Journal of Biosciences and Medicines, 2025, 13(1), 316-337
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Combined Effect of Honey from Central West Brazil on Bacterial Membrane Permeability

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How to cite this paper: Aleixo, M.L.M., da Cruz Mendonça, J., Benevides, R.B.R., Lemos, L.M.S. and Galbiati, C. (2025) Combined Effect of Honey from Central West Brazil on Bacterial Membrane Permeability. *Journal of Biosciences and Medicines*, **13**, 316-337.
https://doi.org/10.4236/jbm.2025.131026

Received: December 18, 2024
Accepted: January 23, 2025
Published: January 26, 2025

Abstract

Honey, an apicultural product with a complex chemical composition, contains numerous bioactive compounds with potential antimicrobial effects. This study investigated the effect of *Apis mellifera* honey from Brazil's Central-West Region, combined with antibiotics, on bacterial membrane permeability, exploring the contributions of bioactive compounds and the botanical origin of honey. Six fresh *Apis mellifera* honey samples and their fractions (hexane and ethyl acetate) were analyzed, for a total of 18 samples. The bacteria *Staphylococcus epidermidis*, *Helicobacter pylori* and *Enterococcus faecalis* were used for antibacterial activity tests, which included minimum inhibitory concentration (MIC) determination and synergistic effect (checkerboard) assays. The total polyphenol and flavonoid contents were quantified, and the botanical origin was determined based on pollen analysis. The tested honey samples significantly affected bacterial membrane permeability when combined with rifampicin and clarithromycin. Although many honey-derived bioactive compounds, when isolated, did not exhibit significant activity against these bacteria, the additive or synergistic effect of multiple compounds acting on different targets appears to potentiate the antibacterial action. Descriptive statistical analysis, including means and 95% confidence intervals, confirmed the relevance of the findings. This study has provided an important discovery: Honey has an effect on bacterial membrane permeability, although the specific mechanisms involved in this process require further investigation.

Keywords

Honey, Mechanism of Action Antibacterial, Bacterial Membrane

Permeability, Bioactive Compounds

1. Introduction

Honey is a natural product produced by *Apis mellifera* bees from the nectar of flowers or plant exudates. After collection, it is transformed and combined with specific substances from the bees' own reserves and stored in honeycombs for maturation [1]. Renowned for its health benefits, honey is considered a complex functional food, primarily due to its high concentration of carbohydrates such as fructose, glucose, and isomaltose [2]. In addition, it contains enzymes (e.g., *α*-amylase and glucose oxidase), proteins, amino acids, phenolic compounds, minerals, vitamins, and organic acids. Its composition is influenced by factors such as the geographic location and flowering season [3] [4].

In vivo and in vitro studies have demonstrated that honey contains bioactive compounds with anti-inflammatory, antiproliferative, antimutagenic, antitumoral, antiglycemic, antioxidant, antibacterial, and antifungal properties [4] [5]. These compounds can prevent chain oxidation reactions associated with respiratory, cardiovascular, inflammatory, and gastrointestinal diseases [6]. The antibacterial activity of honey is attributed to factors such as hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin-1, and phenolic compounds [3]. Its high sugar concentration induces osmotic stress, while its low water activity, acidic pH, and hygroscopic nature inhibit bacterial growth [7].

Phytochemical factors render honey effective against pathogenic bacteria; however, these factors vary depending on the botanical origin, climate conditions, storage, and preservation methods [3]. The most significant antibacterial factor in honey is non-peroxide inhibitors, including polyphenols and certain antimicrobial peptides (e.g., defensin-1, defensin-2, hymenoptaecin, and apidaecin), which can alter bacterial membranes and penetrate pores in their cell walls [8].

Membrane pore penetration is one of honey's mechanisms of action. Substances such as flavonoids and phenolics, which interfere with bacterial growth, contribute to its pharmacological properties and, when combined with drugs, may provide an effective alternative [9].

According to [10] and [8], the mechanisms of action of honey (e.g., Manuka and Hungarian honeys) can induce structural and morphological changes in bacteria, alter bacterial membrane potential, disrupt growth and bacterial cell cycles, inhibit metabolism, affect efflux pump activity, disrupt quorum sensing, inhibit biofilm formation, and affect bacterial stress responses.

While many mechanisms remain incompletely described, studies indicate that these mechanisms vary between gram-positive and gram-negative bacteria, targeting specific cellular structures [8], as shown in **Figure 1**.

According to Tortora *et al.* [11], drugs generally act on five main targets: inhibition of cell wall biosynthesis, inhibition of protein synthesis, nucleic acid (RNA

and DNA) biosynthesis, alterations in cell membrane permeability, and folic acid metabolism. Antibiotics such as clarithromycin and rifampicin act on cell wall biosynthesis by blocking transcription and inhibiting RNA synthesis. Resistance mechanisms may include structural changes in RNA polymerase, reduced cellular permeability to antimicrobials, alterations in the receptor site on the 50S ribosomal subunit, and enzymatic inactivation [12].

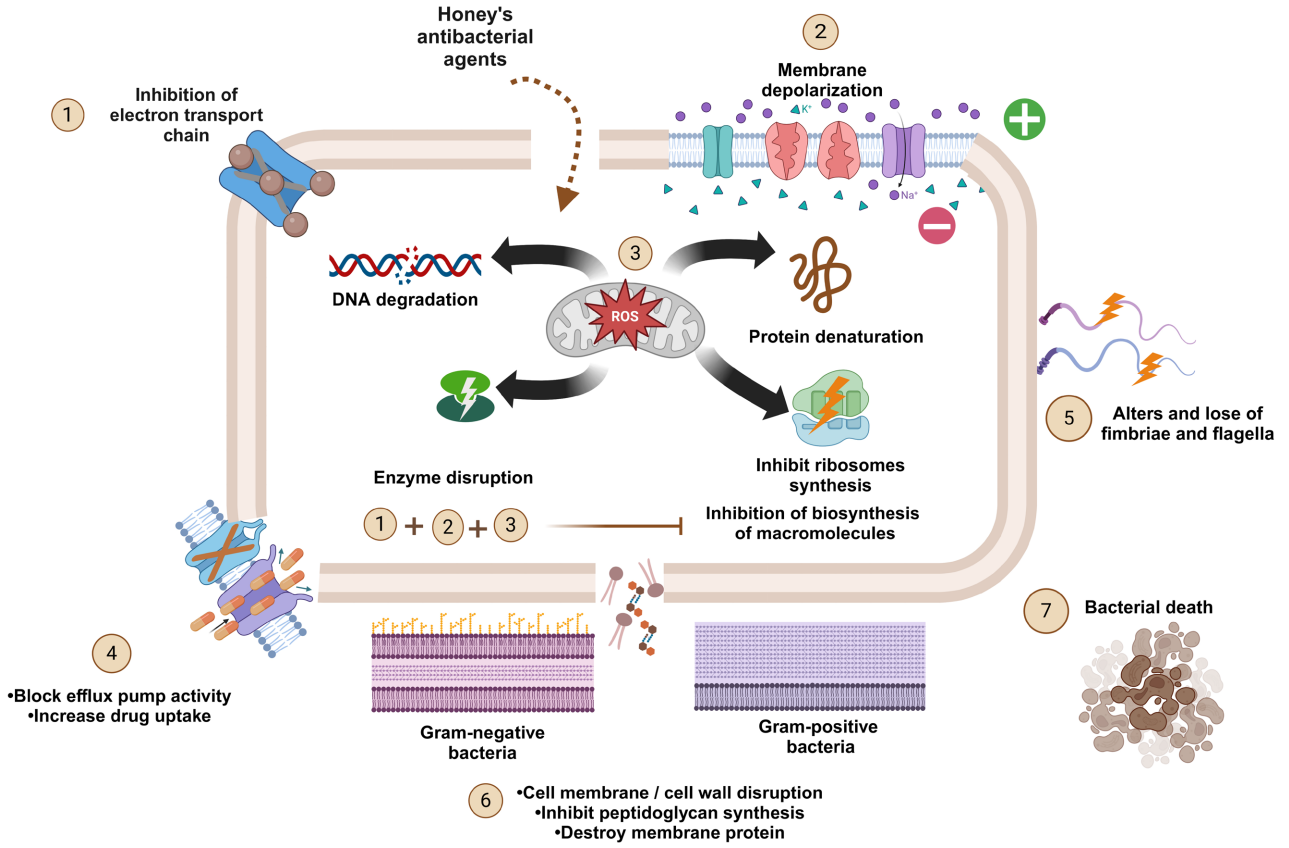

Figure 1. Schematic representation of mechanisms responsible for honey's antibacterial agents against pathogenic bacterial cells. 1 and 2—Methylglyoxal and phenolic compounds increase membrane permeability, depolarize the membrane, and inhibit the electron transport chain. 3—Generation of reactive oxygen species, as illustrated in the figure. These three pathways inhibit macromolecule biosynthesis. 4—MGO and phenolic compounds enhance drug uptake and inhibit efflux pump activity due to membrane potential collapse. 5—MGO reduces motility and interferes with flagella synthesis. 6—Phenolic compounds, MGO, and bee defensin disrupt cell wall and membrane integrity by inhibiting peptidoglycan and membrane protein synthesis. 7—Lysis and death of bacterial cells. Created in BioRender. Menezes Aleixo, M. (n.d.) https://BioRender.com/e47x932.

There is a need to study the ability of natural substances such as honey to assist the action of antibiotics, including bacterial membrane permeability. This approach would offer a promising alternative for treating infections caused by multidrug-resistant bacteria, which represent a growing public health threat [13]. Honey can transfer many therapeutic properties of plants and is considered to be an essential natural resource for new treatments, avoiding the side effects often associated with synthetic chemical drugs [14].

Brazil ranks as the tenth-largest honey producer globally. Its physicochemical

characteristics and quality depend on the climate, maturation stage, bee species, processing and storage technologies, and floral origin [15] [16]. For example, the floral origin influences the composition of phenolic compounds, proteins, minerals, sugars, and other components in honey [16].

Studies conducted in municipalities within Brazil's Central-West Region have shown that local honey exhibits anti-*Helicobacter pylori* potential, with up to 94% bacterial growth inhibition [17] [18]. The phytochemical composition of honey largely reflects the secondary metabolites present in the plants of its botanical origin, such as flavonoids and phenolic compounds, which are well-known for their antimicrobial properties, thereby substantiating the observed antibacterial activity. However, the effect of honey combined with antibiotics has not yet been studied.

Despite this honey antibacterial potential, studies exploring the combined effects of honey and antibiotics remain scarce. In this way, this study aimed to evaluate the bacterial outer membrane permeability mechanisms, by proceeding the synergistic effects of *Apis mellifera* honey from Brazil's Central-West Region and with known antibiotics, as well as the bioactive compounds in the honey samples and its botanical origin. By testing the hypothesis that honey enhances antibiotic efficacy through membrane disruption, this research seeks to propose an innovative strategy for combating multidrug-resistant bacteria. For sure, other mechanisms of action must be evaluated later.

2. Materials and Methods

2.1. Study Design

An experimental study was conducted to evaluate the effect of honey obtained from *Apis mellifera* on the permeability of the bacterial outer membrane against *Staphylococcus epidermidis*, *Helicobacter pylori*, and *Enterococcus faecalis*.

2.2. Study Area and Period

The analyzed honey samples were produced by *Apis mellifera* and were collected in Brazil's Central-West Region from six apiaries located in the municipalities of Sinop and Porto dos Gaúchos (within the Amazon Biome), Cuiabá (96% Cerrado and 4% Pantanal), Santo Antônio do Leverger (62% Cerrado and 38% Pantanal), and Cáceres (86% Pantanal, 8% Cerrado, and 6% Amazon), all located in the state of Mato Grosso (**Figure 2**).

These municipalities were selected for their potential antioxidant and antibacterial properties [18], linked to the total polyphenol content (TPC) and total flavonoid content (TFC) of the honey.

2.3. Sample Colletion and Preparation for Honey Extraction

Six fresh honey samples were studied: honey 1 and 2 (Cáceres), honey 3 (Cuiabá), honey 4 (Sinop), honey 5 (Porto dos Gaúchos), and honey 6 (Santo Antônio do

Leverger). The honey samples were previously diluted in water (1:1). The extraction involved sequential steps using hexane and ethyl acetate (from lower to higher polarity) in separatory funnel, as shown in **Figure 3**.

The collected fractions were concentrated using a rotary evaporator. Subsequently, the extracts were dried in ceramic capsules at 37˚C until a constant weight was achieved. The absence of solvent residues in the final extracts was confirmed through odor and viscosity assessments [19].

The honey extraction process employed a fractionated methodology specifically designed to isolate bioactive compounds according to their polarity. Hexane and ethyl acetate were selected as solvents due to their efficiency in extracting nonpolar and moderate polar compounds, respectively, thereby facilitating the comprehensive recovery of bioactive constituents [19].

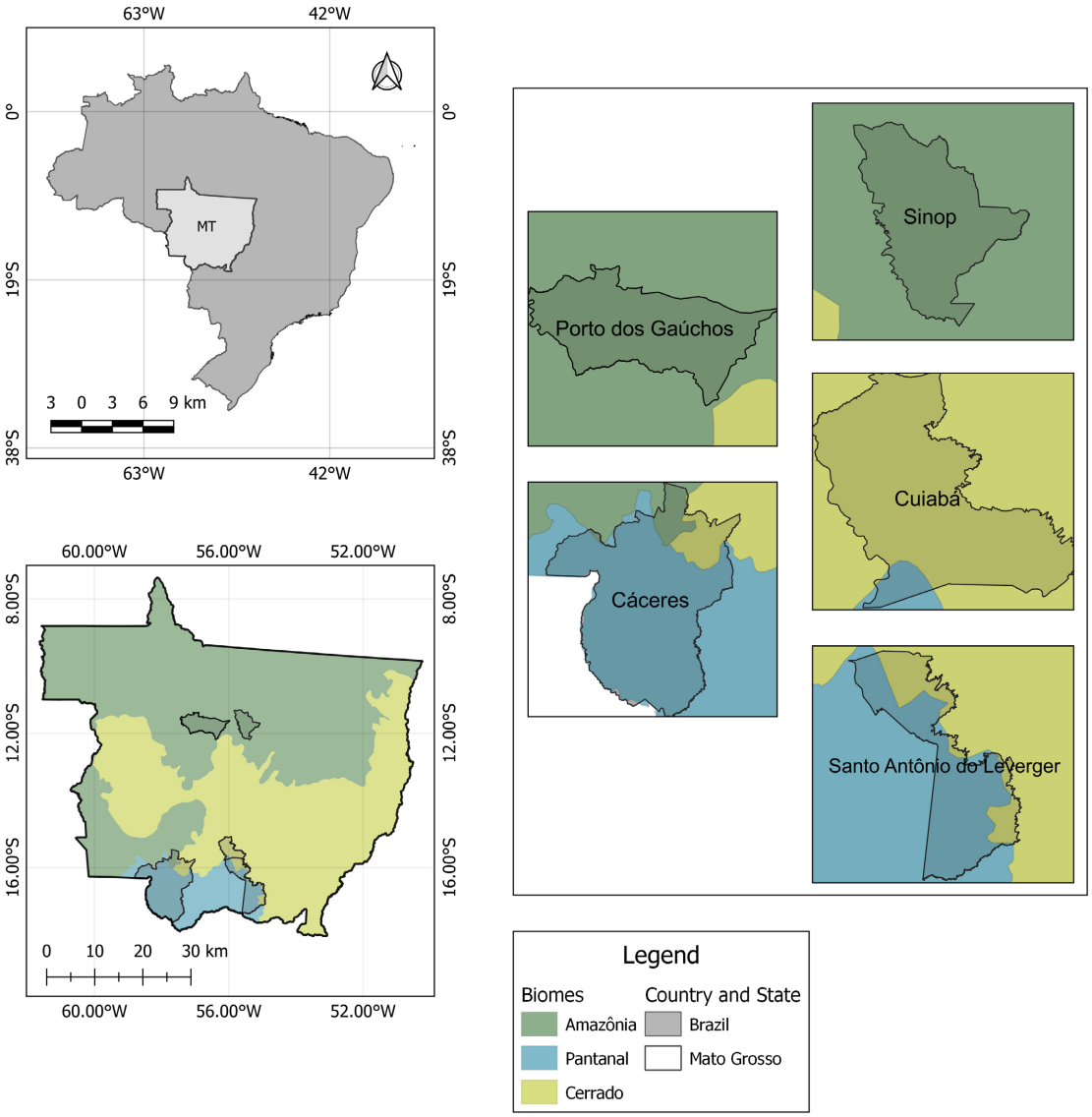

Figure 2. Geographical distribution of selected municipalities in Brazil's Midwest region for this study, categorized by biomes.

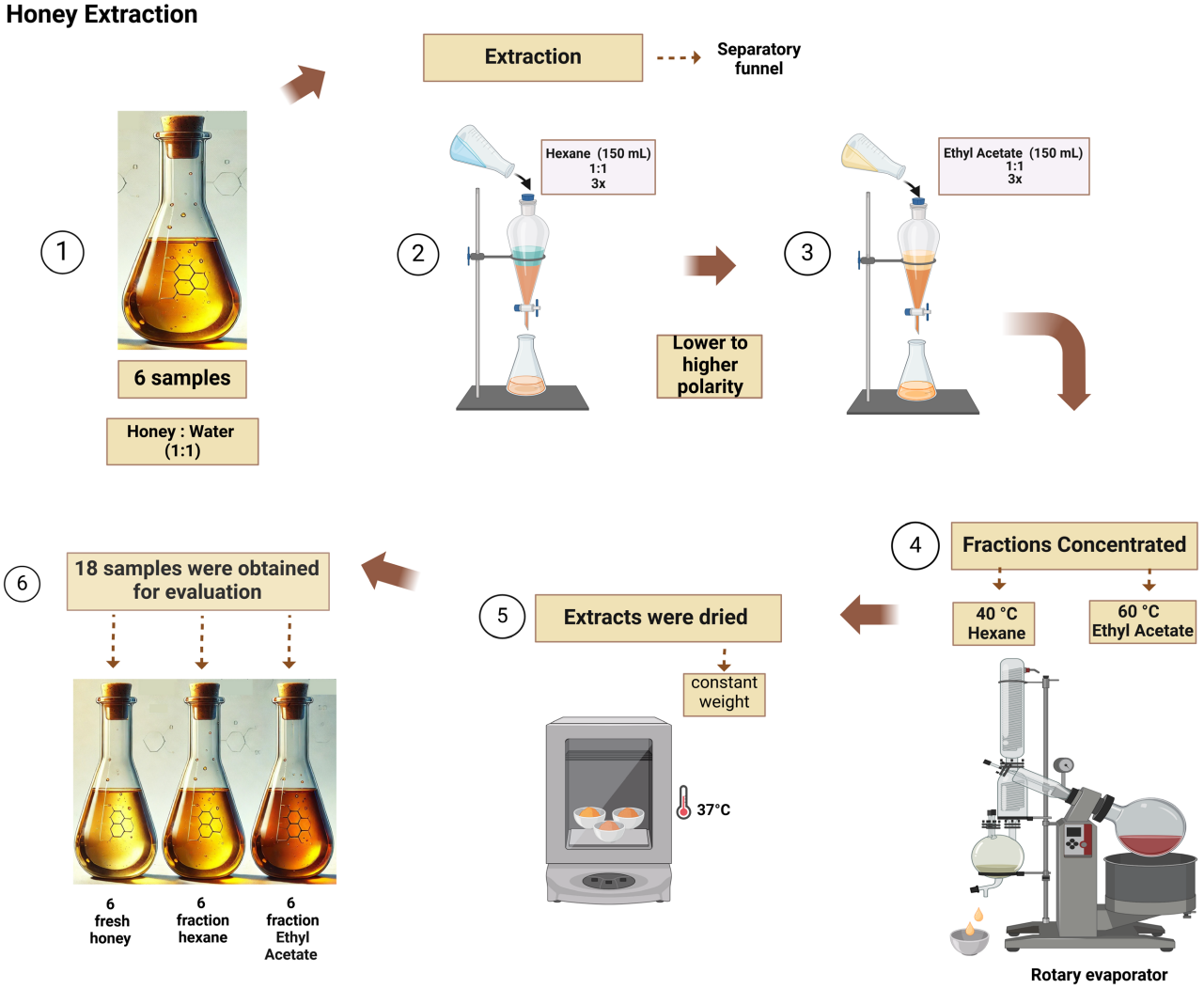

Figure 3. Diagram to summarize the honey extraction process. Created in BioRender, Menezes Aleixo, M. (2024) https://BioRender.com/y78r161.

2.4. Bacterial Strains and Media

The gram-positive bacteria *S. epidermidis* (ATCC 12228, São Paulo, Brazil) and *E. faecalis* (ATCC 29212, São Paulo, Brazil), and the gram-negative bacterium *H. pylori* (ATCC 43504, MD, USA) were used in this study. These strains are part of the collection maintained at the Cet*Apis* Laboratory (Center for Apiculture Studies). The antimicrobial susceptibility of *Staphylococcus epidermidis*, *H. pylori*, and *Enterococcus faecalis*, was determined based on the minimum inhibitory concentration (MIC) method, following the guidelines of the CLSI [16].

2.5. Inoculum Preparation

The bacterial cultures were stored at −80˚C in nonfat milk and reactivated from stock cultures in MH and BHI broths, and cultured on agar for 24 h at 37˚C. For *H. pylori*, the BHI broth was enriched with 5% fetal bovine serum (FBS) and Skirrow supplement (1:500), followed by cultivation for 72 hours at 37˚C under

microaerophilic conditions using a candle jar [20].

The culture age was at the exponential (logarithmic) phase. The bacterial growth was harvested using saline sterile water, its absorbance adjusted at 450 nm to a viable cell count of 10^8 CFU/spot for gram-negative bacterium and 10^7 CFU/spot for gram-positive bacterium using a spectrophotometer (MR-96A Spectrophotometer—Mindray, Brazil).

2.6. Evaluation of the Antibacterial Activity and Effect of Honey on the Permeability of the Outer Membrane

The antibacterial activity of fresh honey and its hexane and ethyl acetate fractions was evaluated based on the MIC method, using serial dilutions from 0.04 to 20 μg/mL [16].

The effect of honey on bacterial outer membrane permeability, in combination with antibiotics, was assessed using a serial dilution and combination assay (checkerboard) as described by Johnson *et al.* [21] with modifications. The positive controls included rifampin, clarithromycin, and vancomycin, each tested at an initial concentration of 20 μg/mL, which was then subjected to serial dilutions (0.04 - 20 μg/mL).

According to Mukherjee *et al.* [22], the concentration of 20 μg/mL (above MIC) for rifampicin, clarithromycin, and vancomycin in antimicrobial activity studies is widely supported by scientific and methodological factors that ensure the efficacy and relevance of the assays. This concentration is ideal for evaluating antimicrobial effects and synergistic potential in combinations, as demonstrated by studies highlighting the efficacy of clarithromycin and vancomycin against *Mycobacterium abscessus* and rifampicin for *S. epidermidis* [22] [23]. The assay was performed in triplicate.

The bacterial inoculum concentrations were adjusted to 1.5×10^8 colony-forming units (CFU)/mL (0.5 McFarland scale) for *E. faecalis* and *S. epidermidis*, and 6×10^7 CFU/mL (2 McFarland scale) for *H. pylori*. Negative growth controls and sterility wells (media only) were included. Plates were incubated for 24 - 72 hours, and readings were taken using an enzyme-linked immunosorbent assay plate reader at 450 nm.

2.7. Bioactive Compounds

Determination of the Total Content of Polyphenols and Flavonoids in Honey

Bioactive compounds quantified in honey included polyphenols and flavonoids. The TPC was determined using the colorimetric method described by Singleton [24], with adaptations. Aliquots of 0.1 mL of honey aqueous solutions (1:1) were mixed with 0.5 mL of 10% Folin-Ciocalteu solution and 2.5 mL of 20% sodium carbonate, diluted to a final volume of 10 mL. After incubation for 2 hours, absorbance was measured at 765 nm. The experiment was performed in triplicate. The TPC was determined by comparison to a gallic acid standard curve (0 - 500 μg/mL), with the results expressed as milligrams of gallic acid equivalents per 100

g of honey (mg GAE/100 g).

The TFC was measured using the colorimetric method reported by Meda [25], with adaptations. Aliquots of 0.05 mL of honey solution were mixed with 1.5 mL of 2% aluminum chloride ($AlCl_3$ in 50% methanol), and the mixture was diluted to 5 mL with 50% methanol. After incubation for 30 minutes, absorbance was measured at 415 nm. The experiment was performed in triplicate. The TFC was calculated based on a quercetin standard curve (0 - 1000 μg/mL), with the results expressed as milligrams of quercetin equivalents per 100 g of honey (mg QE/100 g).

2.8. Botanical Origin of Honey

The botanical origin of honey was determined based on pollen analysis following [26]. Fresh honey samples were prepared for microscopic analysis of the pollen profiles, including qualitative identification of the pollen types and quantitative determination of the relative pollen grain frequency [27]. The pollen frequency was classified as predominant pollen (>45% of total pollen), secondary pollen (16% - 45%), important minor pollen (3% - 15%), and minor pollen (<3%) [27].

2.9. Data Analysis

Statistical analyses were conducted using R software (version 4.1.1, 2021), with the functions available in the CRAN Mirror HOWTO. The Kolmogorov-Smirnov test was used to verify binomial error distribution for percentage data. The results are presented using descriptive statistics, including the mean and standard deviation.

Antibacterial activity was assessed using arithmetic means. Comparisons between honey samples were conducted using descriptive statistics with 95% confidence intervals. The membrane permeability effects were evaluated by considering the mean inhibition of honey combined with antibiotics, which differed from the response pattern of isolated antibiotics, also accounting for the 95% confidence intervals.

3. Results

3.1. Evaluation of the Antibacterial Activity and Effect of Honey on Outer Membrane Permeability

Table 1 shows the effects of the honey samples and their fractions on *S. epidermidis*. When combined with rifampicin, honey 1 and its fractions affected the outer membrane permeability of *S. epidermidis*, with 100% inhibition of bacterial growth ($p \leq 0.05$). Statistical analysis showed this inhibition was significantly higher than rifampicin alone (95.1%). The hexane fraction of honey 5 combined with rifampicin and the ethyl acetate fractions of honey 1 and 6 combined with vancomycin demonstrated 100% bacterial inhibition, surpassing the vancomycin control (90.8%). Conversely, other honey samples and their fractions showed bacterial growth inhibition below the control levels or within the 95% confidence intervals, indicating no statistically significant effect on outer membrane permeability.

Table 1. Effect on the permeability of the outer membrane of the bacterium *Staphylococcus epidermidis* (%) of fresh honey and the hexane and ethyl acetate fractions of each honey from Mato Grosso.

Percentage of inhibition of *Staphylococcus epidermidis* [20 µg/mL]			
Products [20 µg/mL]	Rifampicin	Clarithromycin	Vancomycin
Fresh honey 1	100 ± 0**	98.3 ± 1.6^{ns}	92 ± 0*
Hexane 1	100 ± 0**	100 ± 0^{ns}	45.8 ± 1.1^{na}
Ethyl acetate 1	100 ± 0**	95.3 ± 1.7^{ns}	100 ± 0**
Fresh honey 2	100 ± 0^{ns}	88.5 ± 2.0^{na}	62.2 ± 2.8^{na}
Hexane 2	100 ± 0^{ns}	91.83 ± 0.5^{na}	91 ± 0na
Ethyl acetate 2	100 ± 0^{ns}	87.3 ± 3.9^{na}	59.7 ± 7.9^{na}
Fresh honey 3	54.4 ± 2.4^{na}	88.3 ± 1.1^{na}	59.4 ± 2.0^{na}
Hexane 3	41.6 ± 5.1^{na}	94.5 ± 4.8^{ns}	79.3 ± 1.4^{na}
Ethyl acetate 3	92.4 ± 0.9^{na}	82.7 ± 1.3^{na}	92.3 ± 0na
Fresh honey 4	28.9 ± 1.3^{na}	98.2 ± 1.8^{ns}	60.6 ± 1.7^{na}
Hexane 4	45.3 ± 7na	78.1 ± 1.7^{na}	86.7 ± 0na
Ethyl acetate 4	74.1 ± 3.7^{na}	89 ± 1.1^{na}	89.7 ± 0na
Fresh honey 5	55.9 ± 1.5^{na}	91.8 ± 0.9^{na}	94.5 ± 2.1^{ns}
Hexane 5	100 ± 0**	95.3 ± 3.9^{ns}	91.1 ± 0.6^{na}
Ethyl acetate 5	100 ± 0^{ns}	95.9 ± 2.2^{ns}	95.8 ± 0.1^{ns}
Fresh honey 6	96.9 ± 2.8^{ns}	99 ± 1.2^{ns}	91 ± 2.0^{ns}
Hexane 6	96.3 ± 1.9^{ns}	95.1 ± 2.9^{ns}	87.7 ± 1.5^{na}
Ethyl acetate 6	40.4 ± 0.3^{na}	93.6 ± 0.3^{na}	92.9 ± 1.4*
Rifampicin	95.1 ± 3.10	-	-
Clarithromycin	-	94 ± 2.87	-
Vancomycin	-	-	90.8 ± 3.09

na—Not applicable because the % inhibition of the antibiotic alone is higher than honey combined with the antibiotic. ns—Not significant at the 95% confidence interval. **$p \leq 0.05$—The products differ from each other at the 95% confidence interval. *$p \leq 0.05$—The products differ at the 90% confidence interval.

The other honeys and their fractions showed bacterial growth inhibition below the levels of the controls—Rifampicin (95.1%), clarithromycin (94%), and vancomycin (90.8%), or within the 95% confidence intervals, indicating no significant effect on outer membrane permeability.

Table 2 shows the effects of the honey samples and their fractions on *H. pylori*. When combined with rifampicin, honey 3 and its fractions demonstrated an effect on the outer membrane permeability of *H. pylori*, with a bacterial growth inhibition rate of 97.1%, 99.1%, and 97.3%, respectively, compared with rifampicin alone (95.6%). Statistical analysis confirmed that the differences were significant ($p \leq 0.05$).

Table 2. Effect on the permeability of the outer membrane of the bacterium *Helicobacter pylori* (%) of fresh honey and the hexane and ethyl acetate fractions of each honey from Mato Grosso.

Percentage of inhibition of *Helicobacter pylori* [20 µg/mL]		
Products [20 µg/mL]	Rifampicin	Clarithromycin
Fresh honey 1	91.7 ± 1.5^{na}	62.7 ± 0na
Hexane 1	89.1 ± 0.9^{na}	85.3 ± 3.6^{na}
Ethyl acetate 1	90.3 ± 0na	87.5 ± 2.4^{ns}
Fresh honey 2	87.6 ± 0.9^{na}	84.3 ± 4.7^{na}
Hexane 2	93 ± 3.6^{na}	96.1 ± 1.4*
Ethyl acetate 2	95.1 ± 1.5^{na}	87.3 ± 1.6^{na}
Fresh honey 3	97.1 ± 3.5*	82.7 ± 1.0^{na}
Hexane 3	99.1 ± 0.9*	92.7 ± 2.8^{na}
Ethyl acetate 3	97.3 ± 1.0*	93.4 ± 4na
Fresh honey 4	91.1 ± 0na	66.4 ± 6.2^{na}
Hexane 4	85.8 ± 1.6^{na}	88.9 ± 1.1^{na}
Ethyl acetate 4	89.6 ± 1.9^{na}	88 ± 0.6^{na}
Fresh honey 5	84.8 ± 1.5^{na}	83.4 ± 9.4^{na}
Hexane 5	96.4 ± 2.5*	91.8 ± 0.1^{na}
Ethyl acetate 5	89.8 ± 7.5^{na}	71.3 ± 1.0^{na}
Fresh honey 6	98.5 ± 1.5*	91.1 ± 2.8^{na}
Hexane 6	91.3 ± 6.9^{na}	93.3 ± 4.1^{na}
Ethyl acetate 6	90.2 ± 7.5^{na}	100 ± 0*
Rifampicin	95.6 ± 2.8	-
Clarithromycin	-	90.1 ± 22.6

na—Not applicable because the % inhibition of the antibiotic alone is higher than honey combined with the antibiotic. ns—Not significant at the 95% confidence interval. *$p \leq 0.05$—The products differ at the 90% confidence interval.

Honey 6 and its ethyl acetate fraction, combined with rifampicin (95.1%) and clarithromycin (90.1%), showed an inhibition rate of 98.5% and 100%, respectively. The hexane fraction of honey 2 combined with clarithromycin resulted in a 96.1% inhibition, surpassing the control (90.1%**).** These findings confirm the membrane permeability effect of these combinations on *H. pylori*.

The other honeys and their fractions did not exhibit statistically significant effects, with inhibition rates lower than the controls or within the 90% confidence interval. Fresh honeys and their fractions combined with vancomycin showed inhibition rates ranging from 17.3% to 34%, compared with 30.1% for vancomycin alone, with no significant differences observed.

Table 3 shows the effects of the honey samples and their fractions on *E. faecalis*. When combined with rifampicin, honey 5 and its fractions demonstrated an effect on the outer membrane permeability of *E. faecalis*, achieving 100% bacterial growth inhibition ($p \leq 0.05$), significantly surpassing rifampicin alone (93.6%).

The hexane and ethyl acetate fractions of honey 2, combined with clarithromycin, and the ethyl acetate fraction of honey 3, combined with vancomycin, also inhibited 100% of *E. faecalis* growth ($p \leq 0.05$), showing significant differences compared with clarithromycin (99%) and vancomycin (96.2%) alone.

Table 3. Effect on the permeability of the outer membrane of the bacterium *Enterococcus faecalis* (%) of fresh honey and the hexane and ethyl acetate fractions of each honey from Mato Grosso.

Percentage of inhibition of *Enterococcus faecalis* [20 μg/mL]			
Products [20 μg/mL]	Rifampicin	Clarithromycin	Vancomycin
Fresh honey 1	100 ± 0^{ns}	96.2 ± 4^{ns}	100 ± 0^{ns}
Hexane 1	100 ± 0^{ns}	100 ± 0^{ns}	100 ± 0^{ns}
Ethyl acetate 1	100 ± 0^{ns}	100 ± 0^{ns}	98.3 ± 1.6^{ns}
Fresh honey 2	87.9 ± 5.5^{na}	100 ± 0^{ns}	100 ± 0^{ns}
Hexane 2	100 ± 0^{ns}	$100 \pm 0^{**}$	100 ± 0^{ns}
Ethyl acetate 2	100 ± 0^{ns}	$100 \pm 0^{*}$	100 ± 0^{ns}
Fresh honey 3	100 ± 0^{ns}	100 ± 0^{ns}	22.1 ± 10^{na}
Hexane 3	100 ± 0^{ns}	100 ± 0^{ns}	$100 \pm 0^{**}$
Ethyl acetate 3	100 ± 0^{ns}	100 ± 0^{ns}	31 ± 4.6^{na}
Fresh honey 4	100 ± 0^{ns}	100 ± 0^{ns}	42.3 ± 5.3^{na}
Hexane 4	100 ± 0^{ns}	86.3 ± 2.4^{na}	80.7 ± 1.5^{na}
Ethyl acetate 4	100 ± 0^{ns}	99.2 ± 0^{ns}	30.1 ± 8^{na}
Fresh honey 5	$100 \pm 0^{*}$	100 ± 0^{ns}	100 ± 0^{ns}
Hexane 5	$100 \pm 0^{**}$	98.6 ± 1.2^{na}	99.8 ± 0.1^{ns}

Continued

Ethyl acetate 5	100 ± 0*	94.8 ± 1.6^{na}	100 ± 0^{ns}
Fresh honey 6	100 ± 0^{ns}	98.1 ± 1.5^{na}	97.6 ± 1.2^{na}
Hexane 6	100 ± 0^{ns}	97.5 ± 3.4^{na}	100 ± 0^{ns}
Ethyl acetate 6	82.6 ± 0na	98.8 ± 0na	99.8 ± 0.1^{ns}
Rifampicin	93.6 ± 18.6	-	-
Clarithromycin	-	99 ± 2.26	-
Vancomycin	-	-	96.2 ± 12.2

na—Not applicable because the % inhibition of the antibiotic alone is higher than honey combined with the antibiotic. ns—Not significant at the 95% confidence interval. **p ≤ 0.05—The products differ from each other at the 95% confidence interval. *p ≤ 0.05—The products differ at the 90% confidence interval.

The other honey samples and fractions showed inhibition rates below those of the controls—rifampicin (93.6%), clarithromycin (99%), and vancomycin (96.2%), or within the 95% confidence intervals, indicating no statistical effect on outer membrane permeability.

Considering the *S. epidermidis*, *H. pylori*, and *E. faecalis*, the MIC was not detected at concentrations up to 20 μg/mL for the honey samples and their hexane and ethyl acetate fractions, suggesting that the observed effects are related to enhanced outer membrane permeability rather than direct bactericidal activity.

3.2. Determination of the TPC and TFC in the Honey Samples

Table 4 presents the TPC and TFC. The TPC in the six fresh honey samples ranged from 26.8 to 80.02 mg GAE/100 g of honey, with the highest content in honey 1, 2, 3, and 6. The TFC ranged from 3.4 to 11.3 mg QE/100 g of honey, with the highest content in honey 2, 3, and 4.

Table 4. Total polyphenol and flavonoid content (results expressed as mean ± standard deviation) of *Apis mellifera* honey from the Midwest Region of Mato Grosso, Brazil.

Products [20 μg/mL]	County	Polyphenols[1]	Flavonoids[2]	Registration No. CET*Apis*[3]
Honey 1	Cáceres	40.4 ± 3.1	3.46 ± 2.2	04
Honey 2	Cáceres	72.4 ± 1.9	6.74 ± 0.9	05
Honey 3	Cuiabá	41.0 ± 0.1	1.78 ± 0.2	06
Honey 4	Sinop	26.8 ± 1.3	11.32 ± 2.9	22
Honey 5	Santo Antônio do Leverger	33.7 ± 2.2	5.25 ± 1.1	25
Honey 6	Porto dos Gaúchos	80.0 ± 1.3	5.25 ± 1.1	26

[1](mg equivalent of Gallic Acid/100 g of honey ± standard deviation); [2](mg equivalent of Quercetin/100 g of honey ± standard deviation); [3]Laboratory of the Center for Apiculture Studies—UNEMAT.

3.3. Botanical Origin of the Honey Samples

The pollen profile and concentrations were classified following the methodology described by da Luz *et al.* [27]. *Myracrodruon urundeuva* Allemão (aroeira) was the predominant pollen in honeys 1 and 2 from Cáceres, honey 3 from Cuiabá, and honey 5 from Santo Antônio do Leverger. In honey 4 and 6, the predominant pollen types were *Moraceae* Gaudich and *Protium* sp., respectively (Table 5).

Table 5. Pollen diversity of plant species found in *Apis mellifera* honey from the Midwest region of Mato Grosso, Brazil.

Products	Pollen			
	Predominant	Secondary pollen	Important minor pollen	Minor pollen
Honey 1	*Myracrodroun urundeuva*	*Mimosa pudica* *Protium* sp.	*Astronium fraxinifolium*	*Senna rugosa*; *Marcetia* sp.
Honey 2	*Myracrodroun urundeuva*	-	*Protium* sp.; *Cecropia pachystachya*	*Astronium fraxinifolium*; *Serjania glabrata*; *Bidens gardeneri*; *Myrcia sylvatica*; *Senna rugosa*; *Acacia* sp.
Honey 3	*Myracrodroun urundeuva*	-	*Senna rugosa*; *Mimosa pudica*; *Acacia* sp. *Protium* sp.	*Bidens gardeneri*
Honey 4	Moraceae	*Myracrodroun urundeuva*	*Cecropia pachystachya*; *Mimosa pudica*; *Eupatorium pauciflorum*; *Spermacoce verticillata*; *Brachiaria* sp.	*Alternanthera brasiliana*; *Protium* sp.
Honey 5	*Myracrodroun urundeuva*	-	*Mimosa pudica*; *Marcetia sp.*; *Senna rugosa*; *Protium* sp.	-
Honey 6	*Protium* sp.	*Myracrodroun urundeuva*	*Brachiaria sp.*; *Mimosa pudica*; *Byrsonima* sp.	*Alternanthera brasiliana*; *Miconia ferruginea*; *Bidens gardeneri*; *Cecropia pachystachya*; *Myrcia sylvatica*

Honey 1 presented *Mimosa pudica* (dormideira) and *Protium* sp. as important minor pollens. Honey 4 and 6 shared *M. urundeuva* as secondary pollen, while honey 2, 3, and 5 did not present secondary pollen. Honey 1, 2, 3, and 5 shared *Senna rugosa* (G. Don) H.S. Irwin & Barneby (fedegoso) as important minor pollen, while honey 6 uniquely presented *Byrsonima* sp. (murici) and *Miconia ferruginea* (pixirica) as important minor pollen.

There were 13 additional distinct species across all honeys: *Astronium fraxinifolium* (gonçaleiro), *M. pudica*, *Cecropia pachystachya* Trécul., *Bidens gardeneri* Baker (picão), *Myrcia sylvatica* (purpuna), *Brachiaria* sp., *Eupatorium pauciflorum* (cambará falso), *Alternanthera brasiliana* L. Kuntze (carrapichinho), *Spermacoce verticillata* (vassourinha de botão), *Acacia* sp. (acácia), *Protium* sp. (breu branco), *Serjania glabrata* (cipó-uva), and *Marcetia* sp.

4. Discussion

Our results provide insights into the effects of honey from Mato Grosso, Central-West Brazil, on the membrane permeability of *S. epidermidis*, *H. pylori*, and *E. faecalis*. Honey 1 from Cáceres, honey 3 from Cuiabá, honey 6 from Porto dos Gaúchos, and honey 5 from Santo Antônio do Leverger, when combined with rifampicin, demonstrated an effect on the membrane permeability of *S. epidermidis*, *H. pylori*, and *E. faecalis*, respectively.

Honey 1 presented two unique secondary pollen types: *M. pudica* (16.5%) and *Protium* sp. (24.4%) [28]. Both species are rich in bioactive compounds that contribute to their antibacterial activities (**Figure 4**).

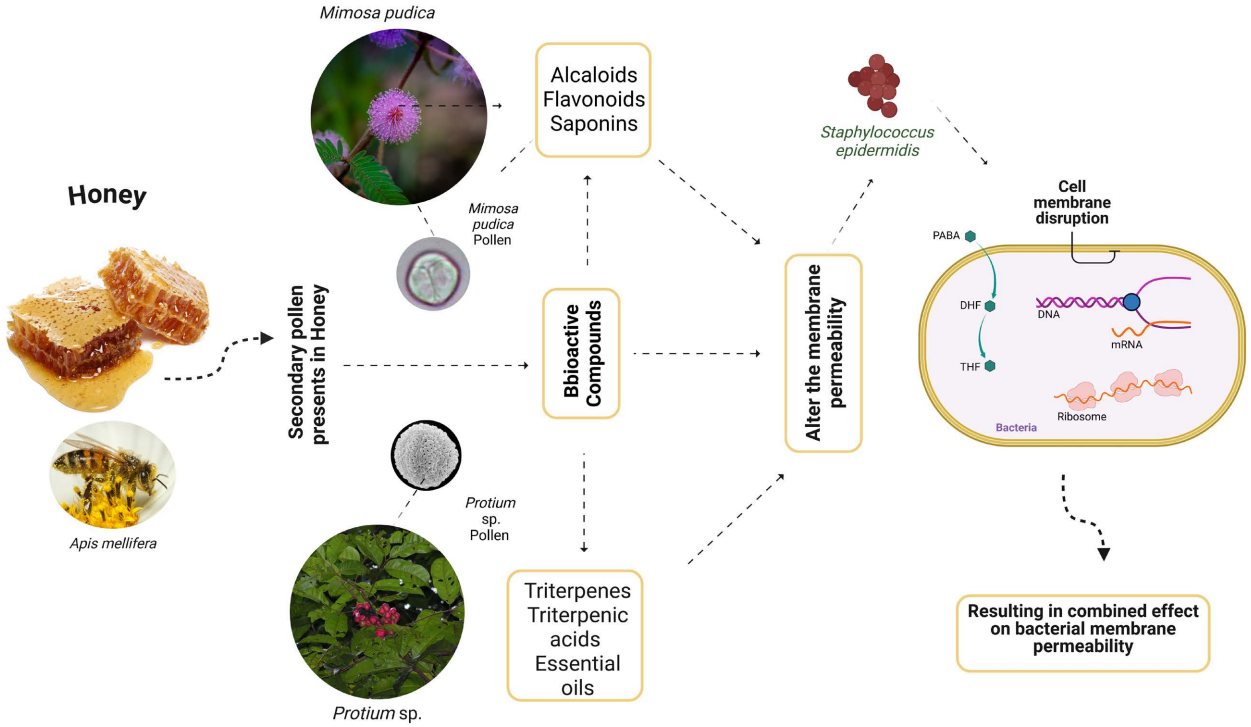

Figure 4. Summary of the potential mechanism of secondary pollen found in Honey 1 with combined effect against *Staphylococcus epidermidis*. Created in BioRender, Menezes Aleixo, M. L. (2024) https://BioRender.com/t03j915.

The resin of Protium sp. contains triterpenes such as α-amyrin and *β*-amyrin, triterpenic acids (ursolic and oleanolic), coumarins, and essential oils, which can destabilize cellular membranes and interfere with protein synthesis in bacteria such as *Staphylococcus aureus* and *Escherichia coli* [29].

Mimosa pudica contains alkaloids, steroids, flavonoids, tannins, and saponins, which affect bacterial membrane permeability, leading to a loss of cellular integrity. These compounds also interfere with nucleic acid and protein synthesis, disrupting bacterial energy metabolism and inhibiting bacterial growth and survival [30].

These effects are consistent with findings from research on Manuka honey, renowned for its high methylglyoxal (MGO) content, which exhibits comparable impacts on bacterial membrane permeability through depolarization and the

inhibition of energy metabolism [7]. Similarly, studies on Hungarian honey emphasize the role of phenolic compounds in destabilizing bacterial membranes and disrupting biofilm formation, particularly in gram-positive bacteria [10]. Collectively, these findings underscore the universal potential of bioactive compounds derived from diverse botanical and natural sources to enhance bacterial membrane permeability and inhibit critical metabolic pathways.

This activity may be justified by the presence of these important minor pollen types, which, although not predominant, are sources of compounds that can act synergistically to enhance antibacterial activity. For example, flavonoids play a crucial role by inhibiting essential bacterial enzymes such as DNA gyrase, preventing bacterial DNA replication. Furthermore, these compounds increase bacterial cell membrane permeability, allowing toxic substances to enter and leading to cell death [31].

Saponins interact directly with the lipids of the cell membrane, resulting in destabilization and eventual cell lysis. Terpenoids interfere with denosine triphosphate (ATP) production, essential for bacterial metabolism, while tannins form complexes with bacterial proteins, destabilizing the cell wall. According to De-Melo *et al.* [15], this diversity in honey composition can influence its chemical profile, enabling various activities based on the predominance of specific individual components. The synergistic action of these compounds aligns with a broader pattern observed in the studies of De-Melo *et al.* [15], demonstrating that the diversity of bioactive constituents in honey and plants directly enhances their capacity to increase bacterial membrane permeability, highlighting their potential as effective antimicrobial agents.

Fresh Honey 3, fresh Honey 6 and its ethyl acetate fraction exhibited an effect on the membrane permeability of *H. pylori* when combined with rifampicin and clarithromycin, respectively. Honey 3 and 6 presented as important minor pollen, *Senna rugosa* and *Byrsonima* sp. with frequencies of 13.7% and 10.3%, respectively [28]. The ability of these honey to affect *H. pylori* membrane permeability may be related to the presence of *Senna rugosa* and *Byrsonima* sp. (**Figure 5**).

Fresh Honey 5 affected the membrane permeability of *E. faecalis* when combined with rifampicin, and also presented *S. rugosa* as an important minor pollen, with frequency of 10.3% [28] (**Figure 6**).

Indeed, the leaf root extracts of this plant include bioactive compounds such as catechins, rutin, epigallocatechin derivatives, kaempferol glycosides, luteolin, dimetric and trimetric procyanidins, stilbenes, naphthopyranones, and flavanones [32]. Other compounds in the genus Senna include alkaloids (piperidine, cassin, and pyridine), anthraquinones (emodin, chrysophanol, aloe-emodin, rhein, physcion, chrysophanol-8-O-glucoside), flavonoids (kaempferol, quercetin, rutin, rhamnetin, cyanidin, hesperidin, gallocatechin, and luteolin), phenolics (tannins, gallic acid, and chlorogenic acid), glycosides, steroids (stigmasterol, β-sitosterol, and daucosterol), terpenoids (β-sitosterol, triterpenoids, sesquiterpenoids, and terpinolene), saponins, and volatile oils [33].

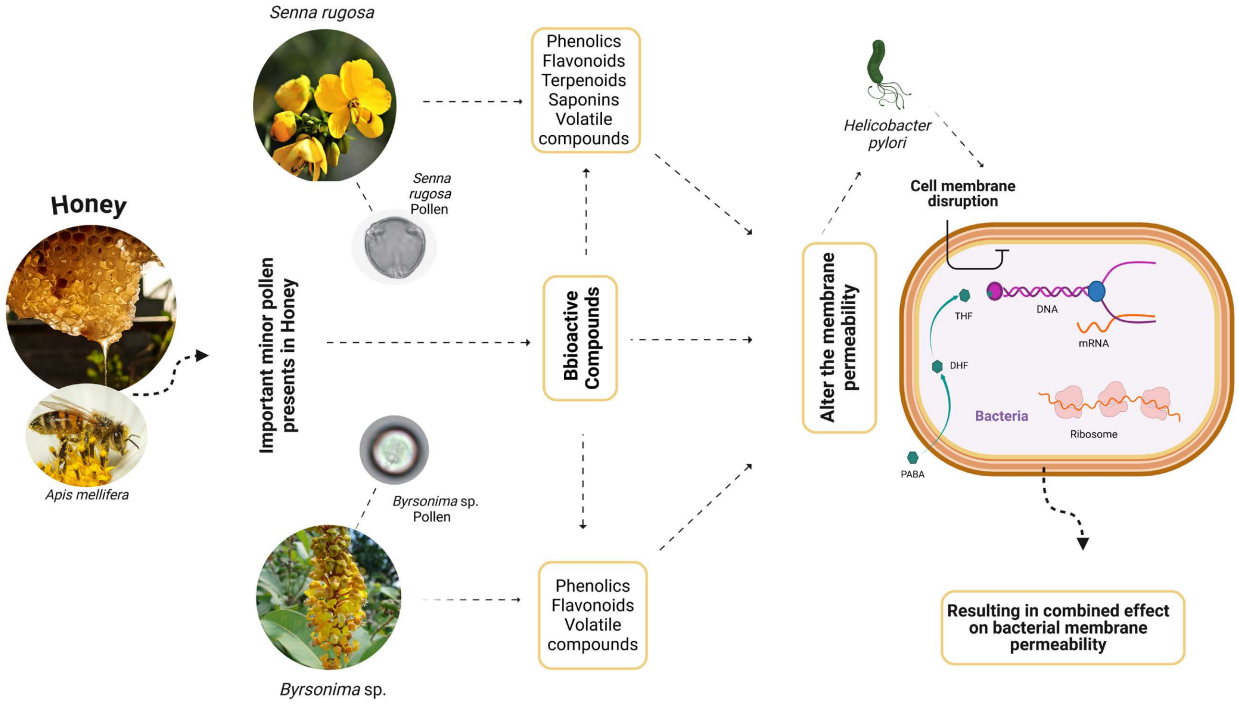

Figure 5. Summary of the potential mechanism of minor pollen found in Honey 3 and Honey 6 with combined effect against *Helicobacter pylori*. Created in BioRender, Menezes Aleixo, M. L. (2024) https://BioRender.com/f84g929.

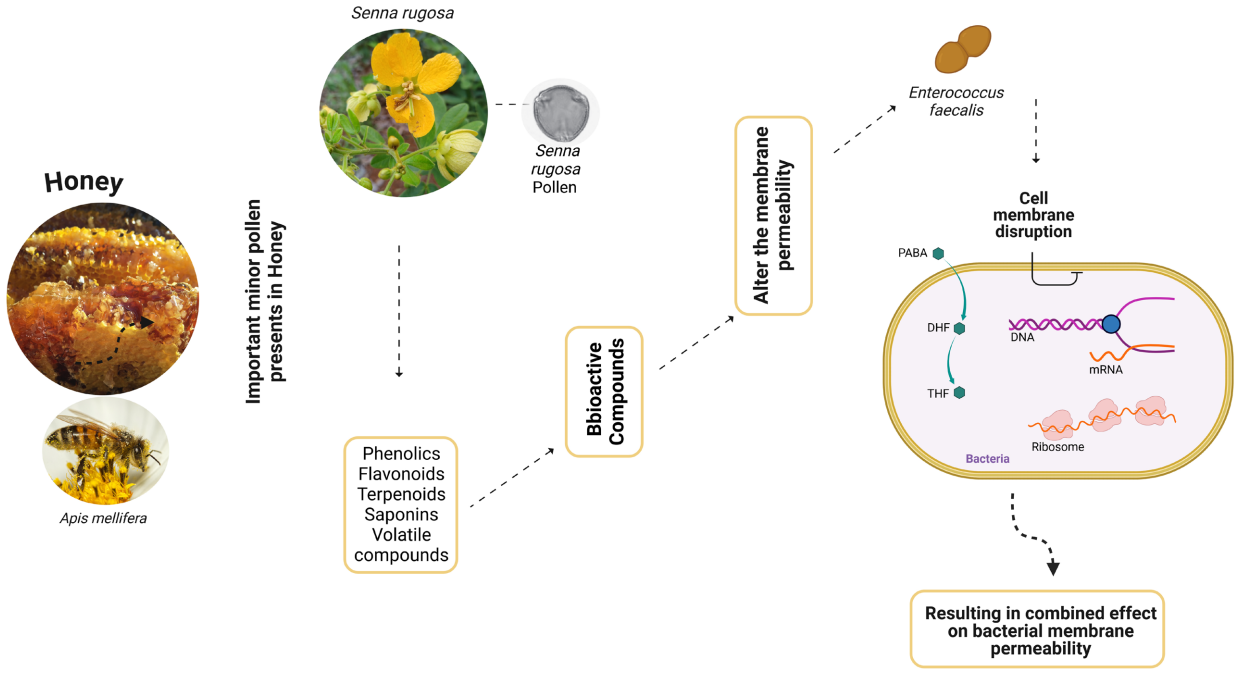

Figure 6. Summary of the potential mechanism of minor pollen found in Honey 5 with combined effect against *Enterococcus faecalis*. Created in BioRender, Menezes Aleixo, M. L. (2024) https://BioRender.com/p88k687.

These bioactive compounds exert antimicrobial activity. Alkaloids and flavonoids such as quercetin and kaempferol interfere with protein synthesis and compromise cell membrane integrity, while tannins and phenolic acids form

complexes with bacterial proteins, inhibiting microbial growth [31].

Anthraquinones, steroids, and glycosides also affect DNA replication and membrane permeability, while terpenoids and saponins primarily target the bacterial cell membrane. Terpenoids disrupt metabolic processes, and saponins cause membrane rupture, leading to cell death. Additionally, glycosides act as prodrugs activated in bacterial cells, and volatile oils disrupt bacterial membrane permeability and inhibit cellular respiration, enhancing antimicrobial efficacy [34].

These mechanisms may explain the promising activity of *S. rugosa* against gram-negative bacteria such as *Proteus mirabilis*, *Salmonella Typhimurium*, *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, and *Shigella flexneri*, as well as gram-positive bacteria such as *S. aureus*, *S. epidermidis*, *Streptococcus agalactiae*, and *E. faecalis* [35] [36].

Honey 6 is an important minor pollen with a frequency of 10.1%. This effect may be related to the presence of *Byrsonima* sp. as an important minor pollen with a frequency of 10.1% [28]. *Byrsonima* sp. contains bioactive compounds such as phenolic acids (gallic, caffeic, coumaric, and chlorogenic), flavonoids (catechin, epicatechin, rutin, taxifolin, quercetin, and kaempferol), carotenoids (lutein, zeaxanthin, β-carotene, and β-cryptoxanthin), and volatile compounds (esters, aldehydes, and ketones) [37].

These compounds are associated with various biological actions, including central nervous system modulation and wound healing, anti-inflammatory, antidiabetic, anticancer, antioxidant, antiviral, and antibacterial activities against *K. pneumoniae*, *H. pylori*, *S. aureus*, *S. epidermidis*, *E. faecalis*, *Mycobacterium phlei*, *Bacillus subtilis*, and *Bacillus cereus* [37].

The compounds in *Byrsonima* sp. have shown promising activity against *H. pylori* through mechanisms such as biofilm disruption, inhibition of urease, DNA damage, and interference with protein synthesis, impairing vital bacterial functions [38] [39].

The ethyl acetate fraction efficiently extracts intermediates such as flavonoids, tannins, phenolic acids, triterpenes, and proanthocyanidins found in both *Byrsonima* sp. and honey, which may contribute to the observed membrane permeability effect in *H. pylori*, highlighting this fraction's antimicrobial role [38].

Another factor that influences the effects of honey is the class of compounds in its different fractions. As demonstrated by Sugiyarto *et al.* [40], ethyl acetate fractions contain phenolic compounds and alkaloids known for their ability to disrupt bacterial cell membranes and to inactivate enzymes, leading to cell lysis. Hexane fractions contain alkaloids and tannins, which also contribute to antimicrobial activity, albeit less intensely than phenolics. The ethyl acetate fractions, in particular, exhibited pronounced effects, aligning with findings by Sugiyarto *et al.* [40] which suggest that intermediate-polarity compounds, such as flavonoids and tannins, are especially effective in disrupting bacterial membranes.

The antibacterial mechanism of honey involves several biochemical factors that enhance its efficacy. Defensin-1, an antimicrobial peptide secreted by the bee

hypopharyngeal glands, is one of the main compounds responsible for honey's activity against both gram-positive and gram-negative bacteria [10].

In addition to defensin-1, enzymes and phenolic compounds play crucial roles in combating bacterial infections. This synergistic action among honey components is particularly significant given the increasing resistance of bacteria to conventional antibiotics, positioning honey as a promising alternative for developing new antibacterial strategies [8] [10].

The honey samples evaluated in this study had a TPC of 26.8 - 80 mg GAE/100 g and a TFC of 3.4 - 11.0 EQ/100 g of honey. Honey 1, 2, 3, and 6 had the highest TPC (40.4 - 80 mg GAE/100 g), comparable to monofloral honey from Minas Gerais in the Southeast Region of Brazil and various regions in Italy (4.88 - 40.7 mg GAE/100 g) [41]-[43], and from Turkey and Tunisia (16.02 - 120.04 mg GAE/100 g) [44].

In contrast, the TFC of the honey samples from the present study is lower than that of honey from the Southeast Region of Brazil and regions in Malaysia (29.17 - 91.25 mg EQ/100 g) [43] [45].

The botanical origin of most of the analyzed honey samples aligns with honey from other areas of Mato Grosso. The samples that presented the best results shared similar floral diversity, particularly among secondary and important minor pollen species. Climate, biome, and botanical diversity play crucial roles in shaping honey's composition, chemical profile, and bioactive properties, directly influencing its quality and potential therapeutic benefits [15].

The bioactive compounds identified in the pollen of the honey samples analyzed in this study are likely to act synergistically, contributing to the observed antibacterial activity. This complex composition underscores the critical role of botanical diversity in shaping the therapeutic properties of honey, emphasizing that its antimicrobial efficacy arises from the integrated action of multiple bioactive constituents rather than the influence of a single compound.

Collectively, these findings strengthen the hypothesis that the unique phytochemical profiles of honey from diverse botanical origins contribute significantly to their antimicrobial potential. Further comparative studies on honey from different regions would provide deeper insights into the role of bioactive compounds in antimicrobial mechanisms, advancing their therapeutic applications.

5. Conclusions

When combined with rifampicin and clarithromycin, four honey samples from the Central-West Region altered the membrane permeability of *S. epidermidis*, *H. pylori*, and *E. faecalis*, an ability that is associated with the presence of bioactive compounds and the botanical origin of the honey. These results confirmed the hypothesis of this study: Combination of honey with antibiotics enhanced the efficacy of these drugs due to the disruption of the bacterial membrane.

The joint action of these bioactive compounds makes them promising candidates for improving the effectiveness of antimicrobial treatments by facilitating

the entry of antibacterial substances into cells. Honey's composition includes various compounds that act on multiple target sites in an additive or synergistic manner.

Additionally, the presence of bioactive compounds related to the species *S. rugosa*, *Protium* sp., *M. pudica*, and *Byrsonima* sp., specifically in honeys 1, 3, 5, and 6, contributed to their effects on membrane permeability.

Of note, the honey samples alone did not exhibit antibacterial activity against the three tested bacteria.

The combined use of honey and antibiotics can reduce the concentrations of drugs needed to achieve efficacy, thereby limiting the likelihood of resistance development and potentially reversing the susceptibility of some antibiotic-resistant bacteria.

The effect of honey on the permeability of the outer membrane of bacteria is undoubtedly the most significant finding of this study, but the mechanism underlying this permeability requires further investigation.

Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) in the form of scholarship grant to Mariana Lenina Menezes Aleixo (CAPES/Demanda Social, Proc. No 1602288). The authors gratefully financial support for this project, Fundação de Amparo à Pesquisa do Estado de Mato Grosso (FAPEMAT—Proc. No. 62437/2013).

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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