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Antimicrobial and antibiofilm activities of Brazilian organic honey against oral microorganisms

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Abstract

Objective To evaluate the antimicrobial activity of Brazilian honeys against oral microorganisms. Design: Organic honeys (OH-1 to OH-8) were diluted (%-w/v) and sterilized by filtration. Antimicrobial activity was defined by determining MIC and CBM against oral *Streptococcus*. The component responsible for the antimicrobial action was defined by a catalase assay. Antibiofilm activity was evaluated against the monospecies biofilm of *Streptococcus mutans* (ATCC 700610).

Results OHs showed antimicrobial activity principally OH-1, OH-2, OH-3, and OH-7 with MIC values ranging between 10 and 25%. The mechanism of action occurs mainly by hydrogen peroxide produced by honey enzymes. OH-1, OH-2, and OH-7 showed total biofilm destruction at low concentrations.

Conclusion Brazilian honeys have promising antimicrobial and antibiofilm activity with the potential to control oral microbiota.

Keywords Honey · Antimicrobial activity · Antibiofilm activity · Oral microorganism

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Introduction

Honey is a naturally occurring substance produced by bees, primarily *Apis mellifera* species. This edible substance comprises sugars, enzymes, amino acids, vitamins, organic acids, carotenoids, aromatic compounds, and minerals. Additionally, honey exhibits a significant presence of flavonoids and phenolic acids, showcasing the rich biodiversity of the ecosystem and potentially accounting for its diverse range of biological activities [1]. Consequently, honey assumes a pivotal role not only as a vital nutritional component but also as a functional food. In other words, honey fulfills nutritional requirements while concurrently serving as a source of physical and mental well-being. Its consumption contributes to the prevention and mitigation of risk factors associated with various diseases or to the enhancement and sustenance of biological functions [2].

Moreover, honey can serve as an exemplary natural resource for the bioprospecting of biological activities associated with injuries and diseases that afflict human beings. Noteworthy attributes such as antimicrobial activity, antibiofilm properties, anti-inflammatory effects, tissue growth stimulation, deodorizing capabilities, and wound debridement have been documented in certain varieties of honey [3].

Dental caries stands out as the most prevalent bacterial disease affecting the oral cavity [4]. This disease is associated with the colonization of oral bacteria, such as *Streptococcus mutans* (*S. mutans*) and other members of the *Streptococcus viridans* group, as well as *Lactobacillus* spp., among others, and consequently, the formation of dental biofilm [5]. Bacteria that thrive within a biofilm often display altered phenotypes, including increased resistance to antimicrobial agents. The stable structural properties and close proximity of bacterial cells within the biofilm create an optimal environment for the horizontal transfer of resistance genes [6].

Additionally, the polysaccharide component of the biofilm matrix itself can facilitate adhesion, provide protection against host immune cells and various stresses, allow stratification of the microbial community, and establish nutrient gradients and residues required to maintain the complex structure [7]. This inherent complexity poses challenges to the efficacy of antimicrobial agents because, apart from creating a favorable environment for genetic mutations, the biofilm forms a physical barrier that hampers the activity of these agents [8].

Some studies have reported certain types of honey as an antimicrobial against cariogenic bacteria, such as *S. mutans* and *Lactobacillus* [9], and gum disease [10], despite being a food rich in sugars. However, there is no record of investigating the antimicrobial activity against other cariogenic bacteria or antibiofilm in mature biofilms, nor is the mechanism by which this action occurs described. Furthermore, the incipient nature of the study and the possibility of distinct composition in honey produced in native forests organically highlight the need for further research. Nevertheless, this study aimed to evaluate the antimicrobial activity of eight honeys produced organically against cariogenic microorganisms and to assess their antibiofilm activity in biofilms of *S. mutans.*

Table 1 Sample collection of organic honeys in southern Brazil

Honey sample	Collection place (city / state)	Type
OH-1	Turvo-PR	Polyfloral honey
OH-2	Turvo-PR	Polyfloral honey
OH-3	General Carneiro-PR	Honeydew honey (*Mimosa scabrella*)
OH-4	General Carneiro-PR	Polyfloral honey
OH-5	General Carneiro-PR	Honeydew honey (*Mimosa scabrella*)
OH-6	Turvo-PR	Polyfloral honey
OH-7	General Carneiro-PR	Honeydew honey (*Mimosa scabrella*)
OH-8	Turvo-PR	Polyfloral honey

Adapted from Silva et al., 2019 [23]

Materials and methods

Obtaining, georeferencing and sample preparation

Eight samples of honey, produced in a certified organic manner, were collected and georeferenced in the preserved Atlantic Forest region of two municipalities in southern Paraná: General Carneiro (26°25'44" South, 51°19'2" West) and Turvo (25°2'47" South, 51°32'32" West). The collection period spanned from December 2015 to February 2016. Table 1 displays the division and nomenclature of the honey samples, their collection sites, and floral variations. To achieve the desired concentrations, the honeys were diluted in culture medium and filtered using 0.22 µm syringe filters [11]. The research has been registered in the National Management System for Genetic Heritage and Associated Traditional Knowledge (SISGEN) under the registration number AE0DBB2.

Microorganisms and microbial susceptibility

In this analysis, the following microorganisms were used: *Streptococcus mutans* ATCC 700610, *Streptococcus mitis* NCTC 12261, *Streptococcus oralis* ATCC 10557, *Streptococcus salivarius* ATCC 7073, *Streptococcus gordonii* Challis ATCC 35105, and *Streptococcus sanguinis* 3K36. The antimicrobial activity was assessed using the microdilution technique in a 96-well plate containing Mueller-Hinton

broth, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012), to determine the minimum inhibitory concentration (MIC). The minimum bactericidal concentration (MBC) was determined by plating aliquots from the MIC onto Mueller-Hinton agar. The honey was diluted in Mueller-Hinton broth to achieve concentrations ranging from 60 to 5% (w/v) in the wells [11]. The bacterial inoculum was standardized spectrophotometrically to obtain a concentration of 5×10^5 cells per well. The results were analyzed visually. Each experiment was conducted in triplicate and repeated three times independently.

Antibiofilm activity

We induced the formation of *S. mutans* biofilms in 96-well plates. The inoculum consisted of 100 µL of approximately 8×10^{11} cells, standardized by optical density at 0.8 at 625 nm, and was added to each well in brain heart infusion (BHI) broth supplemented with 1% sucrose, following the methodology described by Cai et al., 2016 [12], with some modifications. The plates were incubated in a CO_2 atmosphere oven. After 24 h, the culture medium was replaced. After 48 h, the old culture medium was discarded, and a new medium was added with honey concentrations equal to the MIC, double the MIC, and 50% of the MIC. The positive control was performed using 0.12% chlorhexidine, while the negative control consisted of BHI broth without sucrose. After 24 h of treatment, the contents of the wells were removed, diluted, and plated on Mitis Salivarius Agar for subsequent enumeration of colony forming units per mL (CFU/mL).

Catalase treatment

To investigate the mechanism of antimicrobial activity, we treated the honeys with catalase. We diluted the solutions in a similar manner to the MIC test and then subjected them to a two-hour treatment with a catalase solution (at a 1/1 ratio). The catalase solution was prepared by combining 40 mg of catalase (Sigma, C-10: 4000 mg-I units) with 20 ml of culture medium [13].

Scanning Electron Microscopy (SEM)

We formed biofilms on coverslips (Lab-Tek, Nunc, Naperville, IL, USA) following the method described in item 2.3. The biofilms were subsequently treated with samples that exhibited the most promising antibiofilm activity at CIM and 2x CIM concentrations. The cells were fixed in 3% glutaraldehyde at room temperature for 12 h. Afterwards, the biofilm was dehydrated using increasing concentrations of ethanol, coated with gold, and observed using scanning electron microscopy (SEM) (JEOL JSM 5600LV, JEOL Tokyo, Japan).

Statistical analysis

The data were assessed for normality and subjected to one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The significance level was set at 5% ($p < 0.05$) to determine statistical significance.

Results

All samples of organic honeys, in natura, (OH-1 to OH-8), demonstrated the ability to inhibit the growth of oral *Streptococcus* in planktonic form. The honeys that exhibited the most notable activity, with lower minimum inhibitory concentration (MIC) values, were OH-1, OH-2, OH-3, and OH-07, as shown in Table 2.

After catalase treatment, there was a significant increase in the minimum inhibitory concentration (MIC) of all eight honey samples for the six tested bacteria. Among the

Table 2 Antimicrobial activity of fresh organic honeys against cariogenic microorganisms, showing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

	MIC (%, w/v) | MBC (%, w/v)					
Sample	***S. mutans* ATCC 700610**	***S. gordonii* Challis ATCC 35105**	***S. salivarius* ATCC 7073**	*S. oralis* ATCC 10557	*S. mitis* NCTC 12261	*S. sanguinis* 3K36
OH-1	15|20	15|20	15|20	15|20	20|25	15|20
OH-2	20|25	15|25	15|20	15|15	15|20	15|15
OH-3	10|20	20|25	20|25	15|15	15|15	15|15
OH-4	25|25	15|20	20|25	20|25	20|25	15|25
OH-5	30|30	25|30	25|30	20|25	20|20	25|30
OH-6	40|50	20|45	40|45	30|55	30|55	25|50
OH-7	20|25	20|25	25|30	15|30	25|25	25|25
OH-8	30|35	35|40	40|40	35|45	35|40	25|40

Legend: OH - organically produced honey plus sample number. Values expressed as a percentage of honey (w/v) diluted in culture medium.

Table 3 Antimicrobial activity of fresh organic honeys (treated with catalase) against cariogenic microorganisms, showing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

	MIC (%, w/v) | MBC (%, w/v)					
Sample	***S. mutans* ATCC 700610**	***S. gordonii* Challis ATCC 35105**	***S. salivarius* ATCC 7073**	*S. oralis* ATCC 10557	*S. mitis* NCTC 12261	*S. sanguinis* 3K36
OH-1	> 60|>60	55|60	60|>60	45|50	50|50	> 60|>60
OH-2	> 60|>60	> 60|>60	60|>60	45|50	50|55	45|50
OH-3	> 60|>60	> 60|>60	45|50	60|>60	45|50	50|55
OH-4	> 60|>60	60|>60	55|60	>60|>60	>60|>60	>60|>60
OH-5	45|50	45|55	55|60	>60|>60	50|55	55|60
OH-6	> 60|>60	> 60|>60	45|50	45|50	>60|>60	>60|>60
OH-7	45|50	45|50	55|60	>60|>60	55|60	55|60
OH-8	45|50	60|>60	>60|>60	>60|>60	45|50	55|60

bacteria, *S. mutans* exhibited the lowest sensitivity to honey with inactivated peroxide and was affected only by OH-5, OH-7, and OH-8 at high concentrations.

The assay for antibiofilm activity, using a 48-hour-old S. mutans biofilm, demonstrated that all honeys significantly reduced the number of viable cells in the biofilm (Fig. 1). Particularly noteworthy are the samples OH-1 (Fig. 1A), OH-2 (Fig. 1B), and OH-7 (Fig. 1G), which, at a concentration of 2x CIM, were able to eliminate 10 logs of biofilm, effectively eradicating it compared to the untreated control ($p > 0.05$). All other honeys, except OH-6 (Fig. 1F), also reduced the biofilm by 10 logs at a concentration of 50% (w/v).

Scanning electron microscopy (SEM), at a magnification of 5000 times, visually corroborated the UFC/mL counts. In the untreated control (Fig. 2A), a dense S. mutans biofilm with an extracellular matrix was observed. The treatment of the biofilm with honeys exhibiting stronger antibiofilm activity (OH-1, OH-2, and OH-7) resulted in severe disruption of the biofilm structure, characterized by cell debris and empty spaces (Fig. 2B and C, and 2D).

Discussion

Honey, a food that has been part of the human diet since ancient times, has historically been used as a therapeutic agent for wound treatment [3]. In recent years, modern medicine has rediscovered the therapeutic potential of honey and incorporated it into hospital-level therapies [14]. This increased recognition is largely attributed to the antimicrobial activity of honey [3].

Indeed, honey has demonstrated antimicrobial activity against bacteria [15] and fungi [16], including strains that are resistant to various antibiotics [17]. This antimicrobial action can exhibit both bacteriostatic and bactericidal effects, and its potency has been reported to be concentration-dependent [18, 19].

Here, we evaluate the antimicrobial activity of eight samples of Brazilian organic honeys against six oral microorganisms of the genus *Streptococcus*. All samples showed antimicrobial activity against the tested strains. The samples OH-1, OH-2, OH-3, and OH-7 stood out the most, with MIC values of 15%, 10%, 20%, and 20% (w/v), respectively. A previous study assessed the antimicrobial activity of local honey from India against *S. mutans* MTCC using the agar diffusion method. The results indicated that concentrations of 5%, 10%, 20%, and 40% showed no zone of inhibition. Only at the concentration of 60% of the honey solution was an average inhibition zone of 10.0 mm observed [20]. Another study evaluated the antimicrobial activity of Hamadan honey using agar diffusion and found that the honey was able to inhibit the growth of *S. mutans* at concentrations above 20% (w/v) and of *Lactobacillus* at a concentration of 100% [9]. Although we did not evaluate the action of our honeys against *Lactobacillus*, all honeys tested, except for honeys 5, 6, and 8, presented MIC values below 20% for one or more cariogenic bacteria analyzed.

The variation in the results of the previously mentioned studies may be related to both the composition of the honeys and the differences in the methods of analysis. In the present study, the antimicrobial activity was assessed using the broth microdilution method (CLSI, 2012) to determine the minimum inhibitory concentration (MIC). Despite being a rapid and straightforward test, the agar diffusion method for evaluating the antimicrobial activity of honey has several limitations, such as difficulties in diffusing the active components of honey due to its high viscosity, low reproducibility, and inability to distinguish between bactericidal and bacteriostatic activity [21].

Using the broth microdilution technique, a study assessed the antimicrobial activity of Saudi Arabian honey against *S. mutans* [22]. The results demonstrated significant growth inhibition in wells containing honey concentrations of 50%, 25%, 12.5%, and 6.25% compared to the control with TSB medium. Spectrophotometry was employed for reading,

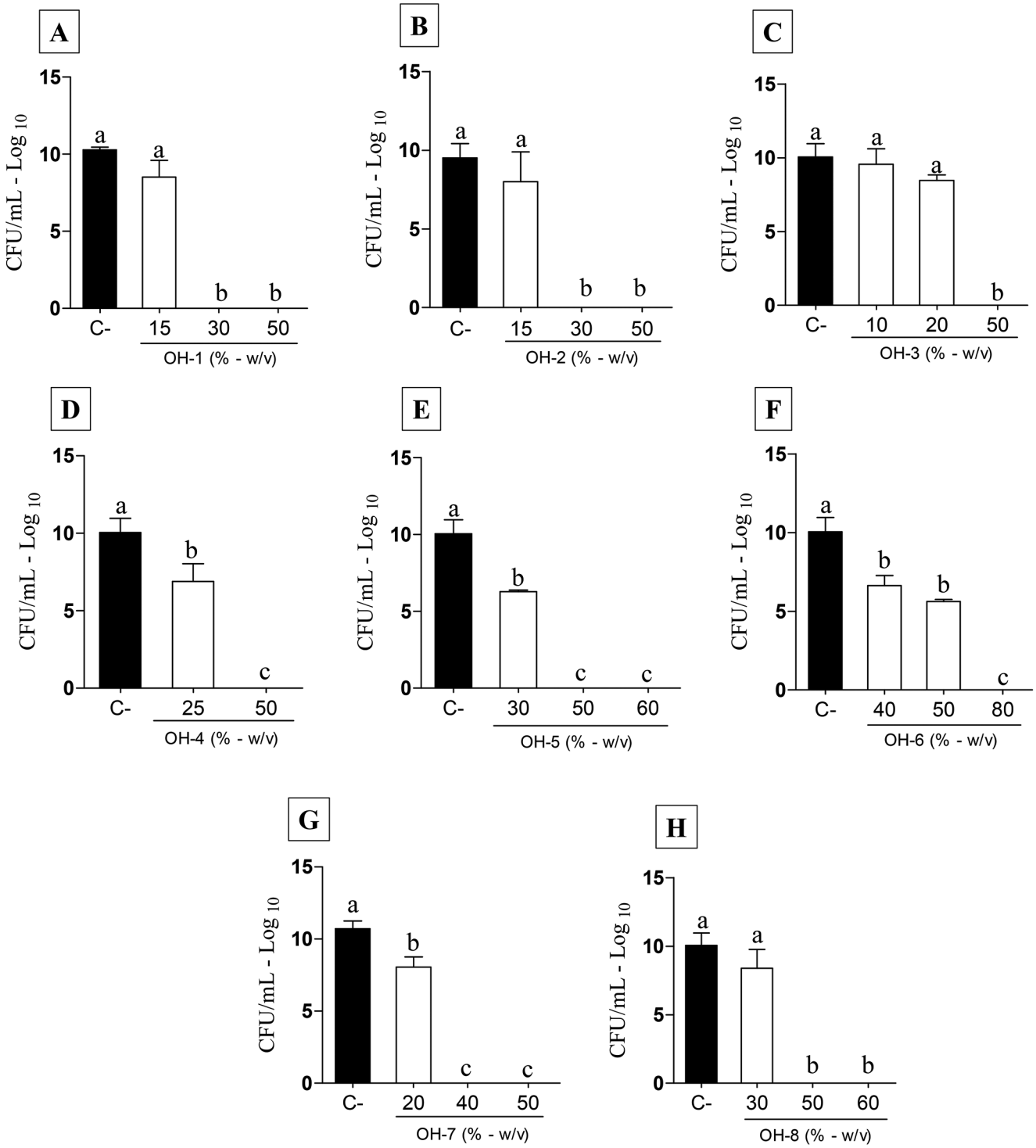

Fig. 1 Antibiofilm activity of eight samples of organic honey (OH-1 to OH-8) at MIC, 2x MIC, and 50% (w/v) concentrations against S. mutans ATCC 700610 monospecies biofilm. Different letters represent statistical differences. Antibiofilm activity of organic honeys

but specific MIC and CBM values were not defined. In our study, we visually identified the MIC as the concentration where no growth (turbidity) was observed in the well. Subsequently, we plated aliquots from this concentration on BHI agar to determine the CBM (Table 2).

The antimicrobial activity of honey is primarily attributed to two main components: hydrogen peroxide, produced by enzymes such as glucose oxidase, and/or phenolic compounds [3]. To determine which component is mainly responsible for the antimicrobial action, honey samples were

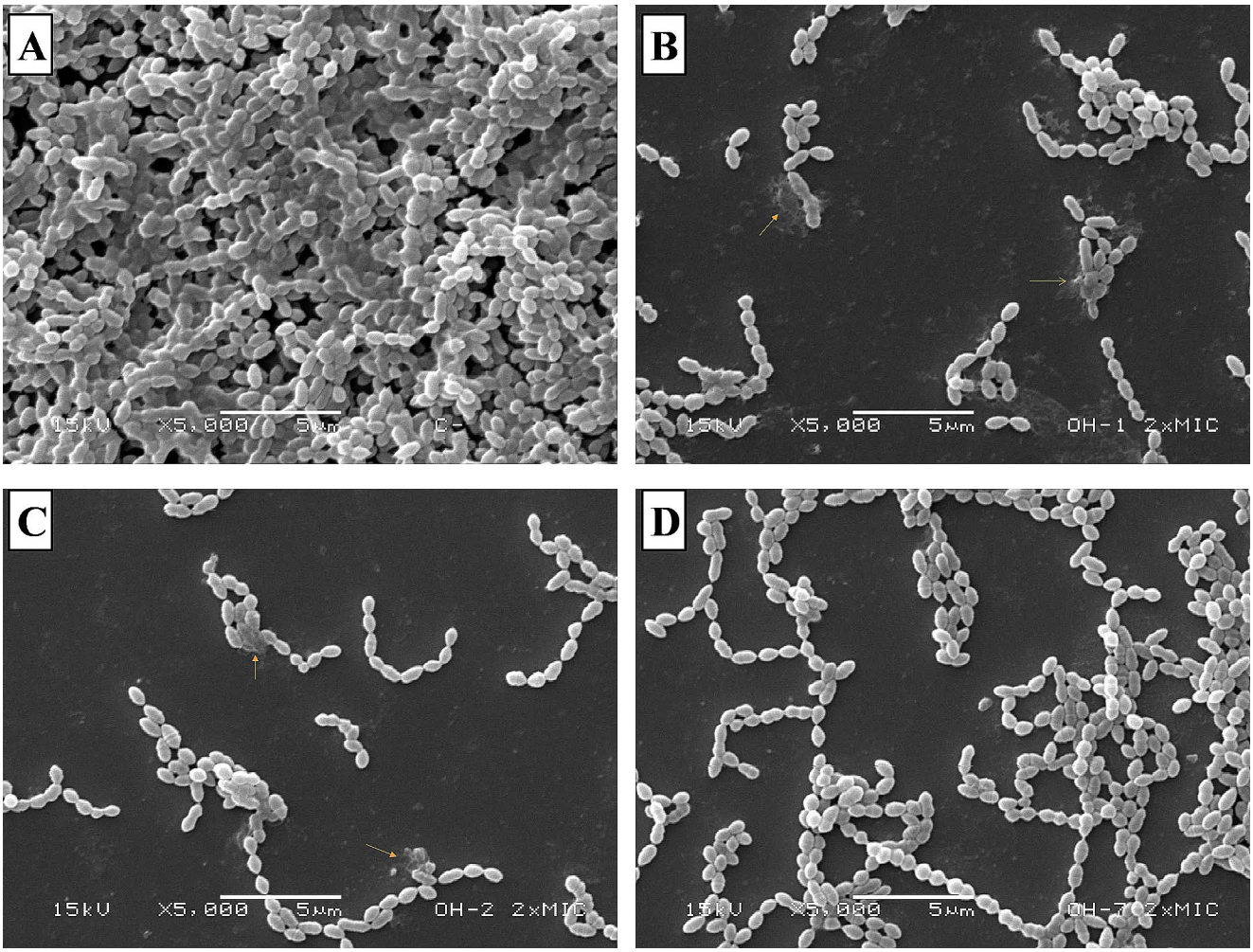

Fig. 2 Scanning electron microscopy images displaying the biofilm of *S. mutans*: untreated (**A**), treated with 2x MIC of OH-1 (**B**), treated with 2x MIC of OH-2 (**C**), and treated with 2x MIC of OH-7 (**D**). Arrows indicate cellular deformations and matrix destruction

treated with catalase [13]. This enzyme degrades peroxide in the sample while leaving the other components intact. A comparison of the MIC and CBM results in Tables 2 and 3 reveals differences in values.

Table 3 presents the outcomes of honeys treated with catalase before exposure, showing mostly higher MIC and CBM values compared to Table 2, which displays the results of honeys without any interference. However, it is noteworthy that in Table 3, some samples exhibited inhibitory capacity at concentrations close to 60% (w/v) for certain bacteria. These findings suggest that the antimicrobial action of Brazilian organic honeys is primarily linked to the peroxide-producing enzyme complex. Nevertheless, at higher concentrations, other honey components, particularly phenolic compounds, may have a slight effect. Additionally, the high osmolarity of honey could also contribute to the persistence of antimicrobial action [21].

In a previous study, our research group demonstrated significant antioxidant activity in the phenolic extracts from these honey samples [23]. We also identified the chemical profile of these honeys, showing phenolic component contents in mg (GAE/g) as follows: 73.15 (OH-1), 59.79 (OH-2), 49.79 (OH-3), 52.20 (OH-4), 117.68 (OH-5), 84.08 (OH-6), 83.19 (OH-7), and 53.03 (OH-8). Among the samples, four phenolic compounds - ferulic acid, caffeic acid, rutin, and hesperidin - were identified and quantified [23]. Previous studies have reported the antimicrobial activity of these molecules against oral microorganisms, which may partly explain the antimicrobial effectiveness of honeys after peroxide inactivation [24–26].

Only a few studies have evaluated the antibiofilm activity of honeys against mature biofilms of oral microorganisms. This study is pioneering in exploring the role of peroxides as responsible for this activity. All evaluated organic honeys demonstrated the capacity to eradicate the 48-hour biofilm of *S. mutans*. According to Albaridi, 2019 [17], honeys with MICs between 12.5% and 50% (w/v) exhibit moderate antimicrobial potency. Hence, the most promising results were

observed in honeys with concentrations at 2x the MIC and below 50%, leading to biofilm destruction. OH-1 (30%), OH-2, and OH-7 (40%) showed the most notable performance. Except for OH-6, all other honeys caused total biofilm death at higher concentrations. Divergences between studies arise from variations in the method of analysis and the units of honey concentration.

A recent review highlights the antimicrobial effects of honey on dental biofilms but concludes that the clinical efficacy of honey in preventing dental caries remains inconclusive, as more robust clinical studies are needed [27]. The present study provides additional data regarding the action of peroxides released by honey enzymes on mature oral biofilms, which may serve as a basis for conducting clinical trials.

One study assessed the antimicrobial activity of two samples of Manuka honey against *S. mutans*. Both varieties weakly inhibited *S. mutans* cell adhesion to a glass surface at sub-MIC concentrations. One of the samples exhibited weak inhibition of biofilm formation at 200 µg/mL but demonstrated greater effectiveness at 500 µg/mL. While the results are interesting, the use of different units of measurement in the study hinders comparison with other studies on honey's antibiofilm activity. The standardized form of honey concentrations for analysis is typically expressed as a percentage (volume/volume or weight/volume). Concentrations in µg or mg per ml can pose challenges for reproducibility due to the viscosity of this food [21].

For visualization purposes, we used SEM to analyze the antibiofilm activity of the most promising honeys (OH-1, OH-2, and OH-7) against *S. mutans*. The SEM images illustrate the results of the graphs depicting the antibiofilm activity. Figure 2A displays a robust biofilm without treatment or any structural changes. Figure 2B and C, and 2D show the biofilm treated with OH-1 (30%), OH-2 (40%), and OH-7 (40%), respectively, at twice the MIC concentration. These images reveal extensive destruction of the biofilm, with some cellular deformations and matrix destruction.

The evidence of antimicrobial activity and antibiofilm properties of Brazilian organic honeys against oral microorganisms suggests that honey has the potential to serve as a substitute for regular sugar, aiding in microbiota control and offering various other benefits known for this functional food in the body.

Conclusion

Brazilian organic honeys display promising antimicrobial and significant antibiofilm activity against oral microorganisms, particularly *S. mutans* mono-species biofilm. Although honey contains sugar, its overall benefits surpass those of common dietary sugars. In addition to its well-documented advantages, such as enhancing physical and mental well-being and improving important biological functions, Brazilian organic honey can effectively control oral microorganisms, potentially reducing the risk of biofilm-dependent diseases.

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Declarations

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Conflict of interest The authors declare no conflict of interest.

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