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Evaluación de la actividad antimicrobiana de mieles de abejas nativas sin aguijón (Tribu: *Meliponini*) en biopelículas

Tesis en torno a una hipótesis o problema de investigación y su contrastación

Fausto Sebastián Cabezas Mera

António Machado Ph.D
Director de Trabajo de Titulación

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EVALUACIÓN DE LA ACTIVIDAD ANTIMICROBIANA DE MIELES DE ABEJAS NATIVAS SIN AGUIJÓN (TRIBU: *MELIPONINI*) EN BIOPELÍCULAS

FAUSTO SEBASTIÁN CABEZAS MERA

Firmas

Antonio Machado, Ph.D.

Director de Trabajo de Titulación

José Miguel Álvarez, Ph.D

Director del Programa de Microbiología

Patricio Rojas, Ph.D

Decano del Colegio de Ciencias Biológicas
y Ambientales

Hugo Burgos, PhD

Decano del Colegio de Posgrados

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Nombre del estudiante:	Fausto Sebastián Cabezas Mera
Código de estudiante:	00215152
C. I.:	1723414932
Lugar, Fecha	Quito, XX de diciembre de 2021

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RESUMEN

Los biofilms se asocian frecuente a condiciones infecciosas más virulentas y resistentes a las terapias convencionales, especialmente en pacientes con heridas, prótesis, quemaduras o pacientes diabéticos. La necesidad de enfoques para combatir las biofilms y considerando la actual crisis de resistencia a los antimicrobianos, nuevamente la miel ha sido considerada como una opción terapéutica. La miel producida por abejas sin aguijón (Tribu: *Meliponini*) alberga una gran variedad de compuestos con potencial antimicrobiano a diferencia de la miel comercial, pero su investigación es limitada debido a su baja producción. El objetivo de este estudio fue evaluar las propiedades antibiofilm de muestras de miel y dar luces sobre los mecanismos implicados. Evaluamos 35 muestras de miel producidas por 10 especies de abejas sin aguijón de diferentes provincias de Ecuador, más exactamente, Tungurahua, Pastaza, El Oro, Los Ríos y Loja. Nuestros hallazgos sugieren un mayor impacto en las etapas iniciales de la formación del biofilm que afectan la macroestructura del biofilm a formarse debido a los cambios morfológicos y metabólicos en las células constituyentes. No se identificó ningún mecanismo específico, por lo que suponemos que el efecto antibiofilm de la miel es el resultado sinérgico de varias condiciones, interacciones y mecanismos. Las mieles de abejas sin aguijón de Ecuador son un candidato prometedor para la investigación y el desarrollo de nuevas moléculas contra los microorganismos formadores de biofilms de interés clínico.

Palabras clave: Antibiofilm, miel, *Meliponini*, abejas sin aguijón, mutidrogoresistencia,

ABSTRACT

Biofilms are frequently associated with infectious conditions that are more virulent and resistant to conventional therapies, especially in patients with wounds, prostheses, burns or diabetic patients. The need for approaches to combat biofilms and considering the current antimicrobial resistance crisis, honey has again been considered as a therapeutic option. Honey produced by stingless bees (Tribe: *Meliponini*) harbors a great variety of compounds with antimicrobial potential unlike commercial honey, but its research is limited due to its low production. The aim of this study was to evaluate the antibiofilm properties of honey samples and to shed light on the mechanisms involved. We evaluated 35 honey samples produced by 10 stingless bee species from different provinces of Ecuador, more precisely, Tungurahua, Pastaza, El Oro, Los Ríos and Loja. Our findings suggest a greater impact on the initial stages of biofilm formation affecting the macrostructure of the biofilm to be formed due to morphological and metabolic changes in the constituent cells. No specific mechanism was identified, so we assume that the antibiofilm effect of honey is the synergistic result of several conditions, interactions and mechanisms. Stingless bee honeys from Ecuador are a promising candidate for research and development of new molecules against biofilm-forming microorganisms of clinical interest.

Keywords: Antibiofilm, honey, *Meliponini*, stingless bees, antimicrobial

TABLA DE CONTENIDO

REVIEW ARTICLE

ANTIMICROBIAL ACTIVITY OF STINGLESS BEE HONEY (TRIBE: *MELIPONINI*) ON CLINICALLY RELEVANT MICROORGANISMS: A SYSTEMATIC REVIEW AND META-ANALYSIS

Fausto Cabezas-Mera[1], Ariana Cedeño[1], Eduardo Tejera[2],*, José Miguel Álvarez-Suarez[3,4] and António Machado[1],*

[1] Universidad San Francisco de Quito USFQ, Colegio de Ciencias Biológicas y Ambientales COCIBA, Instituto de Microbiología, Laboratorio de Bacteriología, Calle Diego de Robles y Pampite, Quito 170901, Ecuador. E-mails: accedenop@estud.usfq.edu.ec (AC); fscabezasm@estud.usfq.edu.ec (FC-M); amachado@usfq.edu.ec (AM)

[2] Facultad de Ingeniería y Ciencias Agropecuarias Aplicadas, Grupo de Bioquimioinformática, Universidad de Las Américas, Quito 170125, Ecuador. E-mail: eduardo.tejera@udla.edu.ec (ET)

[3] Universidad San Francisco de Quito USFQ, Colegio de Ciencias e Ingenierías, Departamento de Ingeniería en Alimentos, Calle Diego de Robles y Pampite, Quito 170901, Ecuador. Email: jalvarez@usfq.edu.ec (JMA-S)

[4] King Fahd Medical Research Center, King Abdulaziz University, Jeddah, 21589, Saudi Arabia.

* Corresponding author: António Machado PhD, amachado@usfq.edu.ec

Co-corresponding author: Eduardo Tejera PhD, eduardo.tejera@udla.edu.ec

ABSTRACT

Honey produced by stingless bees or meliponines (Tribe: *Meliponini*) has been the subject of scientific curiosity for their ancestrally known medicinal properties derived from their diverse content of bioactive compounds. One of the most frequent uses has been as a natural antimicrobial, however, it has not yet been possible to isolate and characterize a particular active principle, probably due to the great taxonomic diversity and therefore to changes in the physicochemical properties of this type of honey and adding to the methodological differences that made it difficult to compare the findings between studies. The present systematic review, based on 117 full-text reviewed articles collected to date, the literature evidence against different pathogens of clinical interest. Our review highlights (i) the need to apply complementary methodologies and consolidate experimental protocols to quantify antimicrobial activity more efficiently (ii) the possibility of isolating and fully characterizing antimicrobial candidates in honeys with known high activity such as those produced by *Heterotrigona itama, Tetragonisca angustula and Melipona beecheii* and ; (iii) opportunities for the search for compounds with activity against multidrug-resistant and/or biofilm-forming pathogens such as strains of methicillin-resistant S. aureus (MRSA) and C. albicans strains. In addition, we performed a meta-analysis of twenty-nine original articles with available quantitative data using diffusion assays and minimum inhibitory concentration; however, the high heterogeneity, mostly due to multiple methodological differences and nuances, limited the depth of our analyses to estimate the pooled antimicrobial capacity of these samples. Despite the obstacles and limitations mentioned in this work, honey and other stingless bee-derived products remain an unexplored source of potential antimicrobial peptides and strategies to confront the current antibiotic crisis.

Keywords: Stingless bee[1], *Meliponini*[2], Antimicrobial[3], Antifungal[4], Antibiofilm[5], Meta-analysis[6]

INTRODUCTION

The progressive ineffectiveness of current antibiotics adding to the alarming appearance of much more virulent and resistant microorganisms has practically forced the scientific community to seek therapeutic alternatives from natural sources. Among one the alternatives are products derived from stingless bees or meliponines (Tribe: *Meliponini*), of which 525 species (48 genera) have been described and are widely distributed in Latin America, Australia, Africa, and parts of Asia (E. K. Nishio et al., 2016; Souza et al., 2021).

The antimicrobial activity of stingless honey bees, besides helping to preserve the honey itself or some foods, has been useful for wounds and burn care, skin, eye, and mucosal infections such as throat diseases and gastrointestinal infections in humans (Almasaudi, 2021; Esa et al., 2022; Jacinto-Castillo et al., 2022; Kimoto-Nira & Amano, 2015; Kwapong et al., 2014; Martínez-Puc et al., 2022; Quezada-Euán, 2018; Tesfaye et al., 2022; G. Zamora, Beukelman, van den Berg, et al., 2015). Since ancient times this knowledge has been used by indigenous communities in tropical regions around the world, as a natural antimicrobial agent against multi-resistant and virulent microorganisms due to its unique biochemical composition and other properties that greatly differ from *Apis mellifera* honey or other commercial honey (Alvarez-Suarez et al., 2018; Domingos et al., 2021; Guerrini et al., 2009; Morroni et al., 2018; W. J. Ng et al., 2017, 2020; Rao et al., 2016; Villacrés-Granda et al., 2021). *Tetragonisca angustula* honey has been one of the most studied, followed by *Melipona becchei* honey and various species of the genera *Scaptotrigona*, *Melipona*, and *Trigona*. Studies have not yet identified a specific active principle, but they have identified numerous candidates in honey and derivatives such as propolis (Al-Hatamleh et al., 2020; Almasaudi, 2021; Duarte et al., 2018; Nogueira et al., 2022; Surek et al., 2021; Yaacob et al., 2018; Yaghoobi et al., 2013).

For this reason, there is a growing interest in stingless bee honey since they are currently a candidate source to find new antimicrobial peptides. Currently, several studies are exploring the huge and diverse composition with the possibility of finding possible applications. (Costa dos Santos et al.,

2022; Dallagnol et al., 2022; Izabely Nunes Moreira et al., 2023; Mokaya et al., 2022; W. J. Ng et al., 2021; Pucholobek et al., 2022; A. C. dos Santos et al., 2021; Wu et al., 2022). However, the diversity of factors of this type of honey difficult their full characterization such as physicochemical properties, geographic origins, melissopalynological origins, climate, phytochemical compounds concentration, species of stingless bees, and associated microbiome. In addition, other non-specific factors hinder consensus in the scientific community due to the heterogeneity of the studies carried out such as applied methodologies, target microorganisms, inoculum, and even storage time, which play a very important role when evaluating the antimicrobial capacity of these samples. The variation of all the above-mentioned aspects originated reports of different organoleptic properties and biological properties in stingless bee honey. The present study aims to consolidate the existing information in a systematic review and carry out statistical analysis (meta-analysis) to better understand the antimicrobial activity of different stingless bee honeys against several relevant pathogenic microorganisms.

MATERIALS AND METHODS

Literature search

This study was conducted following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) strategies (Selcuk, 2019). SpringerLink, Scopus, PubMed, and Google Scholar databases were searched for English/Spanish articles using combinations of Boolean terms and medical subject heading terms (MESH) such as “stingless bee”, “meliponini”,” antibacterial”,” antimicrobial”, “antibiotic”, and “honey”. Articles reporting results on antimicrobial assays of raw honey produced by stingless bee species against bacterial and fungal pathogens of human clinical interest were included. The references to these articles were also checked for finding additional records. All references were compiled into a Zotero Library database (www.zotero.org) and then managed using Excel software.

Screening process

Duplicates were initially identified and removed in Zotero after entering all the recognized studies into an Excel self-created database (see *Meliponini_Meta_Data.xlsx* in the Supplementary section). All articles were assessed by two reviewers (FC-M and AC) by screening titles, abstracts, topics, and finally full texts. At each level, the reviewers independently screened the articles and finally merged their conclusions. An additional examination of the selected articles was realized by a third author (AM) focused on the homogeneity of the eligibility criteria of previous reviewers in the initial data set. Discrepancies were resolved by discussion before finalizing the records for the evaluation of eligibility criteria (see subsection 2.3). In case of disagreements, the third assessor (AM) was assigned to make a final decision.

Eligibility criteria, data extraction, and quality assessment

The main inclusion criteria was the results of antimicrobial assays with stingless bee honey. In addition, data regarding the stingless bee species, the "target" bacterial and fungal species, and the geographical location were extracted if available. Reviews, editorials, congress or meeting abstracts, literature in languages other than English or Spanish, case reports, and letters to editors were excluded from the final data set for meta-analysis. Finally, articles without full text available, duplicate reports on different databases, and studies with missing/incomplete data were also omitted. The extracted information included the first authors' names, year of publication, location, stingless bee specie, target microorganism, assay type, methodology, and quantifiable inhibition parameters (number of replicates, mean and standard deviation). The last three parameters were the most critical methodological criteria in the initial screening. Also, studies involving assays with biofilms were identified for further evaluation. The initial two authors (FC-M and AC) extracted all data, and further confirmation and evaluation were realized by the lead authors (AM, JMA-S, and ET). The final document with the data collected is available upon request from the authors.

Data analysis and statistical methods

Meta-analysis was performed using the RStudio software (Version 1.3.1073; https://rstudio.com/), using several R packages ("meta", "metafor" and "dmetar")(Balduzzi et al., 2019; Harrer et al., 2019; Viechtbauer, 2022) The pooled means were computed, and values were reported with confidence intervals (CI) of 95%. Units were transformed into a single one. In the disk-agar diffusion assays, all data were expressed in millimeters (mm) and, in the MIC assays, the results were converted into percentage values (%) considering positive and negative controls in each study. Studies reporting a standard deviation of zero (±0) were changed to 0.01 for statistical analysis purposes in Rstudio software. The heterogeneity was assessed by the Cochrane Q and I^2 tests. Egger's test was used to assess possible publication bias through funnel plot asymmetry. The effect of possible covariables was evaluated with meta-regression analysis. In statistical analysis, all *p*-values <0.05 were considered significant statistical results. For reproducibility reasons, the R code used in the meta-analysis is found in the supplementary material (see *Meliponini_code.Rmd* in the Supplementary section).

RESULTS

A total of 314 studies were retrieved and 117 full-length articles were reviewed. Thirty-three studies met the inclusion criteria for meta-analysis (see Figure 1). The final data set included studies covering different global regions. All available and relevant data from each study were extracted, more precisely, stingless bee species, honey applied concentration, target microorganism, botanical origin, methodology, biofilm assays, and quantitative inhibition parameters (such as replicates, mean and standard deviation).

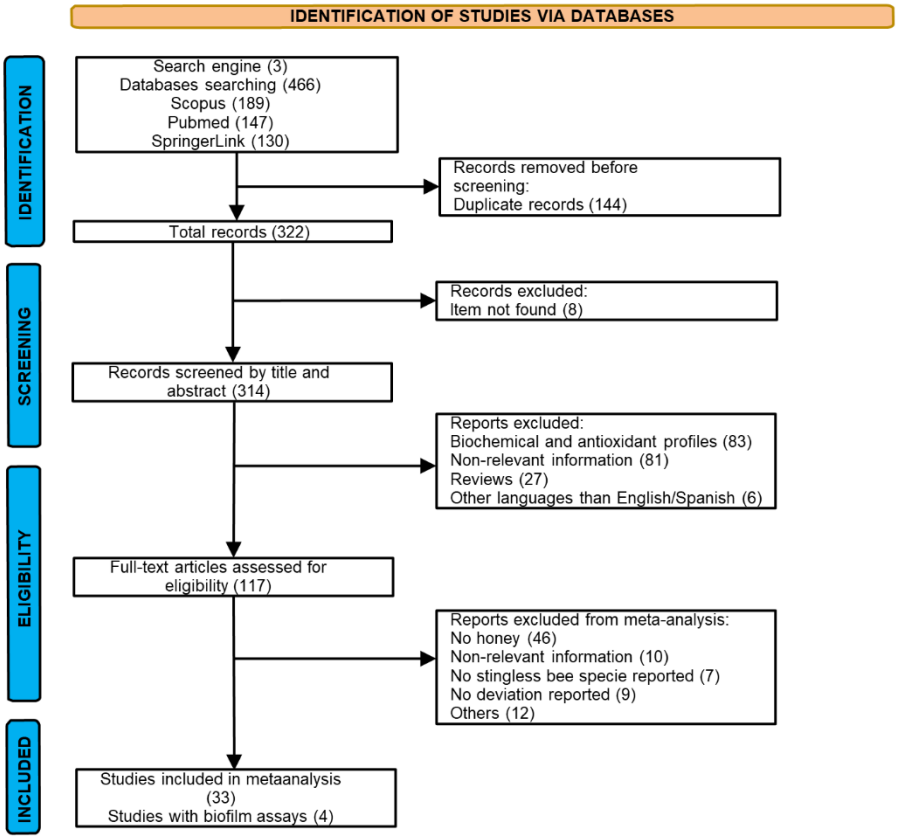


Figure 1 Prisma flow chart of included and excluded studies of the selection process.

As shown in Figure 1, a total data set of 33 studies was achieved for the present meta-analysis following the screening process, eligibility criteria, and quality assessment, in which only 4 studies described the antimicrobial activities against microbial biofilm assays.

Antibacterial activity

Antibacterial activity has been studied mainly for both gram-positive and gram-negative pathogens associated with the wound, skin, and mucosal infections (Abdel-Shafi et al., 2022; Diekema et al., 2019; Mejia et al., 2021; Montero et al., 2021), such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella enterica* ser. Typhimurium. In recent years, attempts have been made to understand which honey compounds or properties are responsible for antimicrobial activity. Although the activity of stingless bee honey is indeed much stronger than the *A. mellifera* honey (Ewnetu et al., 2013; Tenório et al., 2017), the mechanisms involved are not yet known with certainty. Several researchers postulated that the antimicrobial effect can be partially explained by the presence of hydrogen peroxide, low water activity, acidity and hyperosmolarity (Brudzynski, 2020; Esa et al., 2022; W. J. Ng et al., 2020; Nordin et al., 2018;

Yupanqui Mieles et al., 2022), while other authors explained it by the complex interactions with a wide range of phytochemical compounds such as polyphenols, flavonoids, terpenes, methylglyoxal, organic acids, and bee-derived peptides (Proaño et al., 2021; Schuh et al., 2019), among other compounds that are found in much more discrete concentrations like vitamins and a variety of essential minerals (Abd Jalil et al., 2017; Biluca et al., 2016; Ciulu et al., 2011; Habib et al., 2014). There is evidence for the presence of certain compounds (as yet unnamed or uncharacterized) capable of stimulating immune responses such as modulation of cytokine production, triggering of tissue repair cascades, and activation of infections-specific responses (Majtan, 2014; Minden-Birkenmaier & Bowlin, 2018; P. Molan & Rhodes, 2015; Yaghoobi et al., 2013). For example, Tonks and colleagues isolated a 5.8 kDa component of Manuka honey capable of stimulating the production of tumor necrosis factor-alpha (TNF-α) in macrophages through the Toll-like receptor 4 (TLR4) (Tonks et al., 2007). On the other hand, a large number of researchers highlight that the multiple and complex interactions with these compounds could induce an excessive inflammatory immune response by certain cytokines' production, as suggested in studies involving COVID-19 and lipopolysaccharide-induced inflammatory response (Agussalim et al., 2022; Biluca et al., 2020; Mustafa et al., 2020; Ooi et al., 2021; Ranneh et al., 2019).

These potential antibacterial peptides and compounds have been shown to induce changes in transmembrane potential and affect membrane permeability in both gram-negative and gram-positive bacteria. However, their effects varied on the strain rather than the gram-type bacteria (Almasaudi, 2021). Several studies agree that these damages are consequences of disruptive mechanisms of the cell membrane, leaving aside others such as the inhibition of bacterial protein synthesis or the expression of enzymatic activity, in particular, proteases (Brudzynski, 2021; Brudzynski & Sjaarda, 2015). Non-destructive mechanisms of the cell wall have recently been mentioned involving non-glycosylated proteins (Kim & Jin, 2019; Jesús M. Ramón-Sierra et al., 2022; Shen et al., 2021). In a recent study, Ramón-Sierra et al. (2022) demonstrated that non-glycosylated proteins isolated from *Melipona beecheii* honey could be partially responsible for an

antimicrobial effect (Jesús M. Ramón-Sierra et al., 2022). Likewise, some Major Royal Jelly Proteins (MRJPs) showed antihemolytic activity and downregulated the expression of virulence genes (*Stx1*, *Stx2*, and *HlyA*) in *Escherichia coli* O157:H7 (J. M. Ramón-Sierra et al., 2021). *In vivo* assays with guinea pigs with common pathogens in conjunctivitis (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) demonstrated that treatments with stingless bee (*Meliponula* spp.) honey were equivalent to the first-line ocular antibiotics, significantly reducing inflammation and the infection duration (Ilechie et al., 2012).

Certain microbial species of the honey-associated microbiome or other derivatives can produce postbiotic compounds able to control the growth of opportunistic pathogens of bees, as well as, provide symbiotic benefits (Baharudin et al., 2021; de Paula et al., 2021; Ngalimat et al., 2019; Rosa et al., 2003; A. C. C. Santos et al., 2022). There is evidence of great diversity in the *Meliponini*-associated microbiota such as viruses, bacteria, yeasts, and filamentous fungi (Echeverrigaray et al., 2021; Leonhardt & Kaltenpoth, 2014; M. S. Silva et al., 2017; Villegas-Plazas et al., 2018). The honey-associated microbiome has been poorly characterized, even though the isolation of bacteria with potential probiotic effects has been reported, mainly *Bacillota* (Galperin et al., 2022; Oliphant et al., 2022), formerly known as *Firmicutes* (Pucciarelli et al., 2014; A. C. C. Santos et al., 2022; Shanks et al., 2017; Syed Yaacob et al., 2018; Tang et al., 2021), and *Streptomyces* species (Cambronero-Heinrichs et al., 2019; Ngalimat et al., 2019; Promnuan et al., 2009). However, Kimoto-Nira and Amano (2008) suggested that the antimicrobial activity of raw honey could adversely affect beneficial bacteria of the host such as lactic acid bacteria (Kimoto-Nira & Amano, 2015).

Many gram-negative bacteria have been used as targets, mostly members of the *Enterobacteriaceae* family, followed by strains of *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* (Wu et al., 2022), *Haemophilus influenzae* (Brown et al., 2020), and *Alcaligenes faecalis* (Rosli et al., 2020). The multidrug-resistant pathogens used included several carbapenemase-producing strains of *K. pneumoniae* (KPC) and *P. aeruginosa*, obtaining MICs in the range of 3 to 20% w/v (Villacrés-

Granda et al., 2021; G. Zamora, Beukelman, van den Berg, et al., 2015). Of all bacteria previously mentioned, *P. aeruginosa* stands out by the bacteriostatic activity produced by honey samples (Jesús M. Ramón-Sierra et al., 2022). Among gram-positive bacteria, the most studied pathogen is *Staphylococcus aureus* (including methicillin-resistant *S. aureus* strains, MRSA), followed by coagulase-negative staphylococci species, *Enterococcus faecalis, Enterococcus faecium* (Kimoto-Nira & Amano, 2015; Erick Kenji Nishio et al., 2016), streptococci (Domingos et al., 2021; Tuksitha et al., 2018), and occasionally *Listeria monocytogenes* (Mahmood et al., 2021; Suntiparapop et al., 2015), *Micrococcus luteus* (Suntiparapop et al., 2015; Torres et al., 2004), and *Bacillus cereus* (DeMera & Angert, 2004; Ramlan et al., 2021). For all the pathogens mentioned above, MICs ranged from 2 to 15 % depending on the stingless bee species, however, samples produced by *Melipona beecheei* and species of the genus *Scaptotrigona* were able to inhibit the growth of *S. aureus* and *S. epidermidis* at concentrations of less than 2 % (Erick Kenji Nishio et al., 2016; G. Zamora, Beukelman, Van Den Berg, et al., 2015).

No reports of honey assays against wall-less bacteria (Bacilli class and Mollicutes subclass) were found, nonetheless, dos Santos et al. (2017) used propolis extracts and compounds isolated from Brazilian stingless bees *Melipona quadrifasciata* and *Tetragonisca angustula* against *Mycoplasma genitalium, M. hominis, M. pneumoniae* and *Ureaplasma urealyticum*, reporting concentrations between 125 and 1000 μg/mL,,being the only report with this unusual group of bacteria (L. dos Santos et al., 2017).

Antifungal activity

The antimicrobial potential of stingless bee honey against phytopathogenic fungi is also known (Nogueira et al., 2022), but reports involving yeasts and molds isolated from clinical infections are still scarce. Honey is mainly composed of sugars (such as fructose, glucose, sucrose, maltose, and arabinose), constituting around 80% of its weight. However, hyperosmolarity caused by honey is diminished by the presence of these sugars and its consumption by pathogens adapting their

metabolism by different sugar-sensing systems (J. M. B. de Sousa et al., 2016; T. S. Ng et al., 2016; Pemmaraju et al., 2016; Van Ende et al., 2019; Weerasekera et al., 2017).

One of the most studied clinically relevant fungal pathogens is the *Candida* genus, mainly *Candida albicans*, which is the most frequently isolated species due to its ability to produce nosocomial opportunistic infections to life-threatening infections through a wide repertoire of virulence factors such as biofilm formation (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022; Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022; Cangui-Panchi et al., 2022). Little is still known about the antimicrobial efficacy of stingless bee honey on other *Candida* species. Tesfaye et al. (2022) reported that *Meliponula beccarii* honey was not able to inhibit the growth of clinical isolates of *C. albicans*, as well as DeMera and Angert (2004) with *T. angustula* honey against *C. albicans* ATCC 90028, and Jimenez et al. (2016) with *Scaptotrigona mexicana* honey against *C. albicans* ATCC 10231 (DeMera & Angert, 2004; Jimenez et al., 2016; Tesfaye et al., 2022). However, Hau-Yama et al. (2020), Zamora et al. (2015), and Boorn et al. (2010) reported limited activity against the growth of *C. albicans* ATCC 10231 with Mexican *M. beecheii*, Costa Rican *T. angustula*, and Australian *Tetragonula carbonaria* honey samples (Boorn et al., 2010; Hau-Yama et al., 2020; G. Zamora, Beukelman, Van Den Berg, et al., 2015). Similarly, Dardón and Enríquez (2008) reported similar results with Guatemalan *Plebeia* sp. honey against *C. albicans* ATCC 10231 (Dardón & Enríquez, 2008). It is postulated that sporadic effective antifungal activities are observed from a honey set of the same or close geographical origin since some studies suggested a strong link to the melissopalynological nature and their bioactive compounds in honey (Ávila, Hornung, et al., 2019; Biluca et al., 2016). The use of stingless bee honey as traditional or alternative medicine revealed greater anti-*Candida* activity when compared to honey-based products (MediHoney) and commercial honey (Boorn et al., 2010; Souza et al., 2021; G. Zamora, Beukelman, Van Den Berg, et al., 2015), justifying its use in bandages for burns and infected wounds (Abd Jalil et al., 2017; Esa et al., 2022).

Although the exact mechanisms are not completely understood at the molecular level, it is hypothesized that the presence of certain plant flavonoids (such as quercetin, luteolin, chrysin, and galangin) is able to produce morphological damage and affect the hyphal transition by modifying the mitochondrial and vacuolization activity (Candiracci et al., 2011; Canonico et al., 2014). Other flavonoids such as catechin and epigallocatechin gallate in co-treatment with fluconazole might be able to promote early apoptosis in *Candida* cells through externalization of phosphatidylserine at the plasma membrane, mitochondrial depolarization, intracellular accumulation of reactive oxygen species (ROS), cell condensation, and DNA fragmentation. (C. R. Da Silva et al., 2014). Moreover, Kim and Jin (2019) also observed that a peptide member of the MRJPs family called AcMRJP4-15 contained a high content of asparagine and positively charged (hydrophilic) amino acids showing a great inhibition against *C. albicans* KCTC727 and an increment of the membrane permeability with similar action as royalisin and jelleines (Kim & Jin, 2019). Although this peptide was obtained from *Apis cerana* honey, homolog peptides have recently been reported in *M. beecheii* honey, more exactly, non-glycosylated protein fractions between 25 and 95 kDa named MbF1-1,2 and MbF1-3 (J. M. Ramón-Sierra et al., 2021). In a recent study by Dallagnol and colleagues (2022), a positive correlation was revealed between fungal growth and the presence of flavonoids, as well as evident crystallization in samples with high bioactivity, while on the contrary, samples that lacked flavonoids, were rich in phenylethylamides and did not crystallize drastically reduced their activity (Dallagnol et al., 2022). Besides *C. albicans*, other yeasts selected for their medical importance and, in some cases, for their resistance to antibiotics is *Aspergillus flavus*. However, no antifungal activity was evidenced by Jimenez and colleagues (Jimenez et al., 2016).

Antibiofilm activity

A main worldwide concern when treating wounds or burns or handling intravascular devices is the colonization of biofilm-forming microorganisms due to the complexity of biofilms providing robust protection against standard treatments and host immune responses as well as a persistent source of dissemination (Cangui-Panchi et al., 2022; Percival et al., 2015; Rajpaul, 2015; R. E. Thomas &

Thomas, 2021). To date, few studies have been conducted on the possible antibiofilm effects of stingless bee honey. Little is still known about their antimicrobial activity in the inhibition of biofilm formation or even in the disruption of mature biofilms. The failure of standard treatments to eradicate established microbial biofilms represents nowadays a persistent problem in wound healing (Maddocks et al., 2013). Zamora et al. (2017) reported two novel uncharacterized proteins with a molecular weight of 50 kDa and 75 kDa, designated as *Tetragonisca angustula* biofilm destruction factors (TABDFs), from Costa Rican *T. angustula* honey that could inhibit the biofilm formation and destroy mature *S. aureus* biofilms, also allowing antibiotics such as ampicillin and vancomycin to recover their antibacterial activity (L. G. Zamora et al., 2017). Likewise, other studies demonstrated the ability of stingless bee's honey or certain bioactive compounds to act synergistically with antibiotics or other therapies against biofilm-related infections such as *Streptococcus mutans* from active caries (El-Allaky et al., 2020; Hasnamudhia et al., 2017). Ng et al. (2017) demonstrated that the honey of *Trigona* sp. at 20% (v/v) was able to inhibit biofilm formation by 75-90% in several *S. aureus* strains, including clinical isolates from wounds and methicillin-resistant (MRSA) strains. However, only concentrations of *Trigona* sp. honey greater than 60% (v/v) were able to eradicate at least 50% of the preformed biofilms (W. J. Ng et al., 2017). The available literature suggests that these antibiofilm activities derived from a combined or synergic effect of low moisture, acidity, and hyperosmolarity potentiated by non-peroxide compounds, mainly flavonoids and phenolic compounds and other protein-based factors (Proaño et al., 2021; Schuh et al., 2019; Sojka et al., 2016). However, how this synergy works remain yet unknown and undescribed in stingless bee honey. Some authors proposed that these multiple contributions can suppress key quorum sensing genes (Lee et al., 2011) and/or downregulate specific virulence genes in biofilms (Al-Kafaween et al., 2020; J. M. Ramón-Sierra et al., 2021), as well as disrupt extracellular polymeric substances (EPS) of the biofilm exposing persistent cells (L. G. Zamora et al., 2017) and weakening the biofilm structure (Alkafaween et al., 2021). With the incorporation of new next-generation sequencing (NGS) technologies, new findings are currently

being studied on possible mechanisms of the antibiofilm activities. For example, Seder et al. (2021) found that Malaysian honey *Trigona* sp. at 20% (w/v) can down-regulated 470 biofilm-associated genes in *P. aeruginosa* biofilms through microarray, decreasing expression levels of D-GMP signaling pathway and diguanylate cyclase (DGC) genes responsible for cyclic di-GMP formation (Seder et al., 2021).

Recent studies suggested that propolis (a natural resinous mixture produced by honey bees) exhibits greater antifungal and antibiofilm activities than honey, and its use against *Streptococcus mutans* biofilms has already demonstrated to be a prevention strategy or therapy against caries and oral candidiasis (Kolayli et al., 2020; Liberio et al., 2011; Tamfu et al., 2022; Wieczorek et al., 2022). Among the antibiofilm and anti-*Candida* bioactive compounds, flavones in propolis such as baicalein and pinocembrin exhibited the greatest activities. Both flavones decreased the hydrophobicity of the cell surface and inhibited the hyphal transition of *C. albicans* (Rivera-Yañez et al., 2022). Baicalein was associated with the downregulation of CSH1 levels (Cao et al., 2008; Shirley et al., 2017), while pinocembrin was related with the downregulation of ALS3 and ACT1 levels (Manoharan et al., 2017).

Methodologies for quantification of antimicrobial activity

The most used methodologies to quantify antimicrobial activity are the determination of minimum inhibitory concentration (MIC) by broth microdilution assay and diffusion in agar/disk (Boorn et al., 2010; Chan-Rodríguez et al., 2012; Hau-Yama et al., 2020), although other methodologies can be applied such as plate count method (W. J. Ng et al., 2017) and time-kill assay (E. K. Nishio et al., 2016; Erick Kenji Nishio et al., 2016). Therefore, these two methodologies were chosen to continue with the analysis of the honey antimicrobial activity described in the literature. It is important to mention that the natural properties of honey such as viscosity, biological material in suspension, and temperature can modify the sample density (usually not reported in most studies) and therefore influence the outcome of the antimicrobial techniques such as MIC by broth microdilution and diffusion in agar/disk assays. Thus, we divided the data set of each methodology

and analyzed them individually in the present study. Also, all data set was evaluated into percentage values, but the results were splitted again according to honey concentration and administration mode as volume-volume (v/v), and mass-volume (m/v). Twenty-three studies used an agar or disk diffusion method to quantify the antimicrobial effect. While, only 3 and 5 studies applied MIC by broth microdilution administrating samples by volume-volume (v/v) and mass-volume (m/v), respectively. It should be noted that most studies diluted honey samples and these dilutios are still able to inhibit the growth of pathogens due to different factors such as the concentration of several bioactive compounds, the production of hydrogen peroxide (by the enzymatic action of glucose oxidase naturally inactive in honey due to low pH), and hyperosmolarity among others. Simultaneously, other therapeutic properties naturally decrease influenced by the dilution level (Dardón & Enríquez, 2008; Sgariglia et al., 2010; Wavinya et al., 2021; Yupanqui Mieles et al., 2022) and therefore results must be compared by methodology (broth microdilution and diffusion in agar/disk), mode of administration (v/v and w/v) and their dilution (Almasaudi, 2021; P. C. Molan, 2015; Yaghoobi et al., 2013). In addition, low sensitivity of diffusion-based method has been reported because the non-polar constituents may not diffuse well into the agar medium, not allowing the total antimicrobial activity of the honey to be exhibited (Boorn et al., 2010; Hewett et al., 2022; Pimentel et al., 2013). However, this methodology is the most used as exemplified in our study set due to their speed and low cost. Some studies also suggested using diffusion-based methodology as an initial screening procedure to distinguish samples with and without antimicrobial activity and then confirming the initial results by spectrophotometry or fluorometry techniques, standard plate count, and even time-kill assay (Albaridi, 2019; Boorn et al., 2010; Hossain et al., 2022). Yet, each methodology has many variations and modifications to their experimental protocols (such as mode of administration, dilution, and even microbial growth culture time), difficulting the data comparison between studies and representing an important source of the high heterogeneity in our meta-analysis.

Metanalysis

In the initial evaluation of the study set, we observed a large variety of stingless bee species and genera that have been tested against an even wider variety of microorganisms (Supplementary table 3), demonstrating once again another example of the high heterogeneity among studies. After the selection process (Figure 1), the majority of studies were distributed throughout the tropical and neotropical zones (Table 1), where the presence of native stingless bees is already known, mostly in countries of Asia (such as Malaysia and the Philippines), Central America (such as Costa Rica), and South America (such as Brazil, Ecuador, and Argentina). Nonetheless, there are also studies of native bee honey samples of countries from other regions such as Mexico, Tanzania, Ethiopia, and Australia (Table 1). Regarding stingless bee genera, most studies evaluated *Melipona* spp. (21 species), followed by *Scaptotrigona* and *Tetragonula* (both 5 species). Concerning stingless bee species, the *Heterotrigona itama* (9 studies), *Tetragonisca angustula* (6 studies), *Melipona beecheii* (5 studies), *Tetragonula carbonaria*, and *Geniotrigona thoracica* (both 4 studies) were the most frequently evaluated in the group set (Table 1).

Table 1 Stingless bee species (Tribe: *Meliponini*) for each study included in the meta-analysis.

Authors (year)/Reference	Country	N	Stingless bee(s) [1]
(Boorn et al., 2010)	Australia	1	*Tetragonula carbonaria*
(Domingos et al., 2021)	Brasil	4	*Melipona eburnea, M. flavolineata, M. grandis,* and *M. seminigra*
(Chan-Rodríguez et al., 2012)	Mexico	1	*Melipona beecheii*
(DeMera & Angert, 2004)	Costa Rica	1	*Tetragonisca angustula*
(Gopal et al., 2021)	Malasya	1	*Trigona* sp.
(Jibril et al., 2020)	Malasya	1	*Trigona* sp.
(E. K. Nishio et al., 2016)	Brasil	2	*Scaptotrigona postica, S. bipunctata*
	Philippines	1	*Tetragonula biroi*
	Thailand	1	*Tetragonula pagdeni*
(Kimoto-Nira & Amano, 2015)	Mexico	6	*Frieseomelitta nigra, Melipona beecheii, M. colimana, M. solani, Scaptotrigona mexicana,* and *S. pectoralis*
	Australia	2	*Tetragonula carbonaria* and *Trigona australis*
	Paraguay	3	*Melipona quadrifasciata, Scaptotrigona bipunctata,* and *T. angustula*
(Mahmood et al., 2021)	Malasya	1	*Heterotrigona itama*
(Massaro et al., 2014)	Australia	1	*Tetragonula carbonaria*
(Ngaini et al., 2021)	Malasya	1	*Heterotrigona itama*
(Pimentel et al., 2013)	Brasil	1	*Melipona compressipes*
(J. Ramón-Sierra et al., 2020)	Mexico	1	*Melipona beecheii*
(Rosli et al., 2020)	Malasya	8	*Geniotrigona thoracica, H. erythrogastra, H. fimbriata, H. itama, Lepidotrigona terminata, T. apicalis, T. binghami,* and *T. melanoleuca*
(Saputra & Nurlina, 2022)	Indonesia	1	*Heterotrigona itama*
(Suarez et al., 2021)	Philippines	1	*Tetragonula biroi*

(Syed Yaacob et al., 2020)	Malasya	1	*Heterotrigona itama*
(Tesfaye et al., 2022)	Ethiopia	1	*Meliponula beccarii*
(S. C. Thomas & Kharnaior, 2021)	India	1	*Trigona* sp.
(Torres et al., 2004)	Colombia	1	*Tetragonisca angustula*
(Tuksitha et al., 2018)	Malasya	3	*Geniotrigona thoracica*, *Heterotrigona erythrogastra*, and *H. itama*
(W. J. Ng et al., 2020)	Malasya	2	*Geniotrigona thoracica* and *Heterotrigona itama*
(Wu et al., 2022)	Malasya	2	*Heterotrigona itama* and *Tetrigona binghami*
(Ramlan et al., 2021)	Australia	2	*Tetragonula carbonaria* and *T. hockingsi*
	Malasya	2	*Geniotrigona thoracica* and *Heterotrigona itama*
(Suntiparapop et al., 2015)	Thailand	2	*Tetragonula laeviceps*
(Jimenez et al., 2016)	Mexico	1	*Scaptotrigona exicana*
(Villacrés-Granda et al., 2021)	Ecuador	12	*Cephalotrigona* sp., *Melipona cramptoni*, *M. grandis*, *M. indecisa*, *M. mimetica*, *Melipona* sp., *Nannotrigona chapadana*, *Oxytrigona mellaria*, *Paratrigona* sp., *Scaptotrigona polysticta*, *Tetragonisca angustula*, and *Trigona silvestriana*
(G. Zamora, Beukelman, Van Den Berg, et al., 2015)	Costa Rica	4	*Melipona beecheii*, *M. costaricensis*, *Scaptotrigona pectoralis*, and *Tetragonisca angustula*
(G. Zamora, Beukelman, van den Berg, et al., 2015)	Costa Rica	2	*Melipona beecheii* and *Tetragonisca angustula*
(W. J. Ng et al., 2017)*	Malasya	1	*Trigona* sp.
(L. G. Zamora et al., 2017)*	Costa Rica	2	*Melipona beecheii* and *Tetragonisca angustula*
(Alkafaween et al., 2021)*	Malasya	1	*Trigona* sp.
(Seder et al., 2021)*	Malasya	1	*Trigona* sp.

[1]Note: Taxonomic names of the stingless bee species are consistent with the current names in Integrated Taxonomic Information System (ITIS).
*Studies reporting the antimicrobial activity of stingless bee honey samples through planktonic and biofilm assays. These studies were excluded from Table 2 due to the different methodological characteristics of the other studies.

As shown in Figure 2, *S. aureus* and *E. coli* were the most evaluated pathogens in our study set, evidencing 221 and 149 antimicrobial activity assays against honey samples of 36 and 24 stingless bee species, respectively. *P. aeruginosa* and *K. pneumoniae* were also evaluated in 66 and 50 antimicrobial activity assays against honey samples of 28 and 33 stingless bee species, respectively. The remaining evaluated microorganisms include relevant foodborne pathogens (Supplementary Figure 1), due to their virulence and multidrug resistance such as *E. faecium*, *E. faecalis*, Coagulase-negative Staphylococci (CoNS), and other members of *Enterobacteriaceae* family, but the number of antimicrobial activity assays were less than 40 being most of them not suitable for further analysis, except for *Salmonella enterica* and *Enterococcus faecalis* (Table 2). It is also important to mention that the heterogeneity increased in the data set evidencing studies with several honey samples from one or several bee species against numerous pathogens from different origins (such as ATCC collection strains, multidrug-resistant strains, as well as clinical and laboratory isolates).

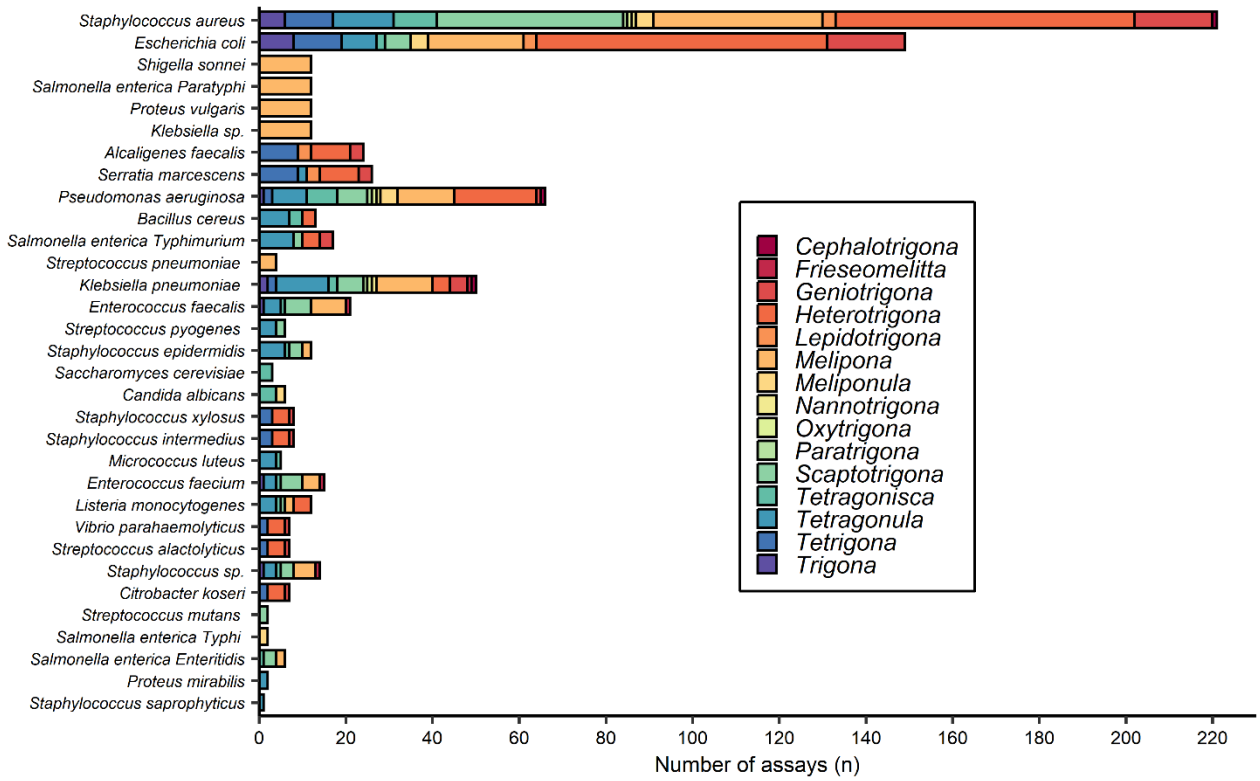


Figure 2 Illustration of the pathogens used in the evaluation of the antimicrobial activity by stingless bee honey samples.

Each y-axis bar showed the target microorganism, while the size of each colored division is proportional to the number of stingless bee species used in the antimicrobial assays within the same genus (color). Finally, the x-axis evidenced the number of assays reported among the data set.

As shown in Table 2, a summary of the information of each study was collected, showing stingless bee species, botanical origin, pathogen, inhibition measures (halos and concentrations), methodology, dilution, and microbial growth time. However, the information about the botanical origin of stingless bee honey samples was poor and very scarce being a shortcoming of the present meta-analysis. Other factors evaluated in some studies in the data set were the comparison of the honey production of the same stingless bee species in different phytogeographic regions (DeMera & Angert, 2004) or biochemical composition in the samples during rainy and dry seasons (Mahmood et al., 2021; Pimentel et al., 2013).

Table 2 A summary of information collected of each study from our data set on the antimicrobial activity by stingless bee honey samples against the selected pathogens in the present meta-analysis.

			Pathogens[2]							Methodology[1,3]		
			Ec	*Sa*	*Pa*	*Sen*[4]	*Ef1*	*Kp*				
Authors (year)/Reference	**Specie (s)**	**Botanical origin**	**Inhibition reported (mean ± standard deviation)[1]**						**MDR**		**Concentration**	**Incubation**
(Boorn et al., 2010)	*T. carbonaria*	Native and exotic cultivated plant species*	8.8 ± 0.2	19.6 ± 6.6	11.8 ± 1.9	6.3 ± 4.2	10.1 ± 0.5	NT	No	DAD	50% (v/v)	24 hours
(Domingos et al., 2021)	*M. eburnea* *M. flavolineata* *M. grandis* *M. seminigra*	NR	NE NE NE NE	9 ± 0.5 (1) 18.3 ± 1.4 (2) 13.3 ± 0.5 (1) 16.6 ± 2.2 (2) 20.3 ± 1.4 (1) 20.6 ± 1.0 (2) NE	NE 10.0 ± 0.9 NE NE	NT	NE 12.3 ± 1.0 12.3 ± 1.4 9.3 ± 1.0	NE 15.3 ± 1.0 NE NE	No	DAD	50% (v/v)	24 hours
(Chan-Rodríguez et al., 2012)	*M. beecheii*	Multifloral*	8.6 ± 0.7	8.9 ± 0.9	NT	NT	NT	NT	No	DAD	Raw honey	12 hours
(DeMera & Angert, 2004)	*T. angustula*	Six different phytogeographic*	NT	NT	(A) 5.0 ± 5.6 (B) 5.0 ± 5.6 (C) 6.0 ± 5.6	NT	NT	NT	No	DAD	Raw honey	17 hours 24 hours 48 hours
(Gopal et al., 2021)	*Trigona* sp.	NR	11.8 ± 0.5	12.6 ± 0.6	NT	NT	NT	NT	No	DAD	22%	24 hours
(Jibril et al., 2020)	*Trigona* sp.	NR	NT	(A) 10.1 ± 0.2 (B) 10.8 ± 0.3 (C) 10.7 ± 0.3 (D) 13.2 ± 0.7	NT	NT	NT	NT	No	DAD	Raw honey	24 hours
(E. K. Nishio et al., 2016)	*S. postica* *S. bipunctata*	NR	(1) 8.0 ± 0.5 (2) 8.0 ± 0.5 (1) 9.0 ± 1.2 (1) 8.0 ± 1.2	(1) 15.0 ± 0.5 (2) 16.0 ± 0.5 (3) 16.0 ± 0.4 (4) 15.0 ± 0.4 (1) 19.0 ± 0.9 (2) 20.0 ± 0.9 (3) 20.0 ± 0.8 (4) 19.0 ± 0.8	(1) 8.0 ± 0.5 (2) 9.0 ± 0.5 (1) 12.0 ± 1.2 (2) 11.0 ± 1.2	(1) 9.0 ± 1.1 (2) 8.0 ± 1.1 (1) 12.0 ± 0.5 (2) 9.0 ± 0.5	18.0 ± 0.6 22.0 ± 1.5	7.0 ± 0.6 11.0 ± 1.5	Yes	DAD MIC (v/v)	50% (v/v)	24 hours
(Kimoto-Nira and Amano, 2008)	*T. biroi* *S. mexicana* *S. pectoralis* *M. beecheii* *T. pagdeni* *F. nigra* *M. colimana* *M. solani* *T. carbonaria* *T. australis* *M. quadrifasciata* *S. bipunctata* *T. angustula*	NR	NT	NT	NT	NT	13.3 ± 1.1 12.5 ± 2.1 12.3 ± 1.5 10.3 ± 0.6 NE	NE	Yes	DAD	50% (w/w)	24 hours
(Mahmood et al., 2021)	*H. itama*	Multifloral* Dry and rainy seasons.Dry season: *S. rebaudiana* and *A. leptopus*.Rainy season:A. *leptopus*, *B. pilosa*, *C. sulphureus*, and *O. stamineus*	(A) 7.3 ± 0.6 (B) 8.7 ± 0.6 (C) 11.3 ± 0.6	(A) 7.7 ± 0.6 (B) 11.3 ± 0.6 (C) 16.3 ± 1.2 (D) 19.3 ± 1.2	NT	(A) 7.3 ± 0.6 (B) 32.3 ± 0.6 (C) 28.0 ± 1.0 (D) 13.7 ± 1.2	NT	NT	No	DAD	Raw honey	24 hours
(Massaro et al., 2014)	*T. carbonaria*	Multifloral* *Leptospermum* species	NT	(A) 15.2 ± 0.1 (B) 19.9 ± 0.1 (C) 17.5 ± 0.8	NT	NT	NT	(A) 15.4 ± 0.01 (B) 17.4 ± 0.01 (C) 15.4 ± 0.07	No	DAD	Raw honey	16 hours
(Ngaini et al., 2021)	*H. itama*	*A. carambola* *A. leptopus*	(A) 9.0 ± 0.4 (B) 10.0 ± 0.4	NE	NT	NT	NT	NT	No	DAD	Raw honey	24 hours

			(C) 19.0 ± 0.7								
(Pimentel et al., 2013)	*M. compressipes*	NR	(A) 9.2 ± 0.1 (B) 15.6 ± 0.1 (C) 16.1 ± 0.1	(A) 12.1 ± 0.2 (B) 12.6 ± 0.3 (C) 18.3 ± 0.4	NT	NE	NT	NT	No	DAD	10- 50 % (v/v) Raw honey	24 hours
(J. Ramón-Sierra et al., 2020)	*M. beecheii*	NR	34 ± 3.4 15.0 ± 1.0	30 ± 2.2 19.00 ± 1.0	NT	NT	NT	NT	No	DAD MIC (w/v)	Raw honey	24 hours
(Rosli et al., 2020)	*G. thoracica,* *H. erythrogastra* *H. fimbriata* *H. itama* *L. terminata* *T. apicalis* *T. binghami* *T. melanoleuca*	NR	NE NE 12.0 ± 0.6 NE NE NE NE 9.0 ± 1.0	11.0 ± 1.0 NE 28.0 ± 0.6 13.0 ± 0.1 11.0 ± 0.6 13.0 ± 0.6 10.0 ± 0.1 22.0 ± 0.6	NT	NT	NT	NT	No	DAD	12–5 - 50 % (v/v)	24 hours
(Saputra & Nurlina, 2022)	*H. itama*	Multifloral*	22.8 ± 4.7	19.2 ± 4.9	NT	NT	NT	NT	No	DAD	Raw honey	24 hours
(Suarez et al., 2021)	*T. biroi*	Coconut, bananas, and mangoes	(1) 4.8 ± 0.6 (2) 3.5 ± 1.0 (3) 12.5 ± 1.0	NT	NT	NT	NT	NT	Yes	DAD	20 % (w/v)	18 hours
(Syed Yaacob et al., 2020)	*H. itama*	Multiflora	(A) 18.0 ± 0.6 (B) 19.0 ± 0.6 (C) 23.0 ± 0.6 (D) 13.0 ± 1.2 (E) 20.0 ± 1.2 (F) 20.0 ± 3.1 (G) 13.0 ± 3.8 (H) 5.0 ± 6.4	(A) 18.0 ± 1.0 (B) 23.0 ± 1.5 (C) 25.0 ± 1.5 (D) 19.0 ± 2.3 (E) 12.0 ± 3.1 (F) 23.0 ± 3.4 (G) 15.0 ± 4.4 (H) 16.0 ± 6.2	(A) 5.0 ± 0.6 (B) 6.0 ± 0.6 (C) 6.0 ± 0.6 (D) 8.0 ± 0.6 (E) 9.0 ± 0.6 (F) 10.0 ± 0.6 (G) 9.0 ± 1.2 (H) 13.0 ± 1.5	NT	NT	NT	No	DAD	75 % (w/v) Raw honey	24 hours
(Tesfaye et al., 2022)	*M. beccarii*	NR	(1) 8.4 ± 1 (2) 13.9 ± 3.4	(1) 12.4 ± 1.6 (2) 15.7 ± 3.2	(1) 8.4 ± 1.2 (2) 15.7 ± 2.4	(1) 11 ± 0.8 (2) 15.8 ± 1.7			No	DAD	75% (v/v)	24 hours
(S. C. Thomas & Kharnaior, 2021)	*Tetragonula* sp.	NR	(A) 12.0 ± 0.2 (B) 14.6 ± 0.3 (C) 17.0 ± 0.4 (D) 17.0 ± 0.5 (E) 14.0 ± 0.6 (F) 15.0 ± 0.6 (G) 16.0 ± 0.7	NT	NT	NT	NT	NT	No	DAD	Raw honey	24 hours
(Torres et al., 2004)	*T. angustula*	NR	21.7 ± 2.9						No	DAD	50% (v/v)	48 hours
(Tuksitha et al., 2018)	*G. thoracica* *H. erythrogastra* *H. itama*	NR	18.3 ± 1.5 10.0 ± 8.7 5.0 ± 8.7	17.0 ± 2.6 15.0 ± 0.1 8.0 ± 7.0	16.0 ± 1.0 15.3 ± 1.5 11.3 ± 1.2	NT	NT	12.3 ± 2.1 13.0 ± 1.7 NE	No	DAD	50% (w/v)	24 hours
(W. J. Ng et al., 2020)	*G. thoracica* *H. itama*	NR (23 honey samples)*	6 strains	2 strains	NT	NT	NT	NT	No	DAD	Raw honey	20 hours
(Wu et al., 2022)	*H. itama*	Polyfloral*	(1) 1.27 ± 0.1 (2) 1.27 ± 0.1	(1) 8.0 ± 0.1 (2) 1.1 ± 0.6 (3) 1.1 ± 0.6	(1) 1.3 ± 0.1 (2) 1.3 ± 0.1	NT	NT	(1) NE (2) 8.0 ± 0.1	No	DAD	35% (v/v)	16 hours
	T. binghami		(1) 13.3 ± 0.6 (2) 14.0 ± 0.1	(1) 12.7 ± 0.6 (2) 12.7 ± 1.2	(1)11.3 ± 0.6 (2) 8.7 ± 0.7	NT	NT	(1) 12.0 ± 0.1 (2) 13.0 ± 0.1				
(Ramlan et al., 2021)	*T. carbonaria*	NR	(A) 20.5 ± 0.1 (B) 5.4 ± 0.1 (C) 6.0 ± 0.1	NT	(A) 5.4 ± 0.01 (B) 5.9 ± 0.01 (C) 8.6 ± 1.1	(A) 7.6 ± 1.1 (B) 6.5 ± 1.6 (C) 13 ± 1.6	NT	(A) 15.7 ± 1.1 (B) 14.1 ± 1.6 (C) 12 ± 3.2	No	MIC (v/v)	Raw honey	18 hours
	T. hockingsi		NT	(A) 8.6 ± 0.1 (B) 6.5 ± 1.6 (C) 8.1 ± 3.8	NT	NT	NT	NT				
	G. thoracica		NT	NT	NT	(A) 9.7 ± 0.1 (B) 9.2 ± 1.6 (C) 7.6 ± 2.2	NT	(A) 6.5 ± 0.1 (B) 8.6 ± 1.1 (C) 13.0 ± 1.1				
	H. itama		(A) 6.5 ± 1.1 (B) 6.5 ± 1.6 (C) 33.5 ± 3.2	(A) 8.0 ± 0.1 (B) 6.0 ± 0.1 (C) 6.5 ± 1.6	(A) 29.7 ± 0.1 (B) 5.5 ± 0.1 (C) 10.8 ± 1.0	NT	NT	NT				
(Suntiparapop et al., 2015)	*T. laeviceps*	Native and exotic plants, such as *Acacia* sp., *Mimosa* sp., and unidentified pollens	(A) 14.7 ± 3.3 (B) 13.1 ± 4.0 (C) 26.2 ± 8.1 (D) 24.0 ± 9.2	(A) 14.2 ± 3.2 (B) 11.8 ± 4.4 (C) 24.0 ± 8.8 (D) 26.7 ± 9.2	(A) 14.8 ± 2.7 (B) 13.3 ± 4.1 (C) 22.6 ± 9.3 (D) 20.0 ± 9.8	(A) 12.6 ± 4.0 (B) 13.3 ± 4.1 (C) 17.4 ± 4.8 (D) 24.0 ± 8.8	NT	(A) 18.7 ± 6.5 (B) 28.0 ± 7.4 (C) 26.2 ± 8.1 (D) NE	No	MIC (v/v)	64% (w/v)	24 hours 18 hours

(Jimenez et al., 2016)	*S. mexicana*	NR	35.0 ± 3.0	25.0 ± 3.0	50.0 ± 5.0	NT	20.0 ± 5.0	NT	No	MIC (w/v)	Raw honey	24 hours
(Villacrés-Granda et al., 2021)	*Cephalotrigona* sp. *M. cramptoni* *M. grandis* *M. indecisa* *M. mimetica* *Melipona* sp. *N. chapadana* *O. mellaria* *Paratrigona.* sp. *S. polysticta* *T. angustula* *T. silvestriana*	NR	NT	7.0 ± 1.0 16.7 ± 1.2 20.0 ± 0.1 14.5 ± 1.1 18.3 ± 1.7 9.3 ± 0.7 16.7 ± 1.7 4.3 ± 0.7 18.3 ± 1.7 10.1 ± 1.4 11.3 ± 5.6 4.3 ± 0.7	3.7 ± 0.7 11.0 ± 3.3 3.7 ± 0.7 9.9 ± 1.4 3.7 ± 0.7 3.7 ± 0.7 8.7 ± 0.7 3.0 ± 0.1 11.7 ± 1.7 7.8 ± 1.2 3.3 ± 0.3 3.0 ± 0.1	NT	8.7 ± 0.7 18.3 ± 10.8 13.3 ± 1.7 19.2 ± 0.5 20.0 ± 0.1 6.7 ± 1.7 20.0 ± 0.1 8.7 ± 0.7 20.0 ± 0.1 10.0 ± 0.9 11.0 ± 5.9 3.7 ± 0.7	NT	Yes	MIC (w/v)	Raw honey	18 hours
(G. Zamora, Beukelman, Van Den Berg, et al., 2015)	*T. angustula* *S. pectoralis* *M. costaricensis* *M. beecheii*	*S. purpurea* and *G. sepium* *Miconia argentea* *Miconia argentea* *T. ochracea* and *A. inermis*	11.5 ± 7.7 7.0 ± 2.4 14.6 ± 4.9 12.9 ± 5.8	8.9 ± 9.3 2.34 ± 0.1 11.2 ± 7.1 8.0 ± 9.7	11.5 ± 7.8 4.7 ± 0.1 13.8 ± 4.8 9.7 ± 8.5	14.8 ± 8.7 4.7 ± 0.1 13.4 ± 4.9 14.4 ± 8.4	NT	NT	No	MIC (w/v)	50 % (w/v)	24 hours 48 hours
(G. Zamora, Beukelman, van den Berg, et al., 2015)	*M. beecheii* *T. angustula*	NR	NT	(1) 14.6 ± 9.5 (2) 14.6 ± 9.5 (3) 16.7 ± 7.2 (4) 18.7 ± 10.8 (5) 20.8 ± 7.2 (6) 20.8 ± 7.2 (7) 20.8 ± 7.2 (8) 25 ± 0.1 (1) 14.6 ± 9.5 (2) 14.6 ± 9.5 (3) 20.8 ± 7.2 (4) 16.7 ± 7.2 (5) 20.8 ± 7.2 (6) 16.7 ± 7.2 (7) 18.8 ± 10.8 (8) 18.8 ± 10.8	(1) 14.6 ± 9.5 (2) 16.7 ± 7.2 (1) 14.6 ± 9.5 (2) 14.6 ± 9.5	NT	NT	NT	Yes	MIC (w/v)	50 % (w/v)	24 hours

Abbreviations: *Escherichia coli* (Ec), *Staphylococcus aureus* (Sa), *Pseudomonas aeruginosa* (Pa), *Salmonella enterica* sv (Sen), *Enterococcus faecalis* (Ef1), and *Klebsiella pneumoniae* (Kp). NE: No effect, NR: No reported, NT: No tested, MDR: Multidrug-resistance, DAD: Disk-Agar Diffusion, MIC: Minimum Inhibitory Concentration. *Please consult the original paper for more details. [1] In DAD assays the unit is (mm), while in MIC assays it is mL/mL or mg/mL as appropriate. [2] The number in parentheses indicates a different strain of the same species, while the capital letters in parentheses symbolize a different honey sample.[3] In case multiple concentrations have been tested, the value shown belonged to the highest concentration. 4 Included serovars: Enteritidis, Paratyphi, Typhi, and Typhimurium.

Due to the high multifactorial heterogeneity observed in the preliminary results added to the methodological variations and parameters attributable to the honey nature, we decided to develop the meta-analytical analyses with the pathogens for which we had at least three different studies for each method, more exactly minimum inhibitory concentration (MIC) by broth microdilution assay and diffusion in agar/disk. As expected, the overall results of the random effects model were consistent and showed a remarkable antimicrobial effect. As shown in Table 3, *S. aureus* is one of the most commonly used organisms in diffusion assays (19 studies), followed by *E. coli* (18 studies) and *E. faecalis* (4 studies), while in MIC assays the most commonly used pathogens are *S. aureus*, *E. coli* and *P. aeruginosa*. The highest pooled mean in diffusion assays corresponded to *S. aureus*, showing a discrete difference between MRSA and non-MRSA strains [14.43 (95% CI: 12.16-16.71) mm *versus* 11.55 (95% CI: 10.22-12.87) mm], while the lowest pooled mean was *K. pneumoniae* 4.80 (95% CI: 2.31-7.29) mm. *E. coli* and *E. faecalis* showed pooled means of 9.09 (95% CI: 7.93-10.25) and 6.59 (95% CI: 3.17-10.00) mm, respectively. Regarding MIC expressed as volume/volume percentage (%, v/v), the estimates were 7.89 (95% CI: 3.94-11.83) mL/100 mL for methicillin-resistant *S. aureus* (MRSA) and 5.60 (95% CI: 2.66-8.55) mL/100 mL considering all *S. aureus* strains. On the other hand, the estimates for MICs expressed as mass/volume percentage (% m/v) showed 15.00 [(95% CI: 12.84-17.16) g/100 mL for *S. aureus*, 16.17 (95% CI: 5.78-26.55) g/100 mL for *E. coli*, and 10.26 (95% CI: 5.64-14.88) g/100 mL for *P. aeruginosa*. However, the results were accompanied by very high values of heterogeneity (I^2), as shown in Table 3. Therefore, no reliable subgroup analysis was possible to obtain due to the large number of subgroups observed in the categorical variables. Overall, no publication bias was identified by Egger's linear regression test (p = 0.4056), but the visual analysis of the funnel symmetry suggested some biases.

Table 3 Pooled means in agar/disk diffusion assays and minimum inhibitory concentration assays

			Inhibition halo (mm)				
Diffusion assays	**n**	**k**	**Pooled mean [95%-CI]**	**τ^2**	**τ**	**Q**	**I^2**
S. aureus	167	19	12.28 [11.13; 13.43]	56.36	7.51	32606935.70	100.0%
MRSA	42	4	14.43 [12.16; 16.71]	52.98	7.28	212303.42	100.0%
Non-MRSA	125	19	11.55 [10.22; 12.87]	55.74	7.47	25183801.81	100.0%
E. coli	133	18	9.09 [7.93; 10.25]	44.79	6.69	54773910.23	100.0%

E. faecalis	20	4	6.59 [3.17; 10.00]	52.97	7.28	7803.29	99.8%
Non-resistant strains	7	3	11.98 [5.54; 18.43]	48.29	6.95	6152.40	99.9%
K. pneumoniae	29	6	4.80 [2.31; 7.29]	42.81	6.54	21956370.79	100.0%
Non-resistant strains	16	5	8.72 [5.19; 12.25]	43.87	6.62	14565536.54	100.0%
Pseudomonas aeruginosa	35	8	8.58 [7.03; 10.14]	19.78	4.45	11846.19	99.7%
Salmonella enterica	23	5	6.62 [2.66; 10.57]	83.59	9.14	16090.87	99.9%

			MIC (mL /100 mL				
MIC assays	**n**	**k**	**Pooled mean [95%-CI]**	**τ^2**	**τ**	**Q**	**I^2**
S. aureus	20	3	5.60 [2.66; 8.55]	27.72	5.26	178206.35	100.0%
Non-MRSA	14	3	7.89 [3.94; 11.83]	33.39	5.78	134815.21	100.0%

			MIC (g /100 mL)				
MIC assays	**n**	**k**	**Pooled mean [95%-CI]**	**τ^2**	**τ**	**Q**	**I^2**
S. aureus	34	5	15.00 [12.84; 17.16]	34.85	5.90	1664456.03	100.0%
Non-MRSA	6	3	12.81 [3.78; 21.84]	71.10	8.43	1563.58	99.7%
E. coli	6	3	16.17 [5.78; 26.55]	94.44	9.72	165.57	97.0%
P. aeruginosa	21	4	10.26 [5.64; 14.88]	95.75	9.78	3024.24	99.3%

*Selected with at least 3 different studies. Variables and parameters: n, Number of assays; k, Number of studies; Q, I^2 and τ, Heterogeneity indexes; MIC, Minimum inhibitory concentration. MRSA, Methicillin-resistant *S. aureus*. The pooled mean was calculated with a 95% CI using a random effects model. The mean, standard deviation, and the number of replicates were used to calculate the pooled effect.

Due to the extreme values of heterogeneity, meta-regression models were used to evaluate the heterogeneity of the data set (see Supplementary table 1 and Supplementary table 2), which showed an abundance of covariates and a small number of studies. Based on meta-regression models, the experimental conditions and honey-associated factors greatly influenced the antimicrobial activity quantified in our study set (Table 4). When comparing individually variance of these variables/moderators, the results on the antimicrobial activity against *S. aureus* were strongly impacted by the type of stingless bee species (48.8% of the variance), followed by the pathogen strain (42.3% of the variance), and the honey concentration/dilution (35.7% of the variance), also the incubation time (7.15% of the variance), as shown in Supplementary table 1. In fact, the multiple meta-regression model considering the previous covariates through an additive model of mixed effects explained more than 70% of the total variance in the results of the data set. Similar results were also obtained on the multiple meta-regression models applied to *E. coli* and *P. aeruginosa* (Table 4, Supplementary table 2).

Table 4 Better explanatory multiple meta-regression models of the disk-agar diffusion methodology.

Microorganism	Moderator(s)	τ^2	τ	I^2	H^2	R^2	*P**
S. aureus	Stingless bee specie + Pathogen strains + Reported concentration + Incubation time	13.16 (SE = 1.76)	3.63	100.0%	76624	76.7%	< 0.0001
E. coli	Stingless bee specie + Pathogen strain + Reported concentration	7.36 (SE = 1.12)	2.71	100.0%	68222	83.6%	< 0.0001
P. aeruginosa	Stingless bee specie + Pathogen strain + Reported concentration	5.79 (SE = 2.22)	2.41	96.4%	28	70.8%	0.0005
E. faecalis	Stingless bee genus + Pathogen strain + Continent	37.07 (SE = 15.94)	6.09	100.0%	303436	30.0%	0.1390
S. enterica	Stingless bee genus	24.00 (SE = 8.04)	4.90	100.0%	440026	71.3%	< 0.0001

τ^2, τ, and I^2, Heterogeneity indexes; H^2 and R^2, Variability indexes *p*, Predictor/model significance.

DISCUSSION

In the last decades, studies on the antimicrobial activity of stingless bee honey had been realized due to the presence of numerous compounds with potential therapeutic properties. However, due to various factors and/or limitations, only a few studies have in detail characterized at the molecular level these compounds by high performance liquid chromatography (HPLC)(Biluca et al., 2020; Pimentel et al., 2013), gas chromatography-mass spectrometry (GC-MS)(Popova et al., 2021), and proton nuclear magnetic resonance (^{1}H-NMR) (Ngaini et al., 2021; J. R. Silva et al., 2022). Nonetheless, the recent implementation of next-generation sequencing (NGS) technologies allow to evaluate changes in gene expression and thus metabolic changes in target pathogens when exposed to certain bioactive compounds (Al-kafaween et al., 2021; Al-Kafaween et al., 2020; Seder et al., 2021). Due to most studies in our group set did not apply these molecular methodologies, little is still known about the contribution of each bioactive compound from stingless bee honey samples in the antimicrobial activity difficulting the assessment of the antimicrobial effect on the present meta-analysis. Further studies must apply these molecular methodologies to fully characterize the potential therapeutic applications of stingless bee honey.

The diversity within the taxonomy of stingless bees, (phyto)geographical origins, biochemical composition in honey samples, microorganisms tested, applied methodology, and procedure settings (such as honey concentration/dilution and microbial growth time) among other less explored factors have not allowed obtaining a straightful outcome in the meta-analysis. However, the present work gathered information on worldwide studies and certain specific stingless bee

species showed antimicrobial activities with potential therapeutic applications, such as *Heterotrigona itama, Tetragonisca angustula*, and *Melipona beecheii. Heterotrigona itama* is a native bee species of the Southeast Asian archipelago and a clear example of how several factors modified its therapeutic effects. Mahmood et al. (2021) showed significantly greater antimicrobial growth inhibition in *H. itama* honey samples collected during the dry season against four foodborne pathogenic bacteria (*E. coli, S. aureus, L. monocytogenes, Salmonella enterica ser.* Typhimurium), when compared to samples collected in the rainy season, suggesting that the rainy season reduces the foraging activity of stingless bees and increases environmental humidity leading to its reduction of antimicrobial activity (Mahmood et al., 2021). Furthermore, certain properties are enhanced by the variety and quantity of nearby flowers, but the role of harvesting in the synchrony of flowering is rarely considered (W. J. Ng et al., 2020; Ngaini et al., 2021; Saputra & Nurlina, 2022; Syed Yaacob et al., 2020; Wu et al., 2022). Most of the studies described little information about the botanical origin of honey samples and merely considered them as multifloral honeys, but future studies should relate the antioxidant and antibacterial properties with the qualitative and quantitative properties of phenols and flavonoids processed or obtained of plant-derived bioactive compounds (Kocsis et al., 2022; Nayaka et al., 2020). Likewise, few studies carried out a complete melissopalynological analysis of the honey samples (Mahmood et al., 2021; Ngaini et al., 2021; G. Zamora, Beukelman, Van Den Berg, et al., 2015), leading to the actual gap in the literature. This aspect has been little explored even though there is strong evidence of the influence of the impact of botanical origin on the abundance of bioactive compounds (Ávila, Hornung, et al., 2019; Majid et al., 2020; Roby et al., 2020). Current literature suggested that stingless bee honeys are usually multifloral (Table 2), although monofloral honeys have been reported such asZamora et al. (2015) reported four Costa Rican stingless bee species (*T. angustula*, *S. pectoralis*, *M. costaricensis*, and *M. beecheii*) that produced monofloral honeys due to local pollinators inducing homogeneous feeding strategies focused on the most profitable nearby floral sources (G. Zamora, Beukelman, Van Den Berg, et al., 2015). More exactly, the cited floral sources were *S. purpurea* and *G. sepium*

(*T. angustula*), *M. argentea* (*S. pectoralis* and *M. costaricensis*), and *T. ochracea* and *A. inermis* (*M. beecheii*) (G. Zamora, Beukelman, Van Den Berg, et al., 2015). Monofloral honeys with prebiotic properties towards *Lactobacillus acidophilus* LA-05 and *Bifidobacterium lactis* BB-12 have also been described in the Brazilian semiarid region produced by stingless bees *M. subnitida* and *M. scutellaris*, where their main floral sources were *Z. juazeiro, C. heliotropiifolius* (velame branco) and *M. arenosa* (jurema branca) (Costa et al., 2018; de Melo et al., 2020). However, other factors influenced the reported antimicrobial activity of stingless bee honey as previously discussed in the present study.

The inhibition halos and MICs estimated by our random-effects models differ markedly from the cut-off points established as reference in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2022; EUCAST, 2023). For example, cefoxitin is a second-generation cephalosporin effective against gram-negative and gram-positive bacteria whose cut-off point in diffusion assays with 30 µg discs is 22 mm, however our estimates are below this value, similar to other cut-off points of other first-line antibiotics. A plausible explanation is the non-polar properties of certain bioactive components with higher antimicrobial activity but they revealed difficulties to correctly diffuse in the agar, so it is suggested to complement the antimicrobial evaluation with other methods such as MIC assays (Mama et al., 2019; Osés et al., 2022; Tomczyk et al., 2020). Concerning MIC values, the differences stand out much more because they must be quantified on logarithmic scales. The antimicrobial action of the bioactive compounds may be attenuated by the interference of other honey components such as sugars, so the full antimicrobial effect of some particular compounds must be individually evaluated after their isolation and purification.

Our meta-regression models with several moderators explained more than 70% of the variance, which is consistent with our very high heterogeneity values, not allowing us to obtain final remarks in our meta-analysis. Higgins et al. (2003) stated that I^2 is useful when assessing inconsistency between studies, besides being a good evaluator of heterogeneity. An I^2 value >75% indicates

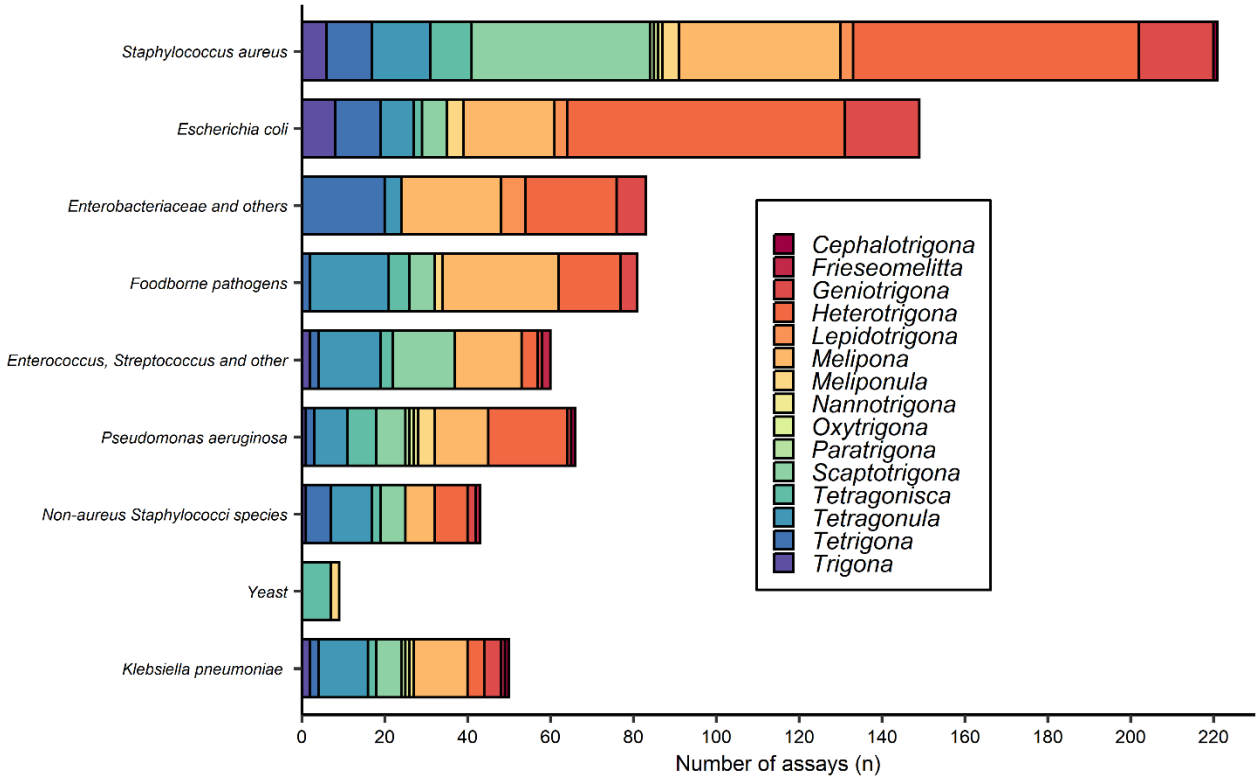
large amounts of heterogeneity. and our study showed I^2 values remained above 97% in most cases indicating that the variability between studies (Fletcher, 2007; Higgins et al., 2003; Imrey, 2020; L. Sun & Feng, 2019). This constitutes the main limitation of the present work, but it does not invalidate the remarks observed in the results collected and analyzed in the systematic review.

CONCLUSIONS

In summary, the present study allowed for the characterization of patterns of the antimicrobial activity of stingless bee honey (Tribe: *Meliponini*) on clinically relevant microorganisms and, consequently, the identification of better approaches for further evaluation. Although the present meta-analysis was performed methodically, some limitations in this study evidenced the high degree of variability in our group set demonstrating a lack of sufficient published data. However, the systematic review suggested that: (1) Applying complementary methodologies compensates for the disadvantages of either method allowing a more accurate quantification of antimicrobial activity and considering the compositional nature of the honey. For example, use as screening of a set of samples a method based on agar diffusion assays and confirm by MIC assay; (2) The honey of *Heterotrigona itama*, *Tetragonisca angustula*, and *Melipona beecheii* were the most studied stingless bee species requiring further analysis through the isolation and full characterization of their antimicrobial candidates for pathogens that require urgent testing such as MRSA strains; (3) The melissopalynological profile allows understanding the compositional changes related to environmental factors, as well as being able to elucidate the origin of certain molecules such as flavonoids. Therefore, it is a pertinent parameter in the complete characterization of a honey sample.; (4) Although there is greater evidence of antibacterial activity against a wide catalog of bacterial species, little is still known about the antifungal and antibiofilm activities of stingless bee honey and future studies must be realized in these areas (5) The therapeutic potential of stingless bee honey is markedly superior to *Apis mellifera* honey, however, its low availability limits the research to be carried out, so efficient augmentation of stingless bee honey production is

recommended. Future studies should also standardize protocols and the multiple experimental variations to evaluate the antimicrobial activity of stingless bee honey and further characterize the bioactive compounds for therapeutic applications

SUPPLEMENTARY INFORMATION



Supplementary Figure 1 Stingless bee genus and grouped target microorganisms. Each y-axis bar represents a grouped target microorganism, while the size of each colored division is proportional to the number of species analyzed within the same stingless bee genus (color). The x-axis indicates the number of assays reported.

Supplementary table 1 Univariate and multiple meta-regression models in assays with *Staphylococcus aureus* (extended version).

Methodology	Moderator(s)	τ^2	τ	I^2	H^2	R^2	*p*
Disk-Agar diffusion	Stingless bee specie	28.8814 (SE = 3.4375)	5.3741	100.00%	207957.54	48.76%	< 0.0001
Disk-Agar diffusion	Pathogen strain	32.5156 (SE = 3.8172)	5.7022	100.00%	244766.95	42.31%	< 0.0001
Disk-Agar diffusion	Reported concentration	36.2440 (SE = 4.2102)	6.0203	100.00%	245590.56	35.69%	< 0.0001
Disk-Agar diffusion	Stingless bee genus	37.6850 (SE = 4.3074)	6.1388	100.00%	281088.94	33.13%	< 0.0001
Disk-Agar diffusion	MRSA strains	44.7275 (SE = 4.9923)	6.6879	100.00%	354095.11	20.64%	< 0.0001
Disk-Agar diffusion	Concentration (cutoff ≤50)	46.1422 (SE = 5.1484)	6.7928	100.00%	356956.82	18.13%	< 0.0001
Disk-Agar diffusion	Concentration (cutoff ≤25)	47.7222 (SE = 5.3228)	6.9081	100.00%	369163.55	15.33%	< 0.0001
Disk-Agar diffusion	Country	50.0471 (SE = 5.6611)	7.0744	100.00%	396797.61	11.20%	0.0004
Disk-Agar diffusion	Continent	50.0558 (SE = 5.6300)	7.0750	100.00%	391968.74	11.18%	0.0001
Disk-Agar diffusion	Incubation time	52.3315 (SE = 5.8857)	7.2341	100.00%	404939.79	7.15%	0.0026
Disk-Agar diffusion	Raw honey	55.0593 (SE = 6.1318)	7.4202	100.00%	425983.50	2.31%	0.0289
Disk-Agar diffusion	Concentration (cutoff ≤50)	56.0031 (SE = 6.2359)	7.4835	100.00%	433285.70	0.63%	0.1692
Disk-Agar diffusion	Stingless bee specie + Pathogen strains	22.9546 (SE = 2.9009)	4.7911	100.00%	159179.51	59.27%	< 0.0001
Disk-Agar diffusion	Stingless bee specie + Pathogen strains + Reported concentration	17.8141 (SE = 2.3424)	4.2207	100.00%	102005.05	68.39%	< 0.0001
Disk-Agar diffusion	Stingless bee specie + Pathogen strains + Reported concentration + Incubation time	13.1623 (SE = 1.7557)	3.6280	100.00%	76623.99	76.65%	< 0.0001
Disk-Agar diffusion	Stingless bee specie + Pathogen strains + Raw honey + Incubation time	16.5411 (SE = 2.1318)	4.0671	100.00%	114411.95	70.65%	< 0.0001
Disk-Agar diffusion	Stingless bee specie + Pathogen strains + Concentration (cutoff ≤75) + Incubation time	16.0757 (SE = 2.0740)	4.0094	100.00%	111192.67	71.48%	< 0.0001
Disk-Agar diffusion	Stingless bee specie + Pathogen strains + Concentration (cutoff ≤50) + Incubation time	13.8953 (SE = 1.7982)	3.7276	100.00%	96111.27	75.35%	< 0.0001
Disk-Agar diffusion	Stingless bee specie + Pathogen strains + Concentration (cutoff ≤25) + Incubation time	13.0883 (SE = 1.6961)	3.6178	100.00%	90530.28	76.78%	< 0.0001
MIC (v/v)	Country/Continent	0.5797 (SE = 0.2557)	0.7614	99.91%	1139.74	97.91%	< 0.0001
MIC (v/v)	Stingless bee specie	0.6244 (SE = 0.2883)	0.7902	99.92%	1299.78	97.75%	< 0.0001
MIC (v/v)	Stingless bee genus	0.7677 (SE = 0.3329)	0.8762	99.93%	1420.35	97.23%	< 0.0001
MIC (v/v)	Incubation time	0.9547 (SE = 0.3961)	0.9771	99.97%	3259.00	96.56%	< 0.0001
MIC (v/v)	Pathogen strain	3.8788 (SE = 1.7470)	1.9695	99.99%	16873.15	86.01%	0.0003
MIC (v/v)	MRSA strains	17.0328 (SE = 6.2107)	4.1271	100.00%	58304.49	38.55%	0.0221
MIC (v/v)	Reported concentration	17.4536 (SE = 6.2929)	4.1777	100.00%	60305.54	37.03%	0.0677
MIC (v/v)	Country/Continent + MRSA strains	0.5663 (SE = 0.2627)	0.7525	99.92%	1179.14	97.96%	< 0.0001
MIC (v/v)	Country/Continent + Pathogen strain	0.4385 (SE = 0.2486)	0.6622	99.91%	1127.64	98.42%	< 0.0001
MIC (v/v)	Country/Continent + Pathogen strain + Reported concentration	0.4264 (SE = 0.2422)	0.6530	99.92%	1196.00	98.46%	< 0.0001
MIC (w/v)	Stingless bee specie	8.7121 (SE = 7.4845)	2.9516	84.34%	6.39	75.01%	< 0.0001
MIC (w/v)	Stingless bee genus	26.4923 (SE = 10.3114)	5.1471	100.00%	30600.66	24.00%	0.0371
MIC (w/v)	**C**ountry/Continent	30.9307 (SE = 10.1595)	5.5615	99.98%	6054.65	11.27%	0.0446
MIC (w/v)	Incubation time	31.7037 (SE = 10.1492)	5.6306	99.98%	6021.03	9.05%	0.0392
MIC (w/v)	Pathogen strain	32.6915 (SE = 11.8434)	5.7176	98.95%	95.41	6.22%	0.3150
MIC (w/v)	Reported concentration	34.9125 (SE = 11.0089)	5.9087	99.98%	6631.36	0.00%	0.2872
MIC (w/v)	MRSA strains	35.7963 (SE = 11.2679)	5.9830	100.00%	33628.06	0.00%	0.3984
MIC (w/v)	Stingless bee specie + Reported concentration	8.4751 (SE = 8.4459)	2.9112	38.92%	1.64	75.69%	< 0.0001
MIC (w/v)	Stingless bee specie + Pathogen strain	0 (SE = 11.2093)	0	0.00%	1.00	100.00%	< 0.0001

MRSA, Methicillin-resistant *Staphylococcus aureus;* MIC, Minimum inhibitory concentration; SE, standard error.

Supplementary table 2 Univariate and multiple meta-regression models with other target microorganisms.

Microorganism	Methodology	Moderator(s)	τ^2	τ	I^2	H^2	R^2	*p*
E. coli	Disk-Agar diffusion	Reported concentration	20.9121 (SE = 2.7613)	4.5730	100.00%	219773.51	53.32%	< 0.0001
E. coli	Disk-Agar diffusion	Pathogen strain	24.1972 (SE = 3.2134)	4.9191	100.00%	277030.61	45.98%	< 0.0001
E. coli	Disk-Agar diffusion	Concentration (cutoff <75)	24.7983 (SE = 3.1272)	4.9798	100.00%	284156.14	44.64%	< 0.0001
E. coli	Disk-Agar diffusion	Stingless bee specie	29.8590 (SE = 4.0493)	5.4643	100.00%	309388.92	33.34%	< 0.0001
E. coli	Disk-Agar diffusion	Concentration (cutoff <50)	30.9986 (SE = 3.8977)	5.5676	100.00%	355201.29	30.80%	< 0.0001
E. coli	Disk-Agar diffusion	Raw honey	31.2898 (SE = 3.9336)	5.5937	100.00%	358539.74	30.15%	< 0.0001
E. coli	Disk-Agar diffusion	Stingless bee genus	34.5990 (SE = 4.4848)	5.8821	100.00%	396862.91	22.76%	< 0.0001
E. coli	Disk-Agar diffusion	Country	36.8079 (SE = 4.7235)	6.0669	100.00%	441940.51	17.83%	< 0.0001
E. coli	Disk-Agar diffusion	Concentration (cutoff <25)	37.2744 (SE = 4.6766)	6.1053	100.00%	427110.42	16.79%	< 0.0001
E. coli	Disk-Agar diffusion	Continent	41.1927 (SE = 5.2219)	6.4182	100.00%	483012.38	8.04%	0.0053
E. coli	Disk-Agar diffusion	Incubation time	42.9477 (SE = 5.4430)	6.5534	100.00%	493586.94	4.12%	0.0598
E. coli	Disk-Agar diffusion	Reported concentration + Pathogen strain	14.8020 (SE = 2.0641)	3.8473	100.00%	159036.97	66.96%	< 0.0001
E. coli	Disk-Agar diffusion	Reported concentration + Pathogen strain + Stingless bee specie	7.3614 (SE = 1.1240)	2.7132	100.00%	68221.46	83.57%	< 0.0001
E. coli	Disk-Agar diffusion	Raw honey + Pathogen strain + Stingless bee specie	10.0265 (SE = 1.4614)	3.1665	100.00%	104323.47	77.62%	< 0.0001
E. coli	Disk-Agar diffusion	Concentration (cutoff <75) + Pathogen strain + Stingless bee specie	7.3044 (SE = 1.0735)	2.7027	100.00%	76003.14	83.69%	< 0.0001
E. coli	Disk-Agar diffusion	Concentration (cutoff <50) + Pathogen strain + Stingless bee specie	9.7046 (SE = 1.4162)	3.1152	100.00%	98099.60	78.34%	< 0.0001
E. coli	Disk-Agar diffusion	Concentration (cutoff <25) + Pathogen strain + Stingless bee specie	10.4854 (SE = 1.5274)	3.2381	100.00%	105989.24	76.59%	< 0.0001
E. coli	MIC (w/v)	Stingless bee specie	0 (SE = 8.0978)	0	0.00%	1.00	100.00%	0.0726
E. coli	MIC (w/v)	Pathogen strain	11.3720 (SE = 12.0467)	3.3722	79.84%	4.96	87.96%	0.0034
E. coli	MIC (w/v)	Country/Continent/ Reported concentration	61.1443 (SE = 48.4567)	7.8195	94.03%	16.75	35.26%	0.1170
E. coli	MIC (w/v)	Stingless bee genus	130.9365 (SE = 111.0096)	11.4427	97.28%	36.74	0.00%	0.7654
P. aeruginosa	Disk-Agar diffusion	Stingless bee specie	9.9749 (SE = 3.2268)	3.1583	97.92%	48.17	49.58%	0.0037
P. aeruginosa	Disk-Agar diffusion	Pathogen strain	12.3450 (SE = 3.6083)	3.5136	100.00%	28540.41	37.60%	0.0074
P. aeruginosa	Disk-Agar diffusion	Stingless bee genus	13.1188 (SE = 3.7536)	3.6220	100.00%	29209.52	33.69%	0.0094
P. aeruginosa	Disk-Agar diffusion	Reported concentration	15.1294 (SE = 4.1839)	3.8897	100.00%	31369.05	23.52%	0.0217
P. aeruginosa	Disk-Agar diffusion	Country/Continent	17.6213 (SE = 4.7217)	4.1978	100.00%	35327.20	10.93%	0.1050
P. aeruginosa	Disk-Agar diffusion	Concentration (cutoff <50)	19.2107 (SE = 4.9319)	4.3830	100.00%	35033.20	2.89%	0.1672
P. aeruginosa	Disk-Agar diffusion	Incubation time	19.7079 (SE = 5.1442)	4.4394	100.00%	38258.30	0.38%	0.4256
P. aeruginosa	Disk-Agar diffusion	Concentration (cutoff <75)	19.8928 (SE = 5.0987)	4.4601	100.00%	36262.60	0.00%	0.4076
P. aeruginosa	Disk-Agar diffusion	Raw honey	20.0864 (SE = 5.1441)	4.4818	100.00%	36620.65	0.00%	0.4126
P. aeruginosa	Disk-Agar diffusion	Stingless bee specie + Pathogen strain	5.8741 (SE = 2.1770)	2.4237	96.63%	29.65	70.31%	0.0003
P. aeruginosa	Disk-Agar diffusion	Stingless bee specie + Pathogen strain + Reported concentration	5.7869 (SE = 2.2167)	2.4056	96.44%	28.06	70.75%	0.0005
P. aeruginosa	Disk-Agar diffusion	Stingless bee specie + Pathogen strain + Raw honey	5.7869 (SE = 2.2167)	2.4056	96.44%	28.06	70.75%	0.0005
P. aeruginosa	MIC (w/v)	Pathogen strain	10.8463 (SE = 4.5805)	3.2934	100.00%	20405.43	88.67%	< 0.0001
P. aeruginosa	MIC (w/v)	Country/Continent	12.1388 (SE = 5.0052)	3.4841	100.00%	20299.65	87.32%	< 0.0001
P. aeruginosa	MIC (w/v)	Stingless bee specie	19.5310 (SE = 23.9311)	4.4194	55.17%	2.23	79.60%	0.0230
P. aeruginosa	MIC (w/v)	Incubation time	70.0393 (SE = 24.7492)	8.3689	100.00%	110958.31	26.85%	0.0131
P. aeruginosa	MIC (w/v)	Resistant strains	79.4722 (SE = 27.8803)	8.9147	100.00%	125901.84	17.00%	0.0509
P. aeruginosa	MIC (w/v)	Reported concentration	98.6555 (SE = 34.1583)	9.9325	100.00%	156292.33	0.00%	0.5229
P. aeruginosa	MIC (w/v)	Stingless bee genus	118.3537 (SE = 50.1817)	10.8790	99.48%	190.69	0.00%	0.7769
P. aeruginosa	MIC (w/v)	Pathogen strain + Stingless bee genus	12.0546 (SE = 7.5840)	3.4720	95.69%	23.18	87.41%	0.0005
P. aeruginosa	MIC (w/v)	Pathogen strain + Stingless bee specie	0 (SE = 25.1111)	0	0.00%	1.00	100.00%	0.0005
E. faecalis	Disk-Agar diffusion	Pathogen strain	31.8595 (SE = 11.3512)	5.6444	100.00%	477955.04	39.85%	0.0115
E. faecalis	Disk-Agar diffusion	Reported concentration/Incubation time/ Resistant strains	38.3780 (SE = 12.8749)	6.1950	100.00%	511886.43	27.54%	0.0108
E. faecalis	Disk-Agar diffusion	Country	39.9104 (SE = 15.1867)	6.3175	100.00%	427825.43	24.65%	0.1083
E. faecalis	Disk-Agar diffusion	Stingless bee genus	47.6592 (SE = 18.1238)	6.9036	100.00%	408758.35	10.02%	0.2728
E. faecalis	Disk-Agar diffusion	Continent	59.3820 (SE = 21.0862)	7.7060	100.00%	668295.10	0.00%	0.8094

E. faecalis	Disk-Agar diffusion	Stingless bee specie	146.2556 (SE = 146.4449)	12.0936	99.96%	2747.37	0.00%	0.9453
E. faecalis	Disk-Agar diffusion	Pathogen strain + Continent + Stingless bee genus	37.0717 (SE = 15.9346)	6.0887	100.00%	303435.78	30.01%	0.1390
E. faecalis	Disk-Agar diffusion	Pathogen strain + Continent	30.8746 (SE = 12.2176)	5.5565	100.00%	356331.47	41.71%	0.0358
E. faecalis	Disk-Agar diffusion	Pathogen strain + Country	26.0516 (SE = 10.7488)	5.1041	100.00%	325713.25	50.82%	0.0216
K. pneumoniae	Disk-Agar diffusion	Pathogen strain	16.9304 (SE = 5.0202)	4.1147	100.00%	398174.23	60.45%	< 0.0001
K. pneumoniae	Disk-Agar diffusion	Reported concentration	17.7241 (SE = 5.1461)	4.2100	100.00%	399479.07	58.60%	< 0.0001
K. pneumoniae	Disk-Agar diffusion	Incubation time	21.0057 (SE = 5.8583)	4.5832	100.00%	485337.05	50.93%	< 0.0001
K. pneumoniae	Disk-Agar diffusion	Resistant strains	24.2985 (SE = 6.6457)	4.9294	100.00%	567585.47	43.24%	< 0.0001
K. pneumoniae	Disk-Agar diffusion	Concentration (cutoff <75)/Raw_honey	28.3348 (SE = 7.7452)	5.3230	100.00%	662116.48	33.81%	0.0006
K. pneumoniae	Disk-Agar diffusion	Country	33.6750 (SE = 10.1941)	5.8030	100.00%	735923.10	21.34%	0.0744
K. pneumoniae	Disk-Agar diffusion	Continent	36.2689 (SE = 10.2936)	6.0224	100.00%	828061.41	15.28%	0.0695
K. pneumoniae	Disk-Agar diffusion	Stingless bee genus	40.8640 (SE = 12.9487)	6.3925	100.00%	920973.42	4.54%	0.3691
K. pneumoniae	Disk-Agar diffusion	Concentration (cutoff <50)	42.3161 (SE = 11.5509)	6.5051	100.00%	988409.80	1.15%	0.2636
K. pneumoniae	Disk-Agar diffusion	Stingless bee specie	42.7714 (SE = 22.8888)	6.5400	100.00%	921502.07	0.09%	0.5401
S. enterica	Disk-Agar diffusion	Stingless bee genus	23.9989 (SE = 8.0375)	4.8989	100.00%	440025.72	71.29%	< 0.0001
S. enterica	Disk-Agar diffusion	Stingless bee specie	25.1840 (SE = 8.6743)	5.0184	100.00%	488910.75	69.87%	< 0.0001
S. enterica	Disk-Agar diffusion	Pathogen strain	31.0288 (SE = 10.4342)	5.5703	100.00%	568906.14	62.88%	0.0002
S. enterica	Disk-Agar diffusion	Serovar*	36.2601 (SE = 11.8569)	6.0216	100.00%	629846.88	56.62%	0.0003
S. enterica	Disk-Agar diffusion	Country/Continent	37.0027 (SE = 12.0435)	6.0830	100.00%	642767.69	55.73%	0.0003
S. enterica	Disk-Agar diffusion	Concentration (cutoff <75)	59.9032 (SE = 18.5836)	7.7397	100.00%	855965.97	28.33%	0.0052
S. enterica	Disk-Agar diffusion	Concentration (cutoff <50)	61.8763 (SE = 19.1945)	7.8662	100.00%	884150.20	25.97%	0.0076
S. enterica	Disk-Agar diffusion	Raw honey	68.9906 (SE = 21.3897)	8.3061	100.00%	985809.63	17.46%	0.0271
S. enterica	Disk-Agar diffusion	Reported concentration	69.4031 (SE = 24.6661)	8.3309	100.00%	781111.86	16.97%	0.1721
S. enterica	Disk-Agar diffusion	Concentration (cutoff <25)	70.6013 (SE = 21.8885)	8.4025	100.00%	1008806.06	15.53%	0.0355

*Include serovars: Enteritidis, Paratyphi, Typhi, and Typhimurium.

Supplementary table 3 Studies considered in the meta-analysis to estimate the antimicrobial activity of stingless bee honey against different pathogens (extended version) with representative data available.

		Target microorganisms[1]																	
Authors (year)	**Country**	***Ec***	***Sa***	***SCN***	***Pa***	***Sen***	***Ef1***	***Ef2***	***Kp***	***Bc***	***Ca***	***Sp***	***Lm***	**Pr**	**Sm**	**Other**	**MDR**	**Methodology[2]**	**Concentration**	**Incubation time**
Boorn et al. (2010)	Australia	X	X	X	X	X	X										No	DAD	50% (v/v)	24 hours
Domingos et al. (2021)	Brasil	X	X		X		X		X							X	No	DAD	50% (v/v)	24 hours
Chan-Rodriguez et al. (2012)	Mexico	X	X														No	DAD	Raw honey	12 hours
DeMera y Angert (2004)	Costa Rica				X					X	X					X	No	DAD	Raw honey	17 hours 24 hours 48 hours
Gopal et al. (2021)	Malasya	X	X														No	DAD	22%	24 hours
Jibril et al. (2020)	Malasya		X														No	DAD	Raw honey	24 hours
Nishio et al. (2016)	Brasil	X	X	X	X	X X	X	X	X			X					Yes	DAD MIC (v/v)	50% (v/v)	24 hours
	Philippines						X	X	X									DAD		
	Thailand						X	X	X									DAD		
Kimoto-Nira et al. (2015)	Mexico						X	X	X								Yes	DAD	50% (w/w)	24 hours
	Australia						X	X	X									DAD		
	Paraguay						X	X	X									DAD		
Mahmood et al. (2021)	Malasya	X	X			X							X				No	DAD	Raw honey	24 hours
Massaro et al. (2014)	Australia		X						X								No	DAD	Raw honey	16 hours
Ngaini et al. (2021)	Malasya	X	X														No	DAD	Raw honey	24 hours
Pimentel et al. (2013)	Brasil	X	X			X								X		X	No	DAD	10- 50 % (v/v) Raw honey	24 hours
Ramon-Sierra et al. (2020)	Mexico	X	X														No	DAD MIC (w/v)	Raw honey	24 hours
Rosli et al. (2020)	Malasya	X	X												X		No	DAD	12.5 - 50 % (v/v)	24 hours
Saputra et al. (2022)	Indonesia	X	X														No	DAD	Raw honey	24 hours
Suarez et al. (2021)	Philippines		X	X													Yes	DAD	20 % (w/v)	18 hours
Syed Yaacob et al. (2020)	Malasya	X	X		X												No	DAD	75 % (w/v) Raw honey	24 hours
Tesfaye et al. (2022)	Ethiopia	X	X		X	X					X						No	DAD	75% (v/v)	24 hours
Thomas et al. (2021)	India	X															No	DAD	Raw honey	24 hours
Torres et al. (2004)	Colombia	X															No	DAD	50% (v/v)	48 hours
Tuksitha et al. (2018)	Malasya	X	X	X	X				X							X	No	DAD	50% (w/v)	24 hours

Ng et al. (2020)	Malasya	x	x													No	DAD	Raw honey	20 hours
Wu et al. (2022)	Malasya	x	x	x	x			x							x	No	DAD	35% (v/v)	16 hours
Ramlan et al. (2021)	Australia	x	x		x	x		x	x							No	MIC (v/v)	Raw honey	18 hours
	Malasya	x	x		x	x		x	x										
Suntiparapop et al. (2015)	Thailand	x	x	x	x	x		x	x		x	x	x	x	x	No	MIC (v/v)	64% (w/v)	24 hours 18 hours
Jimenez et al. (2016)	Mexico	x	x		x		x									No	MIC (w/v)	Raw honey	24 hours
Villacres-Granda et al. (2021)	Ecuador		x		x			x								Yes	MIC (w/v)	Raw honey	18 hours
Zamora et al. (a) (2015)	Costa Rica	x	x	x	x	x				x		x				No	MIC (w/v)	50 % (w/v)	24 hours 48 hours
Zamora et al. (b) (2015)	Costa Rica		x		x											Yes	MIC (w/v)	50 % (w/v)	24 hours

[1]Abbreviations: *Escherichia coli* (Ec), *Staphylococcus aureus* (Sa), *Coagulase-Negative Staphylococci* (SCN), *Pseudomonas aeruginosa* (Pa), *Salmonella enterica sv* (Sen), *Enterococcus faecalis* (Ef1), *Enterococcus faecium* (Ef2), *Klebsiella pneumoniae* (Kp), *Bacillus cereus* (Bc), *Candida albicans* (Ca), *Streptococcus pyogenes* (Sp), *Listeria monocytogenes* (Lm), *Proteus vulgaris and Proteus mirabilis* (Pr), Serratia marcescens (Sm). Others include*: Alcaligenes faecalis*, *Streptococcus alactolyticus*, *Vibrio parahaemolyticus*, *Citrobacter koseri*, *Micrococcus luteus*, *Saccharomyces cerevisiae*, *Shigella sonnei*, *Streptococcus mutans*, and *Streptococcus pneumoniae*. MDR: Multidrug-resistance. [2]DAD: Disk-Agar Diffusion, MIC: Minimum Inhibitory Concentration

ORIGINAL ARTICLE

EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF NATIVE ECUADORIAN STINGLESS BEE HONEYS (TRIBE: *MELIPONINI*) IN BIOFILMS

Fausto Sebastián Cabezas-Mera[1], María Belén Atiencia-Carrera[1], Irina Villacrés-Granda[2,3], Adrian Alexander Proaño[4], Alexis Debut[5], Karla Vizuete[5], Reinier Abreu-Naranjo[6], Eduardo Tejera[3], José Miguel Álvarez-Suarez[7,8*], António Machado [1*]

[1] Universidad San Francisco de Quito USFQ, Colegio de Ciencias Biológicas y Ambientales COCIBA, Instituto de Microbiología, Laboratorio de Bacteriología, Calle Diego de Robles y Pampite, Quito 170901, Ecuador. E-mails: fscabezasm@estud.usfq.edu.ec (F.S.C.-M.); batienciac@estud.usfq.edu.ec (M.B.A.-C.); amachado@usfq.edu.ec (A.M.)

[2] Programa de Doctorado Interuniversitario en Ciencias de la Salud, Universidad de Sevilla, Sevilla, Spain

[3] Facultad de Ingeniería y Ciencias Agropecuarias Aplicadas, Grupo de Bioquimioinformática, Universidad de Las Américas (UDLA), De Los Colimes esq, Quito 170513, Quito, Ecuador. Email: eduardo.tejera@udla.edu.ec (E.T.); irinamaribel@gmail.com (I.V.-G.)

[4] Laboratorios de Investigación, Universidad de Las Américas (UDLA), De Los Colimes esq, Quito 170513, Quito, Ecuador. Email: adrianalexander.proano@udla.edu.ec (A.P)

[5] Center of Nanoscience and Nanotechnology, Universidad de las Fuerzas Armadas (ESPE), Sangolquí 170501, Ecuador. E-mail: apdebut@espe.edu.ec (A.D.); ksvizuete@espe.edu.ec (K.V.)

[6] Departamento de Ciencias de La Vida, Universidad Estatal Amazónica, Puyo, 160150, Ecuador. E-mail: rabreu@uea.edu.ec (R.A.-N.)

[7] King Fahd Medical Research Center, King Abdulaziz University, Jeddah, 21589, Saudi Arabia

[8] Universidad San Francisco de Quito USFQ, Colegio de Ciencias e Ingenierías, Departamento de Ingeniería en Alimentos, Calle Diego de Robles y Pampite, Quito 170901, Ecuador. Email: jalvarez@usfq.edu.ec (J.M.A.-S.)

* Correspondence: jalvarez@usfq.edu.ec and amachado@usfq.edu.ec

ABSTRACT

Biofilms are associated with serious and chronic infections that are resistant to conventional therapies, contributing to the antimicrobial resistance crisis. The need for alternative approaches to combat biofilms is well-known. Although natural products like stingless bee honeys (tribe: *Meliponini*) have been considered as an alternative treatment, much is still unknown. Our main goal was to evaluate the antibiofilm activity of stingless bee honey samples against multidrug-resistant (MDR) pathogens through biomass assays, fluorescence (total cell count and cell viability), and scanning electron (structural biofilm composition) microscopy. We analyzed thirty-five honey samples produced by ten different stingless bee species from five provinces of Ecuador (Tungurahua, Pastaza, El Oro, Los Ríos, and Loja) against 24h biofilms of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Candida albicans*, and *Candida tropicalis*. The five best honey samples showed a range of 63–80% biofilm inhibition through biomass assays. Fluorescence microscopy (FM) analysis evidenced statistical log reduction in the cell count of honey-treated samples in all pathogens (P <0.05), except for *S. aureus* ATCC 25923. Concerning cell viability, *C. tropicalis*, *K. pneumoniae* ATCC 33495, and *K. pneumoniae* KPC significantly decreased (P <0.01) by 21.67, 25.69, and 45.62%, respectively. Finally, scanning electron microscopy (SEM) analysis demonstrated structural biofilm disruption through cell morphological parameters (such as area, size, and form). To the authors' best knowledge, this is the first study to simultaneously analyze stingless bees honey-treated biofilms of susceptible and/or MDR strains of *S. aureus*, *K. pneumoniae*, and *Candida* species. This study confirmed the antibiofilm activity of several stingless bee honeys in Ecuador.

INTRODUCTION

Ecuadorian ancestral medicine maintains a legacy of pre-Columbian entomological knowledge, alongside native culture in art and gastronomy (Vit et al., 2017). In particular, Indigenous peoples considered stingless bees as special, because honey and its derivatives can be used in the treatment of wounds as well as ocular and gastrointestinal infections (Hau-Yama et al., 2020; Rao et al., 2016; Vit et al., 2017). The honey production of stingless bees (Tribe: *Meliponini*) is very small compared to honeybees (genus *Apis*), so its use is more directed to medicinal than food purposes (Rao et al., 2016; Vit et al., 2015; Zulkhairi Amin et al., 2018). The antibacterial activity of stingless bee products has been widely discussed by researchers around the world (Al-Hatamleh et al., 2020; Bouchelaghem et al., 2022; Popova et al., 2021; Zulhendri et al., 2022). The intrinsic properties of honey, such as low pH, high osmolarity, low water activity, and certain antioxidant compounds (still poorly known), inhibit the growth of several microorganisms, including fungi (Proaño et al., 2021; Toaquiza Vilca, 2020). Recent studies have shown that stingless bee honey exhibits a stronger antimicrobial effect compared to honey from domestic bees even in multidrug-resistant strains (Kot et al., 2020; Villacrés-Granda et al., 2021), as well as several fungal species of clinical interest (Fonte-Carballo et al., 2016; Hau-Yama et al., 2020; Manrique & Santana, 2008) and agricultural importance (Albores-Flores et al., 2018). However, little is known about its impact on biofilms, which are frequently associated with more virulent infections and are resistant to conventional therapies, especially in patients with chronic wounds, prostheses, burns, or diabetes (Pinto et al., 2021). Nowadays, more and more research is being carried out regarding antibiofilm agents that can act alone or in synergy and stingless bee honeys have been considered an important source of bioactive compounds with relevant biological properties (Alvarez-Suarez et al., 2018; Biluca et al., 2016). However, the mechanisms involved remain unknown, and only a few possible related bioactive peptides have been described in the literature (Brudzynski, 2021; Brudzynski et al., 2015; Brudzynski & Sjaarda, 2015; Cauich Kumul et al., 2015; Cunha et al., 2013; Proaño et al., 2021). Against this background, the aims of this research were to evaluate the antibiofilm

activity of different native stingless bee honey samples from Ecuador against multidrug-resistant (MDR) pathogens through biomass assays, fluorescence (total cell count and cell viability), and scanning electron (structural biofilm composition) microscopy.

MATERIALS AND METHODOS

Isolates and growth conditions

Two bacterial and two fungal species were selected for the present study. For bacterial species, *Staphylococcus aureus* and *Klebsiella pneumoniae* were chosen as representative examples of well-known gram-positive and -negative pathogens, respectively (Tacconelli et al., 2018). For each bacterial species, one MDR strain and one susceptible strain were used in this study. The two MDR strains were *Staphylococcus aureus* MRSA 333 and *Klebsiella pneumoniae* KPC 609803, while the two susceptible strains were *Staphylococcus aureus* ATCC 25923 and *K. pneumoniae* ATCC 33495. In previous studies, *Staphylococcus aureus* MRSA 333 was isolated from nasal and pharyngeal volunteers from Universidad de Las Americas in Quito (Ecuador) (Proaño et al., 2021; Villacrés-Granda et al., 2021), and *Klebsiella pneumoniae* KPC 609803 was donated from the collection of clinical isolates at Zurita & Zurita Clinical Laboratories (http://www.zuritalaboratorios.com) in Quito (Ecuador) (García-Tenesaca et al., 2017). As previously described, *S. aureus* MRSA 333 is resistant to penicillin and oxacillin (Bastidas et al., 2019) and *K. pneumoniae* KPC 609803 is resistant to imipenem and ertapenem (García-Tenesaca et al., 2017); however, their resistance profiles were confirmed for this study through antibiograms. In addition, *Candida albicans* and *Candida tropicalis* were elected as representative examples of well-known *Candida* species associated with opportunistic infections (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022; Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022), more precisely *C. albicans* ATCC 1023 and *C. tropicalis* isolates from the microbial collection of the Institute of Microbiology, Universidad San Francisco de Quito (designated as IMUSFQ-V546). *C. tropicalis* isolate IMUSFQ-V546 was previously recovered from a patient with invasive candidiasis and identified through DNA sequences at multiple loci and biochemical properties at the National

Institute for Research in Public Health (INSPI). Before biofilm assays, each microorganism was previously cultured in Tryptic Soy Broth (TSB) for 24 hours at 37 °C and then microbial growth was adjusted to 0.5 McFarland with phosphate-buffered saline (PBS) to obtain an estimated cellular density of 1.5 $x10^8$ colony-forming units (CFU)/mL for bacterial strains, $1.5x10^6$ CFU/mL for *C. albicans*, and 3.0 $x10^6$ CFU/mL for *C. tropicalis* (Guinea et al., 2010).

Honey samples

The study set included thirty-five different honey samples produced by ten different stingless bee species from five provinces of Ecuador, namely Tungurahua, Pastaza, El Oro, Los Ríos, and Loja. Samples were donated by stingless bee beekeepers registered at the Ecuadorian Agency for Agricultural Quality Assurance (AGROCALIDAD, Ecuador). The honey samples were collected in sterilized plastic containers and stored at 4–6 °C in the dark until further preparation. The identification of the stingless bee specimens, as well as the physicochemical characterization of honey samples, was determined beforehand as previously reported (Villacrés-Granda et al., 2021). For the anti-microbiological capacity studies developed here, the collected samples were filtered and centrifuged to avoid the development of microorganisms typical of honey (L. P. de Sousa, 2021; Echeverrigaray et al., 2021; Rosa et al., 2003; M. S. Silva et al., 2017). Stock solutions of each sample were prepared at 50% (v/v) diluted in PBS. For all experimental assays, the honey concentration was set up to 15% (v/v), at which point previous studies showed significant biological activity among honey samples (Proaño et al., 2021; Villacrés-Granda et al., 2021). In addition, a solution of artificial honey lacking H_2O_2 was made as osmotic control, which is a normal product of glucose oxidation consisting of 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose, and 33.5 g glucose in 17 mL of deionized water. This osmotic control aimed to evaluate the contribution of the predominant honey sugars to the biofilm inhibition assays and was also evaluated at 15% (v/v) diluted in PBS (Cooper et al., 2002).

Biofilm inhibition and eradication assays

The inhibition and eradication of biofilms by honey samples in the present study were evaluated by biofilm biomass quantification. Further analysis was realized by evaluating the biofilm inhibition assays through the total cell count together with cell viability and the structural biofilm composition. The biofilm biomass quantification was realized through an optical density (OD) assay with crystal violet (CV) staining, as previously reported by (Gulati et al., 2018). The total cell count and cell viability analysis were performed through fluorescence microscopy (FM) using DAPI and LIVE/DEAD assays. Finally, structural biofilm composition was evaluated through scanning electron microscopy (SEM) analysis. Each type of biofilm inhibition assay was performed with at least three replicates per microorganism on different days and, in each replicate assay, two samples of biofilm by microorganism were analyzed.

Optical density assay with crystal violet staining

As previously described, fresh growth cultures of each microorganism were adjusted to 0.5 McFarland with PBS before the preparation of 96-well plates (Guinea et al., 2010). The biofilm inhibition and eradication assays were carried out according to a previous study with slight modifications (Sornsenee et al., 2021). To the 96-well plates for biofilm inhibition, 110 µL of TSB, 100 µL of inoculum 0.5 McFarland, and 90 µL of the honey sample stock solution were added. In addition, positive and negative controls were added in each assay. Positive controls consisted of wells with 110 µL TSB, 100 µL of inoculum, and 90 µL PBS, while negative controls were 110 µL of TSB and 190 µL of PBS. After the initial preparation, the 96-well plate was incubated for 24 h at 37°C under a constant orbital agitation of 120 rpm. To evaluate the honey samples' ability to inhibit biofilm biomass formation, we used an optical density assay with crystal violet (CV) staining using a modified version of the method suggested by Peeters et al. (2008). Briefly, the fixation step was realized with 200 µL of methanol 100% (vol/vol) for 20 min, and the biofilms were stained with 200 µL of CV solution at 1% (vol/vol; Merck, Darmstadt, Germany) for 20 min. Each well

was washed twice with 200 µL of PBS and then decolored with 200 µL of glacial acetic acid at 99.8% (vol/vol; ThermoFisher Scientific, Massachusetts, USA). Finally, the optical density at 630 nm (OD630 nm) of the 96-well plate was read in the spectrophotometer ELISA Elx808 (BioTek, Winooski, USA), removing the absorbance values of the negative controls from the remaining wells and considering positive controls as the total biofilm formation for each microorganism. After the initial evaluation of the potential antibiofilm activity by stingless bee species, the honey samples with the highest inhibition rates on biofilm biomass in each microorganism were selected for further FM and SEM analysis. For biofilm eradication evaluation, similar procedures and controls were realized apart, from previous 24h-biofilm samples grown under the same experimental conditions, followed by washing steps and then the honey samples were added to the fresh medium at 15% (v/v) in the wells. An additional incubation of 24 hours under the same conditions was realized before the 96-well plate was washed and then read in the spectrophotometer at OD630 nm.

Fluorescence microscopy analysis (FM)

The total cell count and cell viability evaluation through FM analysis were performed in 6-well plates containing a sterile coverslip as an abiotic surface for biofilm development (Chandra & Mukherjee, 2015). Each 6-well plate contained honey-treated samples, negative controls, or positive controls. For positive controls, duplicate wells were filled with 100 µL of appropriate microbial inoculum in PBS solution and 2.9 mL of sterile TSB. For negative controls, duplicate wells were set up with 100 µL of PBS and 2.9 mL of sterile TSB. Lastly, honey-treated wells were set up with 100 µL of appropriate microbial inoculum and 2.9 mL of TSB containing 15% (v/v) of the selected honey samples (from previous stock solutions) or a solution of artificial honey (osmotic control). Then, the 6-well plates were incubated for 24 h at 37°C with a constant orbital agitation of 120 rpm. After the realization of 24h-biofilm assays, the medium was carefully removed from the wells and the coverslips were also carefully washed with 3 mL of sterile phosphate-buffered saline (PBS) to remove the growth medium and the planktonic cells. Each coverslip containing the

biofilm sample was then placed in a sterile plastic flask with 3 mL of sterile PBS, scrapped, and vortexed at maximum velocity for five minutes to ensure that the biofilm was removed from the coverslip and entered the PBS solution, as described in the literature (Castro et al., 2022). Finally, the total cell count and cell viability evaluation through FM analysis were realized using 200 µL of the PBS solution containing biofilm cells in a new and sterile coverslip.

For total cell count evaluation, DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; D3571, ThermoFisher Scientific, Massachusetts, USA) fluorescence staining was used in the recovered coverslips by applying a working solution of 300 nM in PBS. A further analysis was also done through DAPI staining to study the structure and composition of biofilm bridges and extracellular DNA (eDNA), as previously described in other studies (Ducret et al., 2016; Lei et al., 2019; Zatorska et al., 2017). Meanwhile, we applied two different LIVE/DEAD kits to evaluate the cell viability, using the LIVE/DEAD Yeast Viability Kit (L7009, ThermoFisher Scientific, Massachusetts, USA) for *Candida* species and LIVE/DEAD BacLight Bacterial Viability Kit (L7012, ThermoFisher Scientific, Massachusetts, USA) for the remaining microorganisms. Working solutions were prepared according to the manufacturer's manuals and stored at -20°C. Briefly, the final concentrations for the bacterial assays were 6 µM of Syto-9 and 30 µM of propidium iodide, and the final concentrations for the fungal assays were 10 µM of FUN-1 and 25 µM of calcofluor white M2R. After the fixation of the previous 200 µL PBS solution containing biofilm cells in a sterile coverslip by drying at room temperature or incubation (at 60 °C), 200 µL of LIVE/DEAD working solution was carefully applied to treated and untreated biofilm samples and incubated at room temperature in the dark for 15 min. The samples were washed twice with 200 µL of PBS to remove excess fluorescent dyes and 200 µL of DAPI working solution was added to the samples, making sure that the biofilms were completely covered. The coverslips were further incubated at room temperature in the dark for 10 min. Finally, the samples were washed twice with PBS to remove the excess DAPI and were dried at room temperature (25 °C) in the dark until EM analysis. Images were obtained with an Olympus BX50 microscope equipped with the MU633-FL

digital camera (AmScope, AmScope, California, USA) and digitized with AmScope version 1.2.2.10. As previously described (Rosenberg et al., 2019), 15 images per sample were taken following a zigzag pattern from top to bottom trying to cover the entire surface of the coverslip. For more reproducible results presentation, the counted cells were given per frame (9600 μm^2) of the visual observation at 1000x on the fluorescence microscope. Images were merged in Fiji-ImageJ version 1.57 (Schindelin et al., 2012). The Fiji-ImageJ software was also used to obtain total cell counts and content of extracellular polymeric substance (EPS) in DAPI images (Lippi et al., 2019). For cell viability, the percentages of dead and alive cells within images were measured using Fiji-ImageJ software, specifically the macros Biofilms Viability checker proposed by the plugin MorphoLibJ (Legland et al., 2016; Mountcastle et al., 2021).

Scanning electron microscopy analysis (SEM)

For SEM analysis, sterile 22-mm circular cover glasses (Heathrow Scientific, Vernon Hills, Illinois, USA) were placed in 6-well plates and 24h-biofilm assays were realized as previously described in EM analysis. Wells containing biofilm samples were fixed with a solution of 4% glutaraldehyde in PBS with adjusted pH (similar to growth broth) for 1 h. A post-fixation step was carried out with 1% osmium tetroxide in cacodylate buffer for 1 h and then samples were treated with 1% tannic acid for an additional 1 h. The samples were dehydrated through a series of immersion steps with different ethanol solutions (30%, 50%, 70%, 80%, 90%, and 100% at HPLC grade; v/v in distilled water) for 30 min for each one. The samples were subsequently frozen with liquid nitrogen and dried for 4 days in a freeze dryer (-50°C, 0.400 hPa). Finally, cover glasses containing biofilm samples were coated with gold through a sputter coating machine (Quorum, Q150R ES, UK) and SEM analysis was performed using a Tescan Mira 3 scanning electron microscope equipped with a Schottky Field Emission Gun (Schottky FEG-SEM, MIRA III TESCAN, Brno, Czech Republic) at the Centro de Nanociencia y Nanotecnología of the Universidad de las Fuerzas Armadas (ESPE), as previously described (Pilaquinga et al., 2019). Morphology parameters and shape descriptors of

the cells were also obtained from all images via Fiji-ImageJ version 1.57 (Schindelin et al., 2012), as reported in other studies (Ducret et al., 2016; Lobo et al., 2016; Prodanov & Verstreke, 2012). At least 150 cells were sampled for comparison between untreated and treated-biofilm samples through different morphological parameters and shape descriptors, as reported by others (Casar et al., 2021; Lobo et al., 2016; Sieniawska et al., 2015; Sridhar et al., 2021). Finally, fractal dimension was estimated from the slopes of cross-correlation functions to describe biofilm morphology, as previously described in other studies (Beyenal et al., 2004; Hermanowicz et al., 1995, 1996; Lewandowski et al., 1999; Picioreanu et al., 1998)

Statistical Analysis

For pairwise comparison between control and honey-treated samples, the Wilcoxon nonparametric test was applied, except for the fractal dimension analysis, where due to sample size, the *t*-test was applied. In the preliminary antibiofilm activity among the honey set screening through OD assays, the Wilcoxon test with Holm–Bonferroni adjustment for multiple comparisons was realized to detect differences between stingless bee species and osmotic controls. All data analysis was carried out through R studio software version 4.0 (RStudio Team, 2015) using several R packages: “ggpubr”, “ggplot2”, “gapminder”, “Rmisc”, “rstatix”, “forcats”, and “tidyverse” (Bryan, 2017; Hope, 2022; Kassambara, 2020, 2021; Wickham, 2016; Wickham et al., 2019). All *P*-values below or equal to 0.05 were considered statistically significant.

RESULTS

The present study analyzed the potential antibiofilm activity of thirty-five different honey samples produced by ten different stingless bee species of five provinces of Ecuador (Tungurahua, Pastaza, El Oro, Los Ríos, and Loja) against susceptible and MDR strains of *Staphylococcus aureus* and *Klebsiella pneumoniae*, as well as *Candida albicans* and *Candida tropicalis* (see Supplementary File S1). The honey samples were evaluated against 24h biofilms through biofilm biomass reduction, total cell count together with cell viability, and structural biofilm disruption.

Initial screening of the honey sample set

The initial sample set revealed a diversity of the antibiofilm activity by stingless bee species in biomass reduction of different pathogens when compared to positive and osmotic controls, i.e., untreated biofilm samples and treated-biofilm samples with artificial honey lacking H_2O_2 (only with sugar products of glucose oxidation), respectively. As shown in Table 5, we initially analyzed the biomass reduction produced by each stingless bee species through their various honey samples and statistically compared each one against the osmotic controls to differentiate biomass variation in every pathogen biofilm induced by the sugars in artificial honey. However, no statistical differences were observed between 24-hour treated biofilms from honey samples of a particular stingless bee species and their osmotic controls among the selected microbial pathogens, except for *Candida albicans*, which had statistical differences in all stingless bee species and the osmotic control, and for *Candida tropicalis* that demonstrated a statistical biomass reduction when treated with honey samples from *Cephalotrigona sp.*

Table 5 Initial evaluation of the potential antibiofilm activity by stingless bee species in biomass reduction through biofilm inhibition assays of different pathogens when compared to positive and osmotic controls.

Stingless bee species and controls	*S. aureus* ATCC 25923		*S. aureus* MRSA 333		*K. pneumoniae* ATCC 33495		*K. pneumoniae* KPC 609803		*C. albicans* ATCC 10231		*C. tropicalis* V546	
	A_{630}	Biomass[1], %	A_{630}	Biomass[1], %	A_{630}	Biomass[1], %	A_{630}	Biomass[1], %	A_{630}	Biomass[1], %	A_{630}	Biomass[1], %
Cephalotrigona sp.	0.169 (0.126)	38.54 (28.71)	0.145 (0.081)	40.38 (22.42)	0.226 (0.017)	55.29 (4.21)	0.239 (0.013)	174.16 (9.24)	**0.086 (0.022)**	**42.53 (10.70)**	**0.044 (0.011)**	**17.49 (4.26)**
Melipona cramptoni	0.162 (0.073)	36.93 (16.73)	0.112 (0.048)	31.07 (13.28)	0.378 (0.106)	92.35 (25.87)	0.122 (0.049)	89.32 (35.88)	**0.062 (0.018)**	**30.74 (8.83)**	0.144 (0.063)	56.76 (24.71)
Melipona fuscopilosa	0.202 (0.017)	46.14 (3.84)	0.224 (0.019)	62.22 (5.20)	0.534 (0.050)	130.63 (12.21)	0.230 (0.057)	167.97 (41.74)	**0.160 (0.007)**	**78.99 (3.54)**	0.326 (0.231)	128.66 (90.97)
Melipona grandis	0.191 (0.047)	43.60 (10.78)	0.250 (0.068)	69.31 (18.74)	0.428 (0.052)	104.57 (12.71)	0.208 (0.062)	151.54 (45.09)	**0.146 (0.084)**	**71.86 (41.12)**	0.220 (0.068)	86.83 (26.69)
Melipona indecisa	0.153 (0.048)	35.09 (10.93)	0.211 (0.051)	58.70 (14.02)	0.283 (0.055)	69.16 (13.52)	0.095 (0.042)	69.32 (30.76)	**0.110 (0.020)**	**54.47 (9.68)**	0.173 (0.053)	68.45 (20.84)
Melipona mimetica	0.112 (0.021)	25.59 (4.74)	0.147 (0.009)	40.78 (2.43)	0.298 (0.021)	72.82 (5.05)	0.191 (0.034)	139.36 (24.63)	**0.110 (0.006)**	**54.34 (2.75)**	0.189 (0.055)	74.79 (21.83)
Melipona nigrifacies	0.154 (0.035)	35.12 (12.75)	0.173 (0.043)	48.14 (11.78)	0.390 (0.059)	95.45 (14.47)	0.159 (0.035)	116.41 (25.68)	**0.113 (0.020)**	**55.89 (9.73)**	0.288 (0.115)	113.41 (45.35)
Melipona sp.	0.154 (0.035)	35.23 (7.87)	0.121 (0.043)	33.64 (11.94)	0.214 (0.079)	52.43 (19.28)	0.069 (0.012)	50.51 (8.97)	**0.149 (0.011)**	**73.61 (5.38)**	0.188 (0.069)	74.27 (27.33)
Scaptotrigona problanca	0.161 (0.076)	36.88 (17.43)	0.150 (0.062)	41.73 (17.20)	0.240 (0.108)	58.67 (26.38)	0.127 (0.043)	92.46 (31.34)	**0.099 (0.040)**	**48.95 (19.46)**	0.131 (0.081)	51.68 (31.98)
Tetragonisca angustula	0.140 (0.038)	31.92 (8.59)	0.142 (0.080)	39.35 (22.10)	0.197 (0.099)	48.35 (24.18)	0.091 (0.039)	66.30 (28.52)	**0.077 (0.030)**	**38.20 (14.53)**	0.166 (0.121)	65.63 (47.67)
Osmotic control	0.112 (0.046)	25.53 (10.48)	0.101 (0.043)	28.08 (11.95)	0.218 (0.034)	53.42 (8.21)	0.105 (0.049)	76.94 (35.82)	0.276 (0.039)	135.94 (19.35)	0.141 (0.030)	55.69 (11.77)
Positive control	0.438 (0.123)	100.00 (28.05)	0.360 (0.070)	100.00 (19.49)	0.409 (0.062)	100.00 (15.06)	0.137 (0.035)	100.00 (25.66)	0.203 (0.048)	100.00 (23.63)	0.253 (0.047)	100.00 (18.40)

Legend: The table illustrated the average results of the optical density at 630 nm (A_{630}) and calculated biofilm biomass percentages (Biomass) with their standard desviation values (SD). All assays were realized in triplicate on different days. [1]Biomass relative of the treated-biofilm sample when compared to the positive control (untreated biofilm assays). Bold values illustrated a significative difference of the treated-biofilm sample when compared to the osmotic control through Mann-Whitney-Wilcoxon test with Holm–Bonferroni adjustment for multiple comparisons illustrating a *P*-values <0.05.

Therefore, further analysis into biomass reduction was realized throughout the honey sample set from every stingless bee species, selecting the most promising honey samples for each microbial pathogen of the present study (see Supplementary File S1). As shown in Table 6, five honey samples showed the highest values of biofilm reduction in inhibition assays out of the six microorganisms, belonging to three specific provinces of Ecuador (Loja, El Oro, and Los Rios) and two stingless bee genera (*Scaptotrigona* and *Melipona* spp.). Furthermore, these honey samples showed statistically significant values in the inhibition of biofilms among pathogens ($P < 0.01$) when compared to the osmotic controls, except for *Staphylococcus aureus* strains. When exposed to honey samples, the biofilm formation was between 19.96 and 36.80%, with *Candida albicans* ATCC 10231 being the most inhibited pathogen from our group set. Likewise, a preliminary analysis was realized with the same honey samples through biofilm eradication assays to evaluate the disruption of pre-established biofilms. The eradication of established 24-hour biofilms showed less efficiency, evidencing biofilm rates of 43.90 to 118.39%, where treated-biofilm samples of *Staphylococcus aureus* strains and *Klebsiella pneumoniae* KPC 609803 showed an increment in biofilm formation (see Table 6). The *Melipona indecisa* species OR24.1 sample demonstrated the highest inhibition values against two pathogen species, *Staphylococcus aureus* ATCC 25923 and *Klebsiella pneumoniae* KPC 609803, showing only 22.95 and 36.80% of biofilm formation, respectively. From the initial assessment of our honey sample set on biofilm biomass reduction by optical density assays, we further evaluated the potential antibiofilm activity of the best honey samples on the biofilm inhibition assays through FM and SEM analysis.

Table 6 Summary of the highest biomass reduction of specific honey samples from the selected stingless bee species through biofilm inhibition and eradication assays in each pathogen and their statistical comparison with the osmotic control.

Biofilm inhibition assays							
Microorganism	Strain	Honey sample	Province procedence	Stingless bee specie	A_{630}	Biomass, %	Pairwise comparison[1], *P*-values
Candida albicans	ATCC 10231	LO40	Loja	*Scaptotrigona problanca*	0.040 (0.016)	19.96 (8.08)	7.14e-05
Candida tropicalis	V546	LO53	Loja	*Scaptotrigona problanca*	0.070 (0.011)	27.74 (4.56)	8.98e-06
Staphylococcus aureus	ATCC 25923	OR24.1	El Oro	*Melipona indecisa*	0.100 (0.014)	22.95 (3.26)	0.571
	MRSA 333	LR34	Los Rios	*Melipona* sp.	0.097 (0.037)	26.99 (10.17)	0.429
Klebsiella pneumoniae	ATCC 33495	LO48	Loja	*Scaptotrigona problanca*	0.0905 (0.011)	23.32 (2.65)	5.86e-10
	KPC 609803	OR24.1	El Oro	*Melipona indecisa*	0.050 (0.012)	36.80 (8.94)	0.000803
Biofilm eradication assays							
Microorganism	Strain	Honey sample	Province procedence	Stingless bee specie	A_{630}	Biomass, %	Pairwise comparison[1], *P*-values
Candida albicans	ATCC 10231	LO40	Loja	*Scaptotrigona problanca*	0.064 (0.013)	61.00 (12.24)	0.0000000573
Candida tropicalis	V546	LO53	Loja	*Scaptotrigona problanca*	0.020 (0.009)	43.90 (18.42)	0.0110
Staphylococcus aureus	ATCC 25923	OR24.1	El Oro	*Melipona indecisa*	0.398 (0.017)	103.99 (4.51)	0.100
	MRSA 333	LR34	Los Rios	*Melipona* sp.	0.387 (0.019)	102.56 (5.01)	0.100
Klebsiella pneumoniae	ATCC 33495	LO48	Loja	*Scaptotrigona problanca*	0.500 (0.061)	118.39 (14.53)	0.100
	KPC 609803	OR24.1	El Oro	*Melipona indecisa*	0.121 (0.017)	92.21 (12.94)	0.700

Legend: The table illustrated the average results of the optical density at 630 nm (A_{630}) and calculated biofilm biomass percentages (Biomass) with their standard desviation values (SD). All assays were realized in triplicate on different days. [1]*P*-values of the treated-biofilm with a certain honey sample when compared to the osmotic control through Mann-Whitney-Wilcoxon test

Total cell count and cell viability on treated-biofilm samples

The FM analysis using DAPI and LIVE/DEAD staining allowed us to evaluate the honey samples' antibiofilm effect on the total cell count, cell viability, and extracellular polymeric substances (EPS) content in 24-hour biofilms (see Supplementary Table S1). As aforementioned, a LIVE/DEAD Yeast Viability Kit was used for *Candida* species (see Figure 3), while a LIVE/DEAD BacLight Bacterial Viability Kit was applied for *S. aureus* (see Figure 4) and *K. pneumoniae* strains (see Figure 5), allowing us to compare the inhibition biofilm assays between these different microorganisms. When looking at the compiled results in Figure 6, the log reductions in the total cell count of honey-treated samples were statistically significant in all pathogens ($P<0.05$), apart from *S. aureus* ATCC 25923 ($P=0.84$), which did not show any reduction. When compared with untreated 24-hour biofilms, the remaining microorganisms evidenced a log reduction of between 8.16 and 28.37%, being again *C. albicans* (26.55%) and *C. tropicalis* (28.37%) the most affected microorganisms, followed by *K. pneumoniae* KPC 609803 (22.39%), and *K. pneumoniae* ATCC 33495 (12.53%). Concerning the viability of the cells within the biofilm, only *C. tropicalis*, *K. pneumoniae* ATCC 33495, and *K. pneumoniae* KPC 609803 demonstrated significant drops in cell viability ($P<0.01$), decreasing by 21.67, 25.69, and 45.62%, respectively. Finally, a preliminary analysis of the content of extracellular polymeric substance (EPS) within the biofilm samples was carried out with ImageJ software through DAPI staining outside the cells in the collected pictures (grays units), showing a statistical EPS diminution in *C. albicans* (24.40%), *C. tropicalis* (34.09%), *K. pneumoniae* ATCC 33495 (34.72%) and *S. aureus* ATCC 25923 (51.76%; all *P*-values <0.01; see Supplementary Table S1).

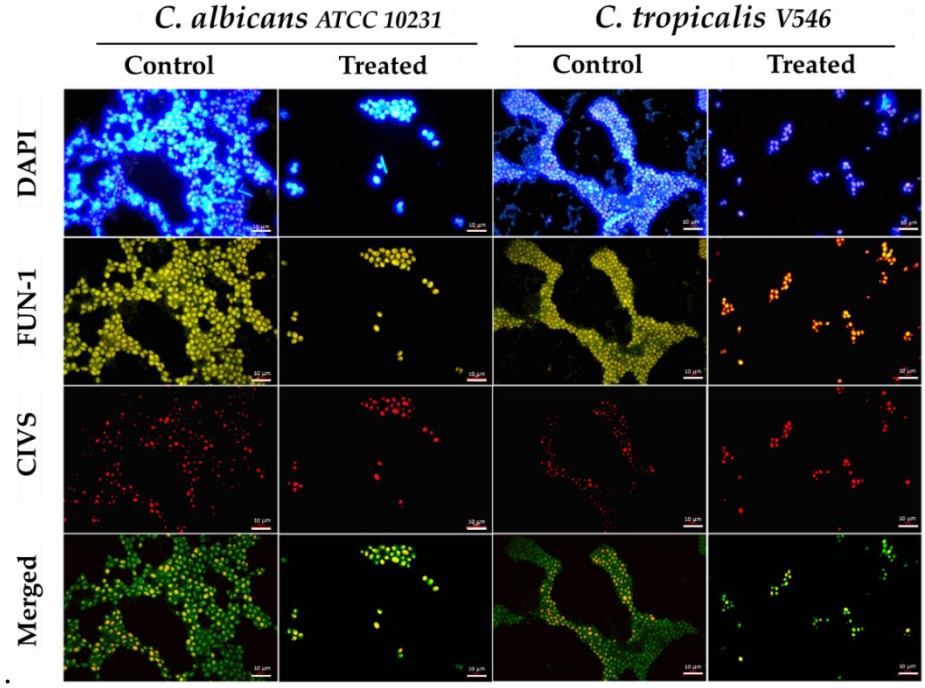


Figure 3 Fluorescence microscopy of the honey-treated and untreated 24-hour biofilms of *C. albicans* and *C. tropicalis*

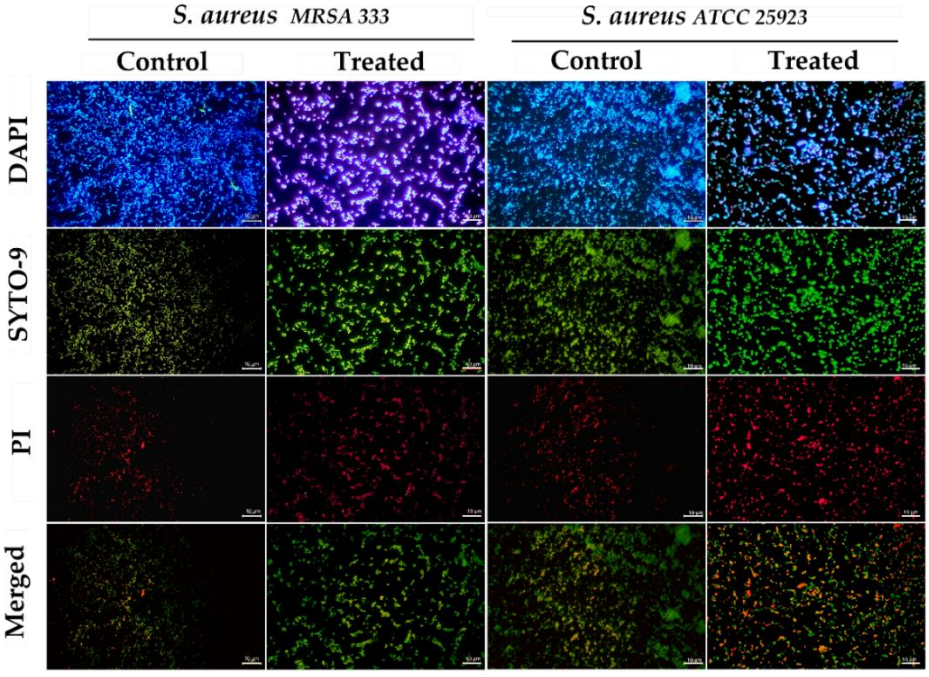


Figure 4 Fluorescence microscopy of the honey-treated and untreated 24-hour biofilms of *S. aureus* MRSA 333 and ATCC 25923

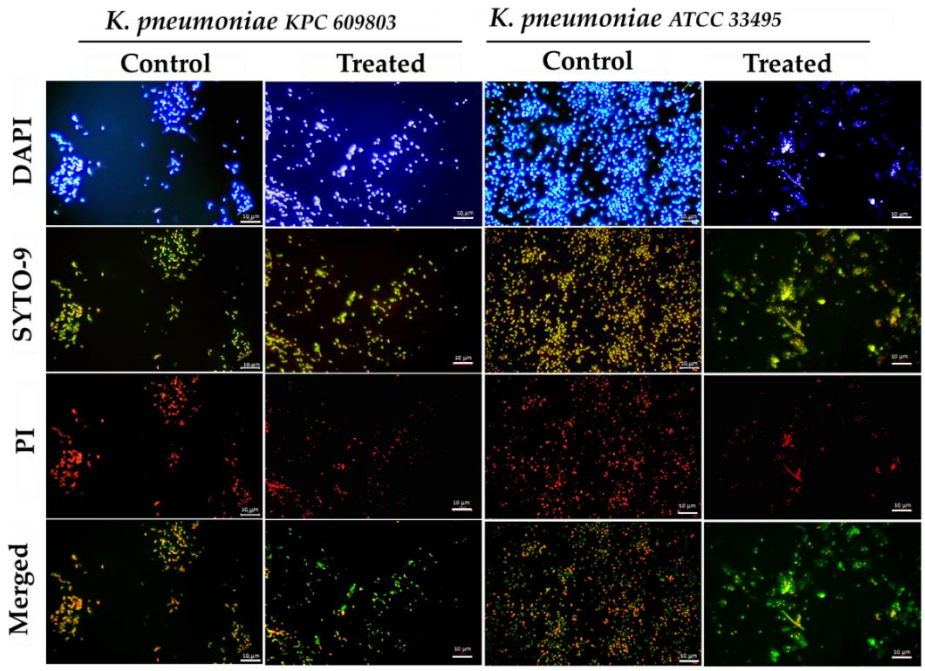


Figure 5 Fluorescence microscopy of the honey-treated and untreated 24-hour biofilms of *K. pneumoniae* KPC 609803 and ATCC 33495

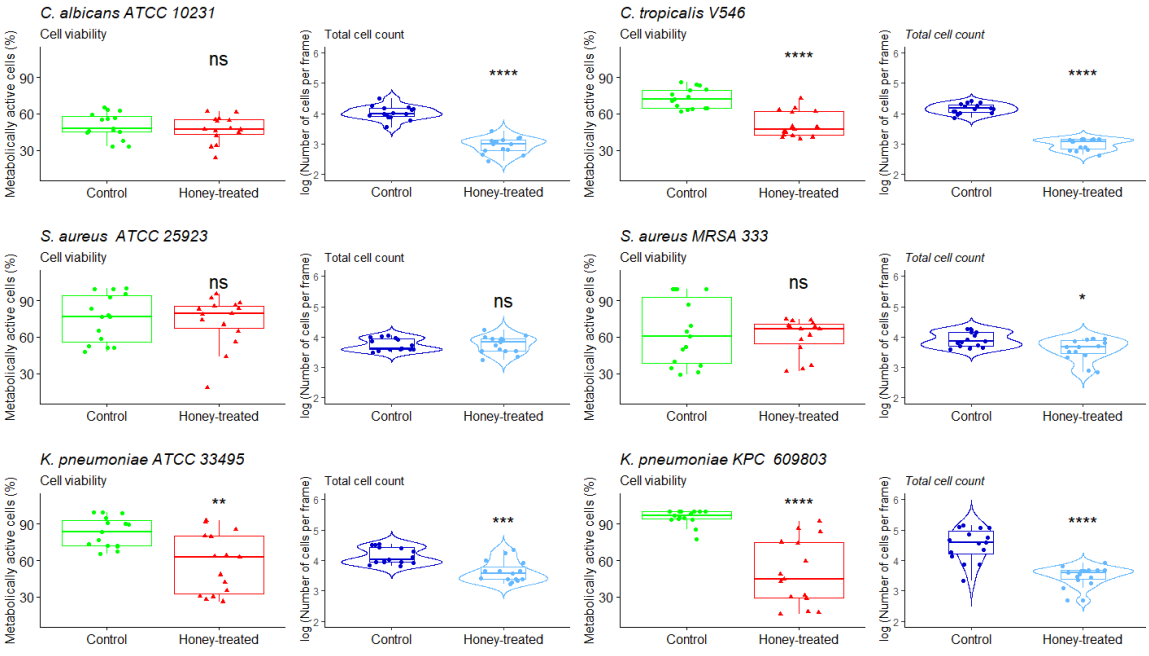


Figure 6 Total cell count and cell viability results of honey-treated and untreated 24-hour biofilms of the pathogens by FM analysis.

Structural composition on treated-biofilm samples

The SEM analysis evaluated the antibiofilm effect of honey samples on the structural biofilm disruption through cell morphological parameters, such as the size, form, shape, and structure of the cells within the biofilm (see Supplementary Table S2). Three different magnifications were used for the imaging evaluation of the *Candida* species (1.67, 3.33, and 16.7 kx; see Figure 7), *S. aureus* strains (10.0, 33.3, and 167 kx; see Figure 8), and *K. pneumoniae* strains (10.0, 33.3, and 167 kx; see Figure 9). This allowed us to study the general disposition of biofilm patterns by fractal dimension index (FDI) under different morphological parameters of the cells by area, circularity, and elongation. The FDI only showed statistical differences between treated and untreated biofilm samples in 3 of the 6 evaluated pathogens: S. aureus ATCC 25923 (P =0.019), S. aureus MRSA 333 (P =0.00099), and K. pneumoniae KPC 609803 (P =0.0038), suggesting a potential disruption of the biofilm pattern. The FDI pattern was reduced by 8.33% in treated S. aureus ATCC 25923, while S. aureus MRSA 333 and K. pneumoniae KPC 609803 showed an increment of 8.33 and 12.05% in the FDI pattern when compared to the untreated biofilm controls (see Supplementary Table S2), respectively.

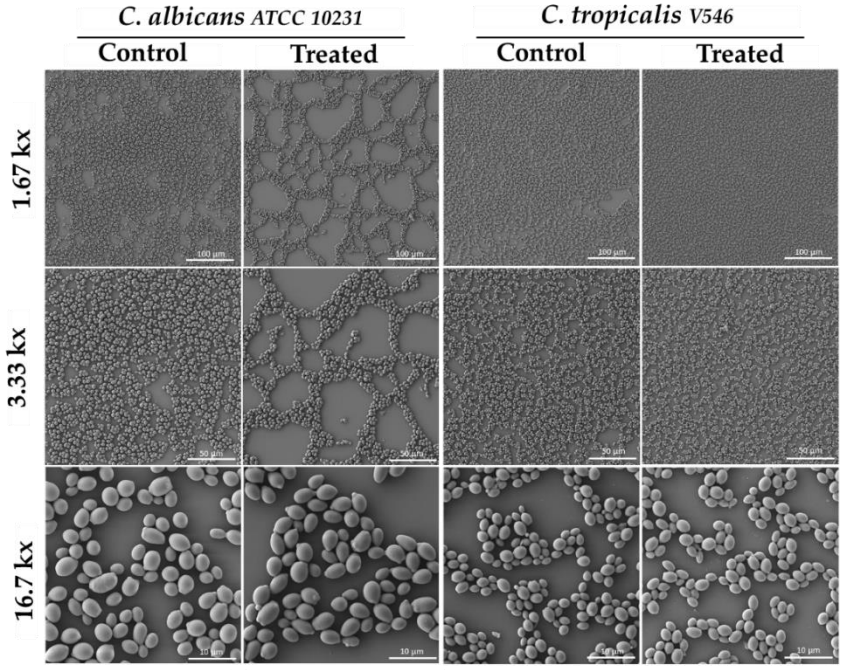


Figure 7 Micrographs of the honey-treated and untreated 24-hour biofilms of *C. albicans* and *C. tropicalis*

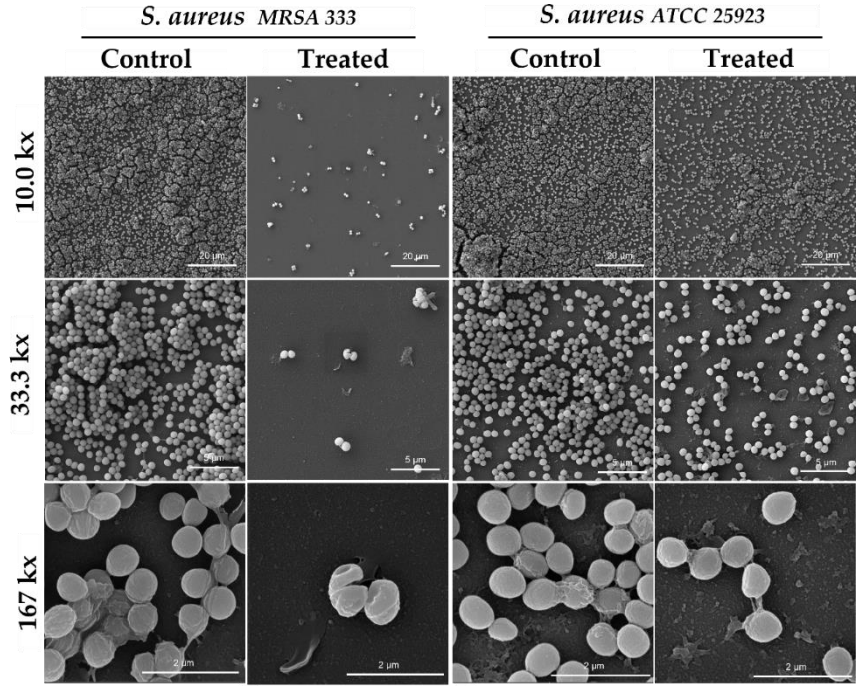


Figure 8 Micrographs of the honey-treated and untreated 24-hour biofilms of *S. aureus* MRSA 333 and ATCC 25923

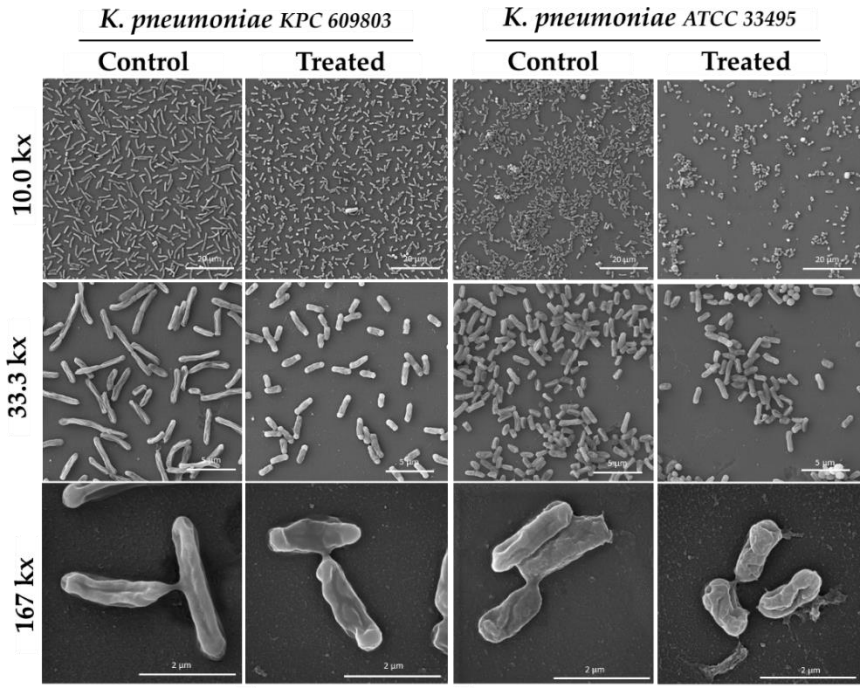


Figure 9 Micrographs of the honey-treated and untreated 24-hour biofilms of *K. pneumoniae* KPC 609803 and ATCC 33495

As shown in Figure 10, when analyzing morphological parameters from cells within the biofilm, the cell area was statistically affected between treated and untreated biofilm samples among *Candida* species and *S. aureus* strains (P <0.01). *Candida albicans*, *S. aureus* ATCC 25923, and *C. tropicalis* showed the greatest increment of cell area values by 25.48, 18.42, and 9.29% when compared to the controls, respectively. However, *S. aureus* MRSA 333 suffered a reduction in the cell area of treated biofilm samples by 7.50%. It is also important to mention that in Figure 10, cell circularity was selected on *Candida* species and *S. aureus* strains as a shape parameter, while elongation was prioritized on *K. pneumoniae* strains due to the morphological nature of the cells between these pathogens. Cell circularity was statistically affected between treated and untreated biofilm samples among *Candida tropicalis* and *S. aureus* strains (P <0.001) with the exception of *Candida albicans* (P =0.87), where cell circularity was reduced between 2.33 and 11.90%. Meanwhile, both *K. pneumoniae* ATCC 33495 and *K. pneumoniae* KPC 609803 showed statistical differences in their cell elongation between treated and untreated biofilm samples (P <0.05), demonstrating a similar reduction of 23.53 and 24.26% on treated biofilms, respectively. However,

other size, form, and shape cell parameters were also found to be statistically significant among treated and untreated pathogens (see Supplementary Table S2), evidencing the importance or usefulness of cell morphological analysis for biofilm samples.

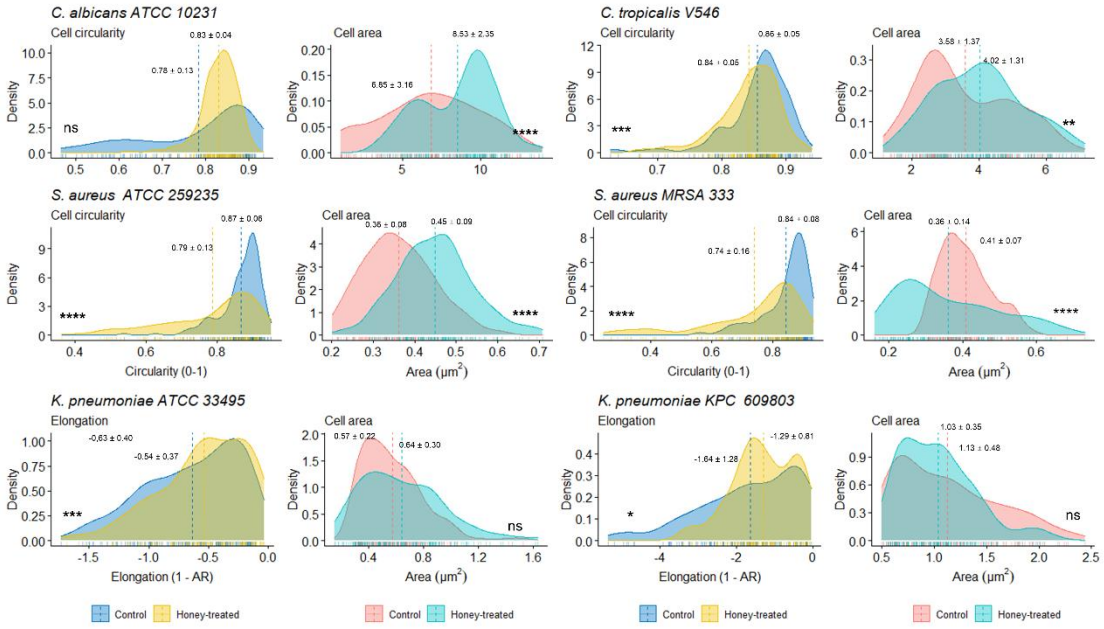


Figure 10 Main cell morphological parameters of honey-treated and untreated 24-hour biofilms of the pathogens by scanning electron microscopy (SEM) analysis.

DISCUSSION

The ability to establish biofilm is a well-known property among several pathogens. It is a major virulence factor among primary or opportunistic infections due to several intrinsic factors, such as antimicrobial resistance, immune system evasion, and horizontal gene transfer (HGT) mechanisms in multispecies biofilms (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022; Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022; Cavalheiro & Teixeira, 2018; de Barros et al., 2020). Given the augmentation of antimicrobial resistance worldwide, alternative treatments have been extensively studied in the last decade. The antimicrobial activity of our honey samples against *S. aureus* appeared in the superficial layers of the biofilm, as observed by SEM analysis, which was unable to reach more active and persistent cells located in deeper layers of the biofilm, as reported with other compounds in previous studies (Lister & Horswill, 2014; Erick Kenji Nishio et al., 2016; Reffuveille et al., 2017). Nonetheless, all studies agree, including our own, that the gradual loss of the hydrogen peroxide content in honey samples could be responsible for the gradual loss of the antimicrobial action over time.

In the antibiofilm activity in this study, no significant relationship was found between stingless bee species and any specific microorganism, aside from *C. albicans* and *C. tropicalis*, where honey samples from *Scaptotrigona problanca* demonstrated a significant antibiofilm activity. In general, little is known about the specificity of any honeybee species with a certain microorganism. However, some studies reported that honey produced by *Tetragonisca angustula* (L. G. Zamora et al., 2017) and *Trigona* spp. (W. J. Ng et al., 2017), and propolis produced by *Tetragonisca fiebrigi* and *Scaptotrigona jujuyensis* (Brodkiewicz et al., 2018) inhibited the biofilm formation of *S. aureus*, including MRSA strains, by 50 to 70% but were notoriously unable to eradicate preformed biofilms. In addition, combined treatments with *Tetragonisca angustula* honeys from Costa Rica and ampicillin or vancomycin allowed the antibiotics to regain their antimicrobial activities on a *Staphylococcus aureus* biofilm (L. G. Zamora et al., 2017). Concerning *K. pneumoniae* and *Candida* spp. biofilms, no literature is available for us to discuss, making them another novelty in

this study to the authors' best knowledge. As previously indicated, not much is known about stingless bee honeys' antibiofilm activity, where there is also a divergence when compared with honey-producing species regarding different bioactive compounds, floral origin (Ávila, Hornung, et al., 2019; Rosli et al., 2020; J. M. Sousa et al., 2016), and even the honey-associated microbiome (Ávila, Lazzarotto, et al., 2019; Baharudin et al., 2021; Julika et al., 2019; Mohammad et al., 2020; Ngalimat et al., 2019; Zulkhairi Amin et al., 2019).

By FM analysis, we were able to confirm a reduction of EPS produced by all biofilms (see Supplementary Table S1). Concerning *C. albicans* and *C. tropicalis*, it can be assumed that the efficacy of our honey samples on these opportunistic species, when compared with non-treated samples, varied due to the chemical composition, particularly differences in phenolic acids, flavonoids, and triterpenes, which can alter the normal metabolism of the fungus and consequently cell viability (Candiracci et al., 2011; Canonico et al., 2014; Estevinho et al., 2011; Liberio et al., 2011; Maghfiroh et al., 2021). However, cell viability did not show statistical differences in *C. albicans* biofilms. Further studies should be realized to improve the evaluation of viability cell data through the fluorescent probe-based methodology (i.e., the LIVE/DEAD Yeast Viability Kit), as widely discussed by previous studies (Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022; Netuschil et al., 2014; Welch et al., 2012).

Last, but not least, we performed SEM analysis and further evaluated the structural biofilm disruption and cell morphological parameters, evidencing statistically significant differences among treated and non-treated pathogens, in agreement with recent studies involving other biofilm-forming microorganisms, such as *S. pyogenes*, *P. aeruginosa*, *Streptococcus pneumoniae* (Al-kafaween et al., 2021; Alkafaween et al., 2021), and *C. albicans* (Hau-Yama et al., 2020). However, it is worth noting that the present study performed a more exhaustive analysis of cell morphologies when compared with the previous studies. Besides the morphological alterations reported in this study, some studies describe the presence of protoplasts, spheroplasts, and septa in treated *S. aureus* strains due to cell wall weakening by peptidoglycan degradation and inhibition of bacterial cell

division (Cushnie et al., 2016; Domingos et al., 2021; E. K. Nishio et al., 2016). These inhibitory mechanisms on *S. aureus* biofilms previously reported could explain the differences observed in cell circularity on treated biofilms of the present study as "deflated balloon-like forms folded on itself" in MRSA strains, probably due to osmotic lysis enhanced by honey flavonoids (Cushnie & Lamb, 2005; Ouyang et al., 2018; Proaño et al., 2021). On the other hand, Jenkins et al. (2011) proposed that the presence of septa and large elongated cells was a consequence of the flavonoid-mediated inhibition of murein hydrolase, which is necessary for bacterial cleavage, as observed in both *S. aureus* strains (Jenkins et al., 2011; Ouyang et al., 2018). In 2017, Ng et al. (2017) reported longer rod and filamentous forms, suggesting inhibition of cell septation and cell division when treating *E. coli* with *Heterotrigona itama* honey. However, in the present study, both treated *K. pneumoniae* strains showed shortened rod and filamentous forms. Moreover, regarding the antifungal effect on *C. albicans*, the identified changes were regarding the size regularity and morphology of the membrane and similar findings were also observed with *Melipona becchei* honey (Hau-Yama et al., 2020) and Jujube honey (Ansari et al., 2013). Although several studies have confirmed that honey's antifungal effect is strongly linked to the floral and entomological origin (Alvarez-Suarez et al., 2018; Boorn et al., 2010; Fernandes et al., 2021; Irish et al., 2006; Morroni et al., 2018; J. Ramón-Sierra et al., 2019; Suntiparapop et al., 2015; G. Zamora, Beukelman, Van Den Berg, et al., 2015), little is known about the antifungal properties of stingless bee honeys. Several studies have found that some phytochemical compounds, especially terpenes and flavonoids, present in natural products including honey, can inhibit morphological transitions in *Candida* species (Al-Ghanayem, 2022; Ansari et al., 2013; Calixto Júnior et al., 2015; T. G. da Silva et al., 2021; Prasath et al., 2020; Priya & Pandian, 2022; Soliman et al., 2017). In our case, the evidence obtained was not sufficient to reinforce this idea. In addition, we found a statistically significant increase in yeast area in both *Candida* species treated with honey, probably due to the availability of sugars. Furthermore, FM analysis evidenced a reduction in viability and cell count, which could explain a possible antagonistic effect between the antifungal effect exerted by osmotic

pressure and the amount of usable sugars, because these pathogens adapt their metabolism according to the available nutrients by different sugar-sensing systems (T. S. Ng et al., 2016; Pemmaraju et al., 2016; Van Ende et al., 2019; Weerasekera et al., 2017).

A new morpho-structural parameter was exploratorily evaluated in the present study, namely the fractal dimension index (FDI). FDI can be considered a structural indicator of the complexity and stability of the biofilm, as well as the degree of response to changes in the environment, allowing us to evaluate biomass variations and changes in surface roughness, cell distribution pattern, and the level of fragmentation (Artyushkova et al., 2015; Grzegorczyk et al., 2018; Kassinger & van Hoek, 2020; Qin et al., 2021). Even so, no statistical differences were found between control and treated samples in both *Candida* species and *K. pneumoniae* ATCC 33495. Both *S. aureus* strains and *K. pneumoniae* KPC 609803 evidenced statistical differences in FDI between treated and non-treated biofilms (see Supplementary Table S2). Nonetheless, to better understand this type of structural biofilm evaluation, further studies are necessary to realize 3D biofilm architecture analysis through atomic force microscopy (AFM) and/or confocal laser scanning microscopy (CLSM), as in other studies (Grzegorczyk et al., 2018).

In summary, the present study provided more detailed information on the antibiofilm activity of stingless bee honey samples against *S. aureus*, *K. pneumoniae*, and *Candida* species, evidencing the ability of biofilm inhibition through biomass, total cell count and viability, and cell morphological parameters. However, this study has several limitations, for example that antibiofilm activity was only studied in monospecies biofilms and there were no analyses based on metabolic or gene expression, flow cytometry, confocal microscopy, and quantitative polymerase chain reaction to assess the differences between control and treated biofilms. Future studies should implement these new analyses in the present biofilm evaluation and develop polymicrobial biofilm models to provide a more detailed picture of the antibiofilm effects of stingless bee honeys (Kucera et al., 2014; Y. Sun et al., 2008; Woods et al., 2012).

CONCLUSIONS

This study achieved its objective to demonstrate the antibiofilm activity of stingless bee honeys against gram-positive, gram-negative, and yeast pathogens, showing biofilm inhibition of 63 to 80% of biomass loss, a significant reduction in the total cell account and viability, as well as differences in cell morphological parameters by SEM analysis. To the authors' best knowledge, this is the first study to simultaneously analyze biofilms of susceptible and multidrug-resistant strains of *S. aureus* and *K. pneumoniae*, as well as different *Candida* species by biomass assays, fluorescence microscopy, and scanning electron microscopy. We were able to validate antibiofilm activity by several stingless bee honey types from different provinces of Ecuador. Further studies should analyze the molecular and metabolic network that influences the inhibition of the biofilm formed by different pathogens by stingless bee honeys. Stingless bee honeys from Ecuador are a promising candidate for the research and development of novel antibiofilm molecules for the treatment of multidrug-resistant bacterial infections and clinically important fungal infections.

SUPPLEMENTARY INFORMATION

Supplementary File S 1 Database of the biomass reduction analysis of honey-treated biofilms through optical density assays at 630 nm (A_{630}).

Supplementary Table S 1 Evaluation of the antibiofilm effect of honey samples on the total cell count, cell viability and extracellular polymeric substances (EPS) content in 24-hour biofilms through fluorescence microscopy (FM) evaluation.

Microorganism	Parameters	Control, Mean (SD)	Honey-treated, Mean (SD)	Pairwise comparison [1], *P*-values
C. albicans ATCC 10231	Total cell count, log (cells/frame)	4.03 (0.23)	2.96 (0.26)	**3.4e-06**
	Surface cell count, log (cells/cm^2)	7.35 (0.23)	6.28 (0.26)	**3.4e-06**
	Viability, % (live cells)	50.12 (10.62)	47.09 (10.79)	0.43629
	Live/dead ratio, log (ratio)	0.002 (0.190)	-0.055 (0.198)	0.44242
	EPS content (grays units)	77.95 (43.43)	58.93 (33.53)	**0.00202**
C. tropicalis V546	Total cell count, log (cells/frame)	4.16 (0.16)	2.98 (0.18)	**3.4e-06**
	Surface cell count, log (cells/cm^2)	7.48 (0.16)	6.30 (0.18)	**3.3e-06**
	Viability, % (live cells)	72.55 (8.29)	50.88 (10.79)	**6.5e-06**
	Live/dead ratio, log (ratio)	0.440 (0.192)	0.018 (0.194)	**5.2e-05**
	EPS content (grays units)	66.71 (27.86)	43.97 (14.81)	**1.7e-11**
S. aureus ATCC 25923	Total cell count, log (cells/frame)	3.75 (0.20)	3.76 (0.28)	0.84
	Surface cell count, log (cells/cm^2)	7.07 (0.20)	7.08 (0.28)	0.81941
	Viability, % (live cells)	75.32 (19.59)	73.19 (20.47)	0.87
	Live/dead ratio, log (ratio)	0.845 (0.943)	0.517 (0.481)	0.87
	EPS content (grays units)	54.70 (9.49)	26.39 (8.97)	**<2e-16**
S. aureus MRSA 333	Total cell count, log (cells/frame)	3.92 (0.24)	3.60 (0.36)	**0.028**
	Surface cell count, log (cells/cm^2)	7.24 (0.24)	6.92 (0.36)	**0.02789**
	Viability, % (live cells)	63.46 (27.32)	60.45 (15.04)	0.967
	Live/dead ratio, log (ratio)	0.614 (1.067)	0.194 (0.277)	0.967
	EPS content (grays units)	72.78 (13.45)	91.15 (19.51)	**6.1e-13**
K. pneumoniae ATCC 33495	Total cell count, log (cells/frame)	4.15 (0.27)	3.63 (0.35)	**0.00053**
	Surface cell count, log (cells/cm^2)	7.47 (0.27)	6.94 (0.35)	**0.00053**
	Viability, % (live cells)	82.99 (12.38)	57.30 (24.56)	**0.00367**
	Live/dead ratio, log (ratio)	0.960 (0.755)	0.186 (0.533)	**0.00479**
	EPS content (grays units)	34.79 (17.31)	22.71 (11.81)	**7.4e-13**
K. pneumoniae KPC 609803	Total cell count, log (cells/frame)	4.51 (0.53)	3.50 (0.31)	**4.8e-05**
	Surface cell count, log (cells/cm^2)	7.83 (0.53)	6.82 (0.31)	**4.8e-05**
	Viability, % (live cells)	95.31 (6.36)	49.69 (26.85)	**9.0e-06**
	Live/dead ratio, log (ratio)	2.186 (1.308)	0.013 (0.573)	**9.1e-06**
	EPS content (grays units)	29.60 (12.98)	37.69 (17.31)	**0.00088**

Legend: [1] Pairwise comparisons were realized through Mann-Whitney-Wilcoxon test. Evaluation of the *in vitro* antimicrobial effect of honey samples on fluorescence-based parameters in 24-hour biofilms. Non-parametric Mann-Whitney-Wilcoxon tests were used to identify significant differences (*P*-values <0.05). The results of the analysis presented in this table were performed in Fiji-ImageJ2 release 2.6.0 software.

Supplementary Table S 2 Evaluation of the antibiofilm effect of honey samples on the cell morphological parameters in 24-hour biofilms through scanning electron microscopy (SEM) analysis.

Microorganism	Cell morphological parameters		Control, Mean (SD)	Honey-treated, Mean (SD)	Pairwise comparison [3], *P*-values
C. albicans ATCC 10231	Size	Length, µm	2.95 (0.80)	3.42 (0.71)	**1.90E-05**
		Cell area, µm^2	6.83 (3.20)	8.57 (2.35)	**1.40E-06**
	Form	Aspect ratio (AR), width/length	1.33 (0.20)	1.41 (0.16)	**2.90E-08**
		Elongation, 1- AR	-0.32 (0.20)	-0.42 (0.15)	**2.90E-08**
	Shape	Perimeter, µm	10.23 (3.05)	11.30 (1.70)	**0.00019**
		Circularity[1]	0.78 (0.13)	0.83 (0.04)	0.87413
		Roundness[1]	0.77 (0.10)	0.71 (0.08)	**3.00E-08**
		Solidity[1]	0.93 (0.04)	0.95 (0.01)	**0.00048**
	Structure	Fractal Dimension Index[2]	1.83 (0.05)	1.78 (0.01)	0.22713
C. tropicalis V546	Size	Length, µm	2.16 (0.44)	2.29 (0.47)	**0.0065**
		Cell area, µm^2	3.66 (1.38)	4.00 (1.32)	**0.02348**
	Form	Aspect ratio (AR), width/length	1.33 (0.15)	1.41 (0.17)	**2.30E-05**
		Elongation, 1- AR	-0.33 (0.15)	-0.41 (0.17)	**2.30E-05**
	Shape	Perimeter, µm	7.20 (1.40)	7.62 (1.28)	**0.00548**
		Circularity[1]	0.86 (0.05)	0.84 (0.05)	**0.00026**
		Roundness[1]	0.76 (0.08)	0.72 (0.08)	**2.40E-05**
		Solidity[1]	0.93 (0.02)	0.93 (0.02)	0.48658
	Structure	Fractal Dimension Index[2]	1.85 (0.03)	1.81 (0.01)	0.19784
S. aureus ATCC 25923	Size	Length, µm	0.70 (0.08)	0.79 (0.11)	**4.10E-14**
		Cell area, µm^2	0.38 (0.08)	0.45 (0.08)	**1.00E-11**
	Form	Aspect ratio (AR), width/length	1.18 (0.12)	1.20 (0.14)	0.1773
		Elongation, 1- AR	-0.18 (0.12)	-0.20 (0.14)	0.1773
	Shape	Perimeter, µm	2.35 (0.28)	2.71 (0.39)	**4.50E-16**
		Circularity[1]	0.86 (0.06)	0.79 (0.12)	**3.30E-06**
		Roundness[1]	0.85 (0.07)	0.84 (0.09)	0.17988
		Solidity[1]	0.93 (0.02)	0.91 (0.05)	**0.00429**
	Structure	Fractal Dimension Index[2]	1.80 (0.04)	1.65 (0.01)	**0.01878**
S. aureus MRSA 333	Size	Length, µm	0.73 (0.08)	0.71 (0.16)	0.03122
		Cell area, µm^2	0.40 (0.06)	0.37 (0.14)	**7.50E-05**
	Form	Aspect ratio (AR), width/length	1.20 (0.13)	1.27 (0.22)	**0.00556**
		Elongation, 1- AR	-0.20 (0.12)	-0.27 (0.21)	**0.00556**
	Shape	Perimeter, µm	2.45 (0.23)	2.52 (0.69)	0.13391
		Circularity[1]	0.84 (0.07)	0.74 (0.16)	**7.00E-14**
		Roundness[1]	0.84 (0.08)	0.80 (0.11)	**0.00543**
		Solidity[1]	0.93 (0.02)	0.91 (0.08)	0.10223
	Structure	Fractal Dimension Index[2]	1.80 (0.01)	1.95 (0.01)	**0.00099**
K. pneumoniae ATCC 33495	Size	Length, µm	0.96 (0.28)	0.94 (0.27)	0.9846
		Cell area, µm^2	0.58 (0.21)	0.62 (0.29)	0.34904
	Form	Aspect ratio (AR), width/length	1.69 (0.43)	1.52 (0.36)	**0.00083**
		Elongation, 1- AR	-0.68 (0.43)	-0.52 (0.36)	**0.00083**
	Shape	Perimeter, µm	3.28 (0.73)	3.28 (0.85)	0.9586
		Circularity[1]	0.67 (0.12)	0.70 (0.11)	**0.03048**
		Roundness[1]	0.63 (0.15)	0.69 (0.15)	**0.00085**
		Solidity[1]	0.89 (0.05)	0.89 (0.05)	0.1181
	Structure	Fractal Dimension Index[2]	1.75 (0.07)	1.86 (0.04)	0.08739
K. pneumoniae KPC 609803	Size	Length, µm	1.63 (0.57)	1.45 (0.43)	0.08345
		Cell area, µm^2	1.15 (0.48)	1.04 (0.37)	0.16041
	Form	Aspect ratio (AR), width/length	2.69 (1.30)	2.28 (0.80)	**0.03638**
		Elongation, 1- AR	-1.69 (1.30)	-1.28 (0.79)	**0.03638**
	Shape	Perimeter, µm	5.65 (1.50)	5.64 (1.52)	0.89358
		Circularity[1]	0.47 (0.16)	0.44 (0.15)	0.08977
		Roundness[1]	0.47 (0.23)	0.49 (0.18)	**0.03602**
		Solidity[1]	0.82 (0.11)	0.79 (0.10)	**0.00077**
	Structure	Fractal Dimension Index[2]	1.66 (0.03)	1.86 (0.04)	**0.00382**

Legend: [1] Shape descriptor/parameter calculated through a classification between 0 and 1. [2] Values of fractal dimension index between control and honey-treated samples were compared through *t*-student test. [3] Pairwise comparisons were realized through Mann-Whitney-Wilcoxon test. Evaluation of the *in vitro* antimicrobial effect of honey samples on cell morphological parameters in 24-hour biofilms through SEM analysis. Parametric *t*-student and non-parametric Mann-Whitney-Wilcoxon tests were used to identify significant differences (*P*-values <0.05). The results of the analysis presented in this table were performed in Fiji-ImageJ2 release 2.6.0 software.

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