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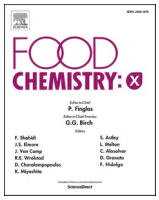

Antimicrobial and antioxidant activity of proteins isolated from *Melipona beecheii* honey

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ABSTRACT

Proteins from *Melipona beecheii* honey were purified by concanavalin A (conA) affinity chromatography and eluted with a stepwise glucose gradient into fractions named F2-F5. The conA-unbound fraction (F1) was further separated by molecular exclusion into fractions named MbF1-1,2 and MbF1-3. All fractions were evaluated for antibacterial activity against foodborne pathogens and antioxidant capacity. F1 fraction possessed highest antimicrobial activity against *S. aureus*, *L. monocytogenes*, *S.* Typhimurium, *E. coli* and *P. aeruginosa* with MIC's 1.4 ± 0.2, 15 ± 1, 39 ± 2, 1 ± 0.1, and 75 ± 2 μg/mL, respectively. F1, MbF1-1,2 and MbF1-3 had bactericidal effect except against *P. aeruginosa*. When the antioxidant capacity of the fractions was determined, F2 had the highest antioxidant activity measured by DPPH radical scavenging activity ($IC_{50} = 2.4 \pm 0.4$ μg/μL) and reducing power of Fe(III) ($IC_{50} = 1.8 \pm 0.2$ μg/μL). We provide evidence that *M. beecheii* honey proteins possess broad spectrum antibacterial and antioxidant activity, the latter probably through their reducing agent and free radical scavenger properties.

Introduction

Melipona beecheii honey is a natural substance produced by the stingless bee *Melipona beecheii,* one of 16 native species of the south-eastern region of the Mexican territory (Quezada Euán, 2005), and is known for being an energy, nutraceutical and medicinal food. Its properties are attributed to the compounds it contains such as carbohydrates, vitamins, minerals, polyphenols, organic acids and proteins (Johnston, McBride, Dahiya, Owusu-Apenten, and Nigam, 2018; Nolan, Harrison and Cox 2019). The honey composition can change depending on the diversity of the floral source used by the bees to harvest the nectar, the climate, storage time and entomological origin. The variation of its components gives rise to its appearance, smell, taste, and biological properties (Meo, Al-Asiri, Mahesar, and Ansari, 2017; Cianciosi et al., 2018; Bocian, Buczkowicz, Jaromin, Hus and Legáth, 2019).

Foodborne diseases caused by bacteria are a significant cause of mortality and morbidity worldwide, and are the focus of great interest to the World Health Organization (Mensah and Ofosu, 2020). Antimicrobial activity is one of the nutraceutical properties attributed to the *M. beecheii* honey, based on reports of its great antibacterial spectrum which causes growth inhibition of both Gram-positive and Gram-negative bacteria (Ramón-Sierra, Martínez-Guevara, Pool-Yam, Magaña-Ortiz, Yam-Puc and Ortiz-Vázquez, 2020). Furthermore, it has been reported that this food could inhibit the growth of pathogens related with foodborne illnesses such as *Pseudomonas aeruginosa, Staphylococcus aureus, Helicobacter pylori, Salmonella* spp., *Campylobacter* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes,* among others (de Queiroz Pimentel, da Costa, Albuquerque, and Junior, 2013; Brown, O'Brien, Georges, and Suepaul, 2020; Domingos et al., 2021). In addition, it has been documented that *M. beecheii* honey has a negative effect on the different virulence factors of some bacteria, diminishing their pathogenicity (Ramón-Sierra, Villanueva, Rodríguez-Mendiola, Reséndez-Pérez, Ortiz-Vázquez and Arias-Castro, 2021). Recently, Almasaudi (2021) reported that the antibacterial efficacy of honey is related to some of its components that work synergistically, which makes it highly effective against a variety of microorganisms; such effectivity is different for each microbial strain. But the honey composition will depend on the source of nectar, bee type, and storage. In fact, the antimicrobial activity of

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M. beecheii honey against *S. aureus* and *E. coli* is attributed to its phenolic compounds and proteins (Ramón-Sierra et al., 2020). These phenolic compound contents, in addition to molecules of protein origin, have been regarded as the source of antioxidants that contribute substantially to this biological activity (Kishore, Halim, Syazana, and Sirajudeen, 2011; da Silva et al., 2013). However, there are no studies on *M. beecheii* honey proteins reporting their antioxidant capacity. The objective of this work was to evaluate the antimicrobial and antioxidant potential of proteins from *M. beecheii* honey, in order to demonstrate their contribution to its antioxidant and antimicrobial properties.

Material and methods

Biological material

Melipona beecheii honey used in this study was collected from Maní, Yucatán, México (20°23′09.8″N 89°23′56.4″W) in the months of April and May 2017, 2018 and 2019. The honey samples were collected in a traditional way, directly from the hive. The collected honey was stored away from light at room temperature (25 ± 2 °C) until its analysis.

The pathogenic strains were *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 13311, *Escherichia coli* ATCC 25,922 and *Pseudomonas aeruginosa* ATCC 27853. The strains were preserved at −80 °C and grown in a Mueller-Hinton medium (Becton Dickinson, Franklin Lakes, NJ, USA).

Extraction and fractionation of the proteins from M. Beecheii honey.

M. beecheii honey was diluted twenty-fold and homogenized in phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7). The proteins from the diluted honey were extracted using the ultrafiltration method reported by Ramón-Sierra et al., 2021. The total protein extract (TPE) obtained was quantified using the Bradford method (Bradford, 1976) and stored at −20 °C for later analysis.

The TPE was fractionated using affinity chromatography with concanavalin A attached to sepharose 4B (Sigma; Burlington, MA, USA) packed in a 2 × 12 cm column. The column was equilibrated with 20 volumes of 25 mM tris buffer, pH 6.8, 0.5 M NaCl, 5 mM $CaCl_2$, 5 mM $MnCl_2$ and 5 mM $MgCl_2$. One aliquot of 2.5 mL containing 15 μg of total TPE protein was diluted in 5 column volumes (40 mL approximately) of the buffer described above and loaded onto the column. The fractionation was carried out by first collecting the flowthrough which was termed conA-unbound F1 fraction. The column was then washed with 2 volumes of the same buffer, followed by the addition of 15 volumes of a stepwise glucose gradient from 0 to 0.1, 0.1–0.2; 0.2–0.5; and 0.5–1 M, and the corresponding fractions named F2-F5, respectively. The fractions thus obtained were concentrated using Amicon® ultra − 15 (3 K) columns (Merck Millipore, Burlington, MA, USA) and subsequently stored at −20 °C for later analysis (Brudzynski and Sjaarda, 2015; Ramón-Sierra et al., 2021).

Antimicrobial activity was determined for each fraction. Then, the fraction F1, which contained the strongest antimicrobial activity, was fractionated on a 2.5 × 75 cm sephadex G-75 Bio-Rad Econo-Column® (Bio-Rad; Hercules, CA, USA); the mobile phase was phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.5). The flow rate was set at 0.02 mL/min; two fractions were obtained and concentrated using Amicon® ultra − 15 (3 K) columns. All samples were analyzed by SDS-PAGE electrophoresis. TPE, protein fractions obtained by affinity chromatography (F1-F5), and protein fractions obtained by molecular exclusion chromatography (0.5 μg/mL) were evaluated using 12% polyacrylamide gels. The run conditions in all cases were 85 V for 115 min at room temperature. After the run, the gels were incubated for 2 h in a fixing solution (10 % acetic acid, 25% methanol and 0.05% formaldehyde). The gels were stained with silver nitrate (Ramón-Sierra et al., 2021) and analyzed in the Bio-Rad Image Lab System (Bio-Rad).

Antimicrobial activity of TPE and fractions obtained from M. Beecheii honey.

The antimicrobial activities of TPE and all fractions were evaluated using the disk diffusion method (Wanger, 2007). An inoculum of 0.5 McFarland scale was used, which is approximately 1×10^8 colony-forming units/mL (CFU/mL); CFU was measured for each strain. Aliquots from 20 to 80 μL taken from each fraction with 0.05 to 0.625 μg/μL of protein, were evaluated. The petri dishes were incubated at 37 °C for 12 h (Wanger, 2007). Determination of the minimum inhibitory concentration (MIC) was carried out through microdilution (Qaiyumi, 2007). Protein aliquots with concentrations from 10 to 80 μg/mL were evaluated. As with the disk diffusion method, an inoculum of 0.5 on the McFarland scale was used. All samples were incubated at 37 °C for 24 h with agitation (250 rpm). Inhibition of bacterial growth was measured spectrophotometrically on a BioPhotometer 6131 (Eppendorf, Hamburg, Germany) at 600 nm. Samples were prepared and analyzed in triplicate. In all cases, amoxicillin (10 μg/mL), ciprofloxacin (1 μg/mL) and tetracycline (10 μg/mL) were used as positive controls, and phosphate buffer (50 mM NaH_2PO_4 /Na_2HPO4, pH 7 as negative control (Schneider et al., 2010). In all cases, the bacteria with the proteins were incubated at 37 °C for 72 h.

Determination of Bactericidal/Bacteriostatic effect of M. Beecheii proteins

The antibiotics, TPE, F1, MbF1-1,2 and MbF1-3 were added at concentrations corresponding to 2X their MIC, determined using the method described by Qaiyumi (2007). The mixtures were incubated at 37 °C for 7 h at 180 rpm and read at 600 nm every hour. In order to determine if the protein fractions had a bactericidal or bacteriostatic effect, growth inhibition curves of the protein fractions were compared to those produced by amoxicillin and ciprofloxacin (bactericidal), or tetracycline (bacteriostatic) and the viable cells were scored after 6 h.

Antioxidant activity of the protein fractions from Melipona beecheii honey

DPPH assay

The capacity of the protein fractions to stabilize the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) was quantified following the methodology proposed by Shimada, Fujikawa, Yahara and Nakamura (1992). One hundred μL of TPE or each protein fraction was mixed with 1 mL DPPH (0.1 mM) prepared in ethanol (96%), and incubated for 30 min; after this time, the absorbance was measured at 517 nm. The percent scavenging of DPPH was given by the formula: percent scavenging (%) = [(A0 − A1)/A0] × 100, where A0 was the absorbance of the control (reactive + distilled water) solution and A1 was the absorbance in the presence of TPE and protein fractions.

Reducing power of Fe(III)

The reducing power of TPE and its protein fractions was determined by the Oyaizu method described by Kumaran and Karunakaran, 2006. Two hundred fifty μL of sample were mixed with 250 μL phosphate buffer (0.2 M, pH 6.6) and 250 μL potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was shaken and incubated at 50 °C, 20 min. Then 250 μL of trichloroacetic acid (10%) were immediately added to the mixture at room temperature, which was then centrifuged for 10 min at 1000 *g*. Five hundred μL from the supernatant were mixed with distilled water (400 μL) and $FeCl_3$ (100 μL, 0.1%), incubated at 50 °C for 10 min. The absorbance was measured at 700 nm. The reducing power was calculated using the formula (%) = [(SA – (CA*SA] × 100, where CA was the absorbance of the control (distilled water instead of sample), and SA was the absorbance in the presence of TPE and protein fractions.

Statistical analysis

All antimicrobial and antioxidant determinations were performed in

triplicate; the data were analyzed by means of one-way ANOVA test using the SPSS v23 program (Statistical package Statgraphics Plus software, version 2.1, Manugistic, Inc., Rockville, MD, USA) and were considered statistically significant ($P \leq 0.05$) under the selected variables.

Results and discussion

Fractionation of M. Beecheii honey proteins

A TPE was obtained from *M. beecheii* honey and analyzed by SDS-PAGE; silver nitrate staining revealed a major band migrating at 25 kDa (Fig. 1a, lane TPE). Five protein fractions denominated F1, F2, F3, F4 and F5 (eluted according to their glycosyl affinity to conA; see methods) were obtained by affinity chromatography from the TPE, and their corresponding polypeptide composition visualized by SDS-PAGE (Fig. 1b). Although the glucose content was not determined, we arbitrarily considered the conA-unbound F1 fraction as non-glycosylated, and F2 to F5 as those from lowest to highest glycosylation degree, respectively (Fig. 1b). The major 25 kDa band was also present in fractions F1 and F2 (Fig. 1b, lanes F1 and F2, respectively), and a doublet of proteins, one with molecular mass slightly higher than 25 kDa and another of 27 kDa were present in fractions F3-F4 (Fig. 1b, lanes F3-F4, respectively). In addition, the presence of minor bands of 61 (Fig. 1b, lanes F4 and F5), and 16 and 9 kDa (Fig. 1b, lanes F3-F5), were observed. With respect to protein concentration, *M. beecheii* honey contained 1.75 ± 0.12 mg of protein per gram; F1, 0.585 ± 0.2 mg/g; F2, F3, F4 and F5, 0.225 ± 0.02 mg/g, 0.291 ± 0.06 mg/g, 0.330 ± 0.03 mg/g, and 0.278 ± 0.02 mg/g respectively. All protein concentrations were referred to gram of honey. It was observed that 34 % of the initial protein remained in the conA-unbound F1 fraction; interestingly, this fraction had the strongest antibacterial activity. This fraction was then separated by size-exclusion chromatography, from which we obtained two fractions named MbF1-1,2 and MbF1-3. MbF1-1,2 showed the presence of the 25 kDa protein (Fig. 1c, lane MbF1-1,2); on the other hand, MbF1-3 showed a major 95 kDa protein (Fig. 1c, lane MbF1-3). It was previously reported that F1 contained three proteins: two 25 and one 95 kDa proteins. Since no 25 kDa band was observed in the MbF1-3 fraction, the MbF1-1,2 fraction contained the two 25 kDa proteins (Ramón-Sierra et al., 2021).

Antimicrobial activity of the TPE and its derived protein fractions from M. Beecheii honey

The total protein extract (TPE) inhibited the growth of the five bacterial strains evaluated in this study (Table 1). *E. coli* and *S. aureus* showed the highest susceptibility to TPE (Table 1); in contrast, *S.* Typhimurium and *P. aeruginosa* were the least susceptible (Table 1). Besides TPE, the conA-unbound F1 fraction also had antibacterial activity against all evaluated strains (Table 1). The inhibition halos were similar to those showed by TPE and the antibiotics (Table 1). Moreover, some inhibition halos produced by TPE or F1 were larger than those produced by the antibiotics (Table 1). MbF1-1,2 and MbF1-3 showed the antibacterial effect against the five strains, but the inhibition halos were smaller than those of TPE and F1. *S.* Typhimurium, *P. aeruginosa* and *L. monocytogenes* were the least susceptible (Table 1). F2, F3, F4, and F5 did not show inhibition halos. The broad spectrum of antibacterial activity presented by the honey protein fractions may be caused by proteins such as MRJP (Major Royal Jelly Proteins) and proteases which could disrupt and contribute to the loss of the bacterial membrane integrity (Kim and Jin, 2019).

The MIC was evaluated for each protein fraction that had antibacterial activity with the disk diffusion test. The results are summarized on Table 2; it can be observed that, in the case of TPE, MICs range from 50 ± 0.1 μg/mL for *E. coli* to 200 ± 1 μg/mL for *P. aeruginosa* as the most and least susceptible bacteria, respectively. MICs from F1 were 41 to 50 times lower than TPE at inhibiting the growth of *S. aureus* and *E. coli* (Table 2). MICs from the same fraction were 2.6 to 5 times less than TPE for *S.* Typhimurium, *L. monocytogenes* and *P. aeruginosa* (Table 2). MICs from MbF1-1,2 and MbF1-3 for the strains tested were similar to those from F1, except for *L. monocytogenes*, where the MICs were twice lower than that from F1.

It is important to mention that the MICs obtained in this study were lower compared to those reported for other honey proteins. For

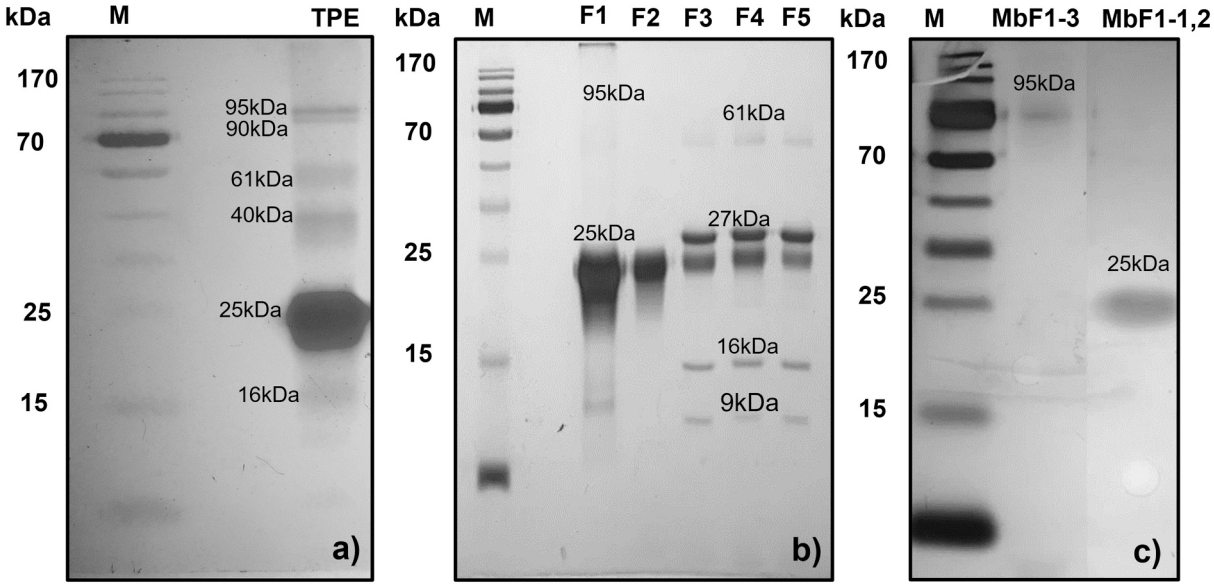

Fig. 1. Analysis by SDS-polyacrylamide gel electrophoresis of different protein fractions from *Melipona beecheii* honey. The protein profile from the total protein extract (TPE) after SDS-PAGE and silver nitrate staining (a) shows a major band migrating at 25 kDa (lane TPE). Upon fractionation on concanavalin A-sepharose (b), the major 25 kDa band was observed in fractions F1 and F2 (lanes F1 and F2, respectively); a doublet of proteins, one with molecular mass slightly higher than 25 kDa and another of 27 kDa was observed in fractions F3-F4 (lanes F3-F4, respectively). In addition, the presence of minor bands of 61 (lanes F4 and F5), and 16 and 9 kDa (lanes F3-F5), were observed. Further fractionation of F1 (c) resolved two fractions named MbF1-3 and MbF1-1,2, which separated a 95 (lane MbF1-3), and a 25 kDa protein (lane MbF1-1,2), in each fraction. Lanes M show the position of migration of molecular weight markers.

Table 1
Antimicrobial activity of the protein fractions from *Melipona beecheii* honey.

Inhibition Halos (mm) Compounds	Bacterial strain *S. aureus* ATCC 25923	*L. monocytogenes* ATCC 15313	*S.* Typhimurium ATCC 13311	*E. coli* ATCC 25,922	*P. aeruginosa* ATCC 27853
AMC	18.10 ± 0.20b	14.20 ± 0.40a	14.50 ± 0.20b	19.50 ± 0.30b	14.10 ± 0.30c
CIP	21.90 ± 0.10a	16.20 ± 0.30b	17.60 ± 0.30c	24.00 ± 0.30a	19.20 ± 0.40d
TET	18.20 ± 0.20b	14.30 ± 0.40a	14.70 ± 0.30b	18.20 ± 0.20c	13.20 ± 0.30c
TPE	21.40 ± 0.80a	14.50 ± 0.75a	08.10 ± 0.50a	23.50 ± 0.75a	10.10 ± 0.50a
F1	20.60 ± 0.75a	12.30 ± 1.5c	07.20 ± 0.75a	22.90 ± 0.80a	08.90 ± 1.25b
MbF1-1,2	15.10 ± 1.25c	08.50 ± 0.75d	04.10 ± 0.50d	18.10 ± 0.50c	05.20 ± 1.50e
MbF1-3	14.30 ± 0.90c	09.10 ± 0.50d	05.40 ± 0.25d	17.20 ± 0.75d	06.10 ± 1.20e

Note: Values with different letters in the same column have statistically significant differences according to the one-way ANOVA test ($p \leq 0.05$).

Table 2
Minimum inhibitory concentration of the protein fractions from *Melipona beecheii* honey.

Minimum inhibitory concentration (μg/mL) Protein fraction	Bacterial strain *S. aureus* ATCC 25923	*L. monocytogenes ATCC* 15313	*S.* Typhimurium ATCC 13311	*E. coli* ATCC 25922	*P. aeruginosa*ATCC 27853
TPE	58 ± 2a	85 ± 4a	140 ± 8a	50 ± 1a	200 ± 1a
F1	1.4 ± 0.2b	15 ± 1b	39 ± 2b	1 ± 0.1b	75 ± 2b
MbF1-1,2	3.8 ± 0.2c	6.6 ± 1.1c	40 ± 1c	2.5 ± 0.1c	50 ± 3c *
MbF1-3	2.1 ± 0.2c	7 ± 0.3c	38 ± 2c	1.8 ± 0.2d	55 ± 4c*

*MIC_{60}
Note: Values with different letters in the same column, have statistically significant differences according to the one-way ANOVA test ($p \leq 0.05$).
Amoxicillin MICs used for: *S. aureus* (4 μg/mL), *L. monocytogenes* (1 μg/mL), *S.* Typhimurium (4 μg/mL), *E. coli* (2 μg/mL), *P. aeruginosa* (8 μg/mL).

example, some reported MICs range from 15.2 to 75 μg/mL protein to inhibit Gram-positive and Gram-negative bacteria (Brudzynski and Sjaarda, 2015; Kim and Jin, 2019; Park et al., 2019). MICs for *E. coli* and *S. aureus* from the F1 fraction were 1 ± 0.1 μg/mL and 1.4 ± 0.2 μg/mL, respectively. These values are ten times lower than those reported by these authors, except for *P. aeruginosa*, whose MIC was 75 μg/mL, meaning that this was the least susceptible species to growth inhibition by the honey protein fractions. Gram-negative bacteria (such as *E. coli, P. aeruginosa* and *S.* Typhimurium) are reported as less susceptible to antibiotics and antimicrobial proteins due to their outer membrane that protects the peptidoglycan layer. Specially, *P. aeruginosa*, that had the higher MIC, exhibits 100 times more restricted membrane permeability than *E. coli* and possesses efflux pumps, which makes it less susceptible and thus, it can avoid bacterial death (bacteriostatic effect; Poole, 2001) On the other hand, Gram-positive bacteria (such as *S. aureus* and *L. monocytogenes*) could be less susceptible due to the hydrolytic enzymes they produce such as proteases that could affect the honey proteins (Schneider et al., 2010; Kim and Jin, 2019). Interestingly, F1 and MbF1-3 had MIC-values slightly lower than amoxicillin (Table 2), which varied from 0.25 to 1 μg/mL against *E. coli* (McGannon, Fuller and Weiss, 2010; Delgado-Valverde et al., 2017; Hubbard, Feasey and Roberts, 2018; Stohr, Kluytmans-van den Bergh, Verhulst, Rossen and Kluytmans, 2020). On the other hand, it was observed that MbF1-1,2 and MbF1-3 had MICs higher than F1 for *S. aureus* and *E. coli*, suggesting an additive effect of the proteins against these bacteria. This phenomenon was assessed using the methodology of the inhibitory fraction, which allowed us to observe an additive effect of MbF1-1,2 and MbF1-3 for *S. aureus* and *E. coli* growth inhibition. (Supplementary Fig. 1) (Ekambaram, Perumal, Balakrishnan, Marappan, Gajendran, and Viswanathan, 2016; Sun et al., 2017). There are no previous reports about additive antimicrobial effects of honey proteins; thus, this would be the first report on such antibacterial effect among proteins isolated from *M. beecheii* honey. This would also be consistent with the fact that at least one of the two 25 kDa proteins from MbF1-1,2 (MbF1-2) was previously identified as a homolog of the MRJP family (Ramón-Sierra et al., 2021). The protein homologs from this family are known to be the main antimicrobial activity components from honey (Chua, Lee and Chan, 2015; Bucekova and Majtan, 2016; Kim and Jin, 2019; Park et al., 2019).

Bactericidal/bacteriostatic effect of the TPE and protein fractions from M. Beecheii honey

Fig. 2 shows the various growth inhibition curves resulting from the isolated protein fractions from *M. beecheii* honey, which allowed us to assess their bactericidal or bacteriostatic effect and thereby calculate the percent lethality. Thus, TPE and F1 showed a bactericidal effect against all strains since TPE exerted > 99 % lethality against *S. aureus* and *E. coli* after 6 h, and F1 exerted 100 % lethality against *S.* Typhimurium, and *L. monocytogenes* (Supplementary figure 2; supplementary Table 1), and > 92% lethality against *P. aeruginosa* after 6 h (Fig. 2e; supplementary Table 1). In comparison, ciprofloxacin exerted > 99% lethality against *S. aureus*, *E. coli, S.* Typhimurium, and *L. monocytogenes* after 3 h, and against *P. aeruginosa* after 4 h. Amoxicillin also exerted > 99% lethality after 6 h for all strains. Finally, tetracycline exerted only 88 % (bacteriostatic effect) lethality after 6 h against all strains. MbF1-1,2 and MbF1-3 fractions (that contained 2 and 1 proteins), respectively, showed a bactericidal effect against all strains tested (Fig. 2b, 2d; supplementary Table 1), except against *P. aeruginosa*, where the effect was bacteriostatic (Fig. 2f; supplementary Table 1). This is consistent with previous studies which report that proteins from *M. beecheii* honey and other stingless bee honeys exhibited bactericidal effects (Kim and Jin, 2019; Ramón-Sierra et al., 2020, 21). It has been demonstrated that some antibacterial proteins or peptides induce changes in the transmembrane potential that may also affect membrane permeability. Although Gram-negative and Gram-positive bacteria could be affected by these antibacterial compounds (Wiese, Gutsmann and Seydel, 2003), the bactericidal or bacteriostatic effect will depend on the strain rather than their Gram classification. The bactericidal effect of conA-unbound proteins could be due to non-destructive cell wall mechanisms such as bacterial protein synthesis inhibition or the expression of enzymatic activity such as proteases that lyse the bacteria. A recent study demonstrated that non-glycosylated lysostaphins had highest antimicrobial activity than glycosylated lysostaphins (Shen, W., Yang, N., Teng, D., Hao, Y., Ma, X.,

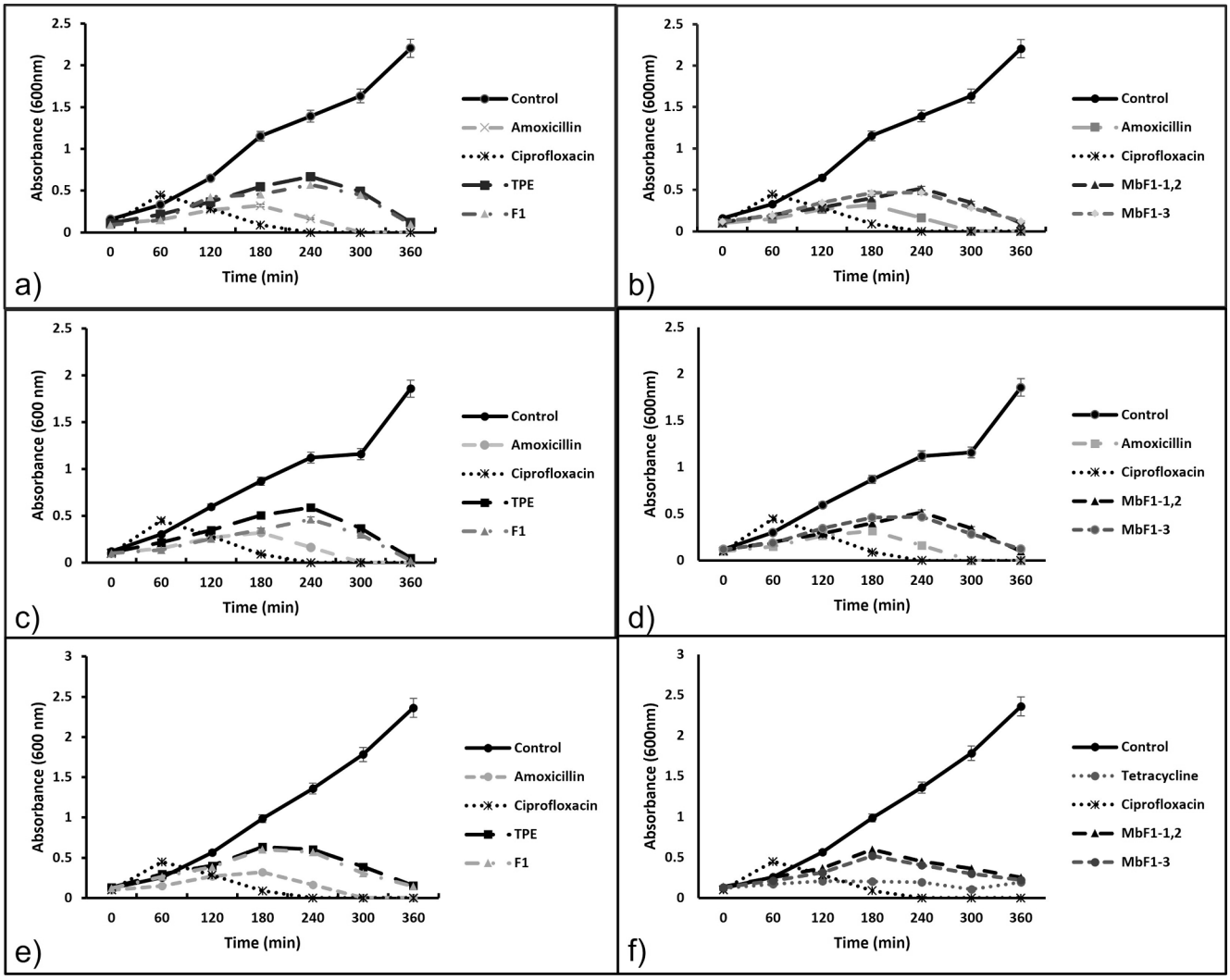

Fig. 2. Growth inhibition curves of bacteria. Growth of *Staphylococcus aureus* ATCC 25,923 (a), *Escherichia coli* ATCC 25,922 (c), and *Pseudomonas aeruginosa* ATCC 27,853 (e), were evaluated in the presence of total protein extract (TPE), the fraction F1, and amoxicillin. In addition, the growth of *S. aureus* ATCC 25,923 (b), *E. coli* ATCC 25,922 (d), and *P. aeruginosa* ATCC 27,853 (f), were evaluated in the presence of MbF1-1,2, MbF1-3, amoxicillin, and tetracycline. The positive control was the profile of bacterial growth in the absence of *Melipona beecheii* honey proteins or antibiotics.

Mao, R., & Wang, J., 2021) Altogether, the results presented in this study show the presence of a broad-spectrum of antimicrobial activity from the conA-unbound protein fraction of *M. beecheii* honey.

Antioxidant activity of the TPE and its protein fractions

Scavenging activity on the DPPH radical

Table 3 shows the results of the assays of determination of scavenging activity on the DPPH radical. TPE, F2 and F3 presented a remarkable antioxidant activity. It was observed that F2 had the lowest IC_{50} (1.8 ± 0.2 μg/μL) while TPE had an intermediate IC_{50} of 2.67 ± 0.1 μg/μL. On the other hand, F3 displayed the highest IC_{50} of 6 ± 0.9 μg/μL. All these had a higher IC_{50} value than ascorbic acid (IC_{50} of 0.01804 μg/μL), which was used as a control. Chua et al. (2015), determined antioxidant activity in Acacia, Tualang and Gelam honeys from Malaysia using the DPPH method and obtained values of IC_{50} 0.78 to 1.08 μg/μL, suggesting that phenolic compounds and proteins from those honeys conferred such antioxidant activity. The IC_{50} value (1.8 ± 0.2 μg/μL) from F2 was 1.6 times higher than that reported by Chua et al. (2015), which is thus less effective as an antioxidant. Conversely, Nagai and Inoue (2004) analyzed the antioxidant capacity of protein extracts from *A. mellifera* royal jelly using the DPPH assay and found that 5 μg/μL protein were required for reducing the DPPH radical by 14%. In comparison with F2, we obtained 0.4 ± 0.1 μg/μL which is more effective than this previously reported value. The MRJP homologs play an important role as antioxidants, since they constitute up to 90% of the total protein of the royal jelly (Schmitzová et al., 1998; Sano, Kunikata, Kohno, Iwaki, Ikeda, and Kurimoto, 2004; Scarselli et al., 2005). Taking into account that the *M. beecheii* honey contains proteins homologous to MRJP, it is highly likely that these proteins are the ones responsible of

Table 3
Free radical scavenging activity and ferric reducing antioxidant activity of protein fractions from *Melipona beecheii* honey.

Protein fraction	DPPH radical scavenging activity at 50% (IC_{50} μg/μL protein)	Ferric reducing antioxidant power (IC_{50} μg/μL protein)
TPE	2.7 ± 0.1b	24.0 ± 2.3b
F1	——	4.7 ± 0.8a
F2	1.8 ± 0.2a	2.4 ± 0.4a
F3	6.0 ± 0.9c	36.0 ± 3.2c
MbF1-1,2	————	4.3 ± 0.6a
MbF1-3	———	————

Note: Values with different letters in the same column, have statistically significant differences according to the one-way ANOVA test ($p \leq 0.05$).

the antioxidant activity (Chua et al., 2015; Ramón-Sierra et al., 2021).

Reducing power of Fe(III)

It was observed that TPE, F1, F2, F3 and MbF1-1,2 had ferric reducing activity, with F1, F2 and MbF1-1,2 displaying the highest reducing power. Their IC_{50} were 4.7 ± 0.8, 2.4 ± 0.4, and 4.3 ± 0.6 μg/μL, respectively (Table 3). MbF1-3 did not show ferric reducing activity. Ascorbic acid was used as a control with an IC_{50} of 0.003 μg/μL. Some authors have reported that 25 to 60 kDa proteins from honey and honeybee royal jelly had the capacity to reduce other molecules (Chua et al., 2015; Park et al., 2019). F1, F2 and MbF1-1,2 fractions, which contain proteins within the aforementioned MW interval, could contribute significantly toward the observed antioxidant effect. The antioxidant activity has been attributed to various mechanisms which include reductive capacity, radical scavenging, and binding of transition metal ion catalysts (Kumaran and Karunakaran, 2006).

Conclusions

The present study showed that some proteins from *M. beecheii* honey contribute to the antibacterial activity of this food against foodborne pathogens. The fact that the proteins contained in fraction F1 did not bind to conA suggests that they are non-glycosylated proteins. The protein fractions that had the most antimicrobial activity contained 25 and 95 kDa proteins whose molecular mass was similar to those of antimicrobial proteins from honeybee and royal jelly (Brudzynski and Sjaarda, 2015; Kim and Jin, 2019). The analyzed fractions showed an antibacterial broad spectrum which suggests the contribution of several bioactive proteins since *M. beecheii* honey contains at least 24 proteins (Ramón-Sierra et al., 2021). A possible antibacterial additive effect among the proteins from the isolated fractions was observed in this work.

In addition, the isolated fractions showed antioxidant activity. Based on previous reports on the capacity of MRJP to exert this action, we suggest that some proteins including the *M. beecheii* MJRP homolog (MbF1-2) (Ramón-Sierra et al., 2021), contribute to this activity. This is the first study reporting the antioxidant activity of honey proteins isolated from the stingless bee honey. Based on the results, it was determined that *M. beecheii* honey proteins exert their antioxidant activity as reducing agents and free radical scavengers, transferring an electron (e^-) or a hydrogen (H^+) atom.

In summary, the TPE and protein fractions obtained from *M. beecheii* honey possess antioxidant and antibacterial activities against foodborne pathogens. *M. beecheii* honey proteins evaluated in this study could be potentially expressed in other biological systems and used as nutraceuticals.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2021.100177.

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