of 29.17 mg QE/100 g of honey[40], as well as honey from Southeast Brazil, with a TPC of 55.59 mg QE/100 g[23]. Thus, the effect of honey 1 on the outer membrane of *E. faecalis* may be related not to the total flavonoid content, but rather to a specific flavonoid.

The flavonoids in honey 1 may be common to the other samples due to their similar botanical origin, but honey 1 differs from the others in that it is the only one containing *S. rugosa* pollen[27].

S. rugosa is a shrub/subshrub belonging to the Fabaceae family that is found in the North, Northeast, Central-West, Southeast, and South of Brazil[5]. The Senna genus contains important metabolites, such as alkaloids, anthraquinones, flavonoids, tannins, glycosides, steroids, terpenoids, saponins, and volatile oils[30]. In the crude ethanolic extract of *S. rugosa*, the majority of phenolic compounds, including flavonols, anthraquinones and anthrone derivatives, were detected[11]. Flavonoids are also present in other species of the same genus, such as Senna reticulata, in which catechin and epicatechin were found. The presence of catechin was also evidenced in *Senna alata* (L.) Roxb., *Senna macranthera* (DC. ex Collad.) H.S. Irwin & Barneby[29]. Epicatechin has also been found in *Acacia catechu*, a member of the *S. rugosa* family (Fabaceae), which has demonstrated antibacterial activity against *E. faecalis*[15].

Epicatechin, a flavonoid also presents in *Camellia sinensis* (L.) Kuntze, disrupts the membrane lipid bilayer by decreasing fluidity and altering membrane permeability[33]. The membrane effect of epicatechin has been explained by amphiphilic phytochemicals, with greater membranotropic activity compared to their hydrophilic and hydrophobic counterparts. Therefore, it is suspected that epicatechin may be present in honey 1 and is associated with the effect

on the membrane permeability of *E. faecalis*, a hypothesis to be verified in future studies.

Honey 3 (fresh honey and its ethyl acetate and n-butanol fractions), the n-butanol fraction of honey 4, and fresh honey 5, combined with clarithromycin, did not reduce the growth of *S. epidermidis*, maintaining the same antibiotic effect at 4 and 8 h. However, the bacteria showed growth at 24 h, suggesting that, at this time point, honey may promote the growth of *S. epidermidis*, as observed in a previous study[4]. However, fresh honey 3 and as the hexane fraction of honey 4 showed greater inhibition of *S. epidermidis* without antibiotic combination (85% and 71%, respectively). This raises the hypothesis that the bioactive compound responsible, at least in part, for their antibacterial effect is likely one (or more) nonpolar compounds concentrated in the hexane fraction.

Fresh honey 4 and its n-butanol fraction, as well as the hexane fraction of honey 5, combined with clarithromycin and erythromycin, reduced the growth of *E. faecalis* between 4 and 8 h, compared to the antibiotics alone. Given this result, it would be interesting to study the effect of offering honey at intervals shorter than 24 h, administering honey in a dosing regimen similar to that of some antibiotics, such as clarithromycin (12/12 h) and erythromycin (6/6 h)[38], in order to maintain the synergistic effect of honey and antibiotics. To investigate this, it is essential to analyze the kinetics of honey's impact on *E. faecalis*, assessing whether more frequent administration of honey at shorter intervals produces positive effects. Such an approach could identify the best way to achieve the synergistic effect of honey with antibiotics.

The increase in outer membrane permeability of *E. faecalis*, promoted by fresh honey 1 combined with the antibiotics, began after 4 h and lasted up to 24 h, suggesting that it may not be necessary for this honey to be administered at shorter intervals. In this sense, honey 1 represents an important finding in this study, with potential use as a complement to drug treatments, such as clarithromycin and erythromycin, for *E. faecalis*.

Although honeys 1, 4, and 5 increased the membrane permeability of *E. faecalis*, their MIC was not found up to 80 mg/ml. On the other hand, honey samples at a concentration of 80 mg/ml considerably increased the inhibition of *S. epidermidis* by up to 70% compared to the concentration of 1 mg/ml. The literature shows complete growth inhibition of various bacteria at honey concentrations higher than those used in this study. For example, honey samples from western regions of Croatia showed MICs ranging from 100 to 400 mg/ml against Gram-negative bacteria *Pseudomonas aeruginosa* and *E. coli*[18]. Honey from the Amazon region of Brazil exhibited a MIC of 100 mg/ml against *S. epidermidis*[10]. This indicates that, at concentrations above 80 mg/ml, the media evaluated in this study may achieve complete inhibition of the bacteria.

Conclusion

Honey and its fractions showed no antibacterial activity up to 1 mg/ml. When combined with clarithromycin, fresh honey 1 and the n-butanol fraction of honey 1, from the city of Cáceres, Brazil, increased the membrane permeability of Gram-positive *E. faecalis*. A synergistic effect was observed in the antibacterial activity of the combined products, with a shorter incubation time compared to the products alone. Further studies are needed to confirm the mechanism of action of honey from Cáceres (Brazil) on the membrane of *E. faecalis*. The isolation of bioactive flavonoids will help to realize the potential of this honey from the Central-West region of Brazil (Mato Grosso).

Authors' contributions

Rithiely Conceição Silva: Formal analysis, investigation, data curation, roles/writing – original draft, writing – review and editing, visualisation. Larissa Maria Scalon Lemos: Methodology, validation and writing – review. Carla Galbiati: Conceptualisation, methodology, validation, resources, supervision, project administration.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2026.100724.

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