Characterization of Bioactive Constituents from Honey Produced by COSTA RICAN STINGLESS BEES

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Characterization of Bioactive Constituents from Honey Produced by Costa Rican Stingless Bees
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Characterization of Bioactive Constituents from Honey Produced by Costa Rican Stingless Bees

Karakterisering van biologisch actieve inhoudsstoffen in honing geproduceerd door Costa Ricaanse angel-loze bijen
(met een samenvatting in het Nederlands)

Proefschrift

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Characterization of Bioactive Constituents from Honey Produced by COSTA RICAN STINGLESS BEES

Voor mijn broer Alejandro Zamora
(1978 - 1997)

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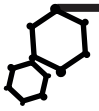
Chapter 1



General introduction

A brief history of honey and wound healing

The Codex Alimentarius defines honey as the natural sweet substance produced by honey bees from the nectar of flowers or extrafloral nectaries, or from excretions of plant sucking insects, which the bees collect and transform by adding specific substances of their own, dehydrate and store in the honey comb to ripen and mature[1].

Human kind has used this nutritious food since ancient times as a source of energy, as delicatessen, and for medical purposes[2,3,4,5,6,7]. The use of *Apis mellifera* (honey bee) honey as a medicine dates back to more than 4000 years, and has its origins in the ancient Syrian and Egyptian cultures. Evidence of this practice can be found in the Egyptian Ebbers Papyrus (circa 1550 B.C.); among the medical applications for honey, its use as a wound dressing is described[2,8]. In fact, honey wound dressings were common in the medical practice up to the early 1940s of the 20th century. The advent of the antibiotic era reduced the use of honey for wound healing[3,8].

The extensive use and misuse of antibiotic therapy led to the appearance of antibiotic resistant microorganisms[7,9,10]. Nowadays, one of the most studied mechanisms for resisting antibiotics is the ability of a great variety of bacteria and fungi to form biofilms[11,12]. A biofilm is formed by two subpopulations of cells: free living or planktonic, and a population of sessile cells growing attached to a surface, and enclosed in an extracellular polymeric substance (EPS) matrix[13,14]. Biofilms are associated with persistent infections like chronic wounds, since biofilms provide a protective environment against the host immune system, antibiotics, and antiseptics[11,12,15,16,17,18,19,20,21,22].

The resistance phenomenon, has negatively affected the treatment of infectious diseases to such degree, that wounds and burns infected with antibiotic resistant strains can seriously compromise the physical integrity of the patient; leading to a chronic condition, surgical excisions, amputations or even death[7,9,10,11,15,22]. At the present time, there is a growing demand for alternative topical treatments for the healing of burns and wounds infected with antimicrobial resistant microorganisms[23,24,25].

Recently, the use of honey for medical purposes has resurged[4,5,6,7,26,27,28]. Many studies have proven that *Apis mellifera* honeys possess antimicrobial properties and their employment in dressings favors the healing of wounds and burns[3,5,6,7,26,27,29,30,31,32,33,34].

There are four main factors involved in the antimicrobial activity of honey: osmolarity, pH (between 3.4 and 3.8), H_2O_2 produced by the enzyme glucose oxidase, and the presence of phytochemicals[3,5,6,26]. When honey is applied to a burn or wound, corporal fluids dilute it. The antimicrobial action, firstly due to osmolarity and pH, is then enhanced by the action of glucose oxidase and phytochemicals that disseminate in the tissues[3,5,6]. Honeys that present a higher antimicrobial activity mostly owe this quality to the efficiency of glucose oxidase and the presence of phytochemicals, which are strongly related to the botanical origin of the honey. It is this fraction, mainly composed of organic acids and phenolic compounds, to which the effectiveness of honey when used as a medicine is attributed[3,5,6,27,30,31,32,33].

A limiting factor for the use of honey in topical treatments is the possible presence of *Clostridium botulinum* spores. The absence of this microorganism must be stated a priori, since its spores could find in the affected tissues a proper environment for their reproduction and could eventually liberate their toxin, complicating the infection and therefore the healing process[5,6,29,34,35].

The best example of a medical grade honey is given by the Manuka honey from Australia and New Zealand[3,36]. *Apis mellifera* bees produce this honey from the nectar foraged from the Manuka tree (*Leptospermum scoparium*) [37,38]. This honey provides a strong antimicrobial activity and has proven to be clinically effective in the healing of burns and wounds infected with microorganisms resistant to conventional antibiotic therapy[3,6,27,33,39]. In 2008, Medihoney™ (Derma Sciences, Inc.), a Manuka honey-based wound dressing set a milestone in the history of honey and healing; it received the United States Federal Drug Administration approval for its marketing and application as a dressing for burns and wounds[40]. In addition, Medihoney™ is considered to date the only antioxidant therapy that has been specifically approved for wound healing[41].

The highly regarded stingless bee honeys of Mesoamerica

The Mesoamerican region is the habitat for native stingless bees (family Apidae, tribe Meliponini), acknowledged as indispensable pollinators with a key role in the tropical forest conservation[42,43,44,45,46,47]. Among them, the most commonly domesticated species are *Melipona beecheii* and *Tetragonisca angustula* (see figure 1).

Figure 1. *Tetragonisca angustula* in flight (left) and *Melipona beecheii* guarding the entrance to the hive (right). Photos courtesy of Christian Reichle.

The ancient Mayan culture started the keeping of stingless bees (meliponiculture) and used their honeys for nutritional, religious and medical purposes[2,48,49,50,51,52,53]. The impact of these insects in the Mayan society is clearly acknowledged in the ancient manuscripts that detail their rituals, astronomic events and prophecies: the Maya codices[48,54]. At present, the use of Meliponini honey for the treatment of infected wounds, digestive disorders, respiratory tract infections, and eye problems like cataracts and conjunctivitis is still common practice in Mesoamerica[2,6,28,48,50,51,52,53].

Meliponiculture in Costa Rica is practiced mostly in the rural areas along the Pacific coast[55,56]. *Melipona beecheii* and *Tetragonisca angustula* are the stingless bee species of most commercial interest. Their honey is their main commercialized product [28,49,55]. Costa Rican Meliponini honey is sold at a substantially higher price than the *Apis mellifera* honey. This is due to the healing properties attributed to stingless bee honeys by traditional medicine[28,48,49,56,57]. Costa Rican ethnopharmacology describes the use of Meliponini honey as a dressing for wounds and burns, and a treatment for eye illnesses like pterygium, cataracts, and conjunctivitis[28,49,55,56,58]. Stingless bee honeys are locally considered to have stronger healing effects than the honeys from *Apis mellifera* of the same regions[28,48,49,57,59]. However, there are no studies that evaluate the real potentials and risks of using Meliponini honey as a dressing for the treatment of infected burns and wounds.

Aims and outline

Given the growing problem of antimicrobial resistance, it is of vital importance to find new topical treatments for wound healing. This thesis comprises a series of investigations that evaluate the medicinal properties of Costa Rican stingless bee honeys; focused on their potentials as a burn and wound dressing agent.

The first step taken was a screening and comparison of the antimicrobial activity of Meliponini honeys versus Costa Rican *Apis mellifera* honeys (Chapter 2).

Chapter 3 presents a microbiological safety analysis as a means to evaluate the health risks associated to the application of stingless bee honeys as a wound dressing. Following, this study reports an antimicrobial activity comparison between Meliponini honeys and Medihoney™ against a range of microorganisms that are relevant to the wound healing practice. Finally, the botanical origin of the stingless bee honeys under study is reported.

The basis of antioxidant therapy for wound healing is the interference with the signal transduction pathways of inflammation. A milestone that can be achieved by the administration of a dressing agent with an antioxidant capacity that scavenges reactive oxygen species, restores redox homeostasis by antagonizing oxidative stress, and consequently enhances wound healing[60,61,62]. Chapter 4 discloses the antioxidant capacity and immunomodulatory activities of Costa Rican stingless bee honeys.

Infections with antibiotic-resistant strains represent a major challenge to wound healing therapy[63,64]. Chapter 5 reports the results for minimum inhibitory concentrations assays performed with stingless bee honeys against clinical isolates of antibiotic-resistant bacteria.

Biofilms constitute a major obstacle for wound healing[65,66,67,68]. Biofilms are nowadays considered one of the main reasons why antimicrobial resistance is present in the clinical setting[69,70]. Chapter 6 details a screening for antibiofilm effects in Costa Rican Meliponini honeys, the discovery of antibiofilm activity, and the subsequent activity guided isolation of two antibiofilm constituents.

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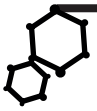
Chapter 2



Costa Rican stingless bee honey: ethnopharmacology and antibacterial effect

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Abstract

An evaluation of the antimicrobial activity of honey samples of *Tetragonisca angustula*, *Melipona beecheii* and *Apis mellifera* was performed in order to determine if the traditional value given to stingless bee honey from Costa Rica over *A. mellifera* honey is valid. The stingless bee honeys were more active than *A. mellifera* samples against *Staphylococcus aureus* and *Pseudomonas aeruginosa*; currently the most relevant causes of burn and wound infections. The results presented herein are the first steps towards further studies on the nature of the antimicrobial activity exerted by Costa Rican Meliponini honeys.

Introduction

Honey is the natural sweet substance produced by honey bees from the nectar of flowers or extrafloral nectaries, or from excretions of plant sucking insects, which the bees collect and transform by adding specific substances of their own, dehydrate and store in the honey comb to ripen and mature[1]. Many studies have proven that *Apis mellifera* honeys possess antimicrobial properties and its employment in dressings favors the healing of wounds and burns[2,3,4,5]. Nevertheless, stingless bee honeys are locally considered to have stronger healing effects than the honey from *Apis mellifera* of the same regions[6,7,8,9].

The Mesoamerican region is the habitat of native stingless bees (Meliponinae), acknowledged as indispensable pollinators with a key role in the tropical forest conservation[10,11,12,13,14]. Among them, the most commonly domesticated species are *Melipona beecheii* and *Tetragonisca angustula*. The Mayan and Aztec cultures started the keeping of these bees and used their honeys for medicinal purposes[6,15]. At present, the use of Meliponini honey for treatment of infected wounds, digestive disorders, respiratory tract infections and eye problems like cataracts and conjunctivitis is widespread[15,16,17]. However, there are no studies that evaluate the medicinal properties of honey from stingless bee species proceeding from Costa Rica.

The traditional use of honey produced by stingless bees as a medicine is part of Costa Rica's ethnopharmacology. These natural product remains as a traditional medicine since pre-Columbian times. At present, it is still highly regarded as a burn and wound dressing and a topical treatment for cataracts and conjunctivitis[6,7,18].

Honey produced by the stingless bee species *T. angustula* and *M. beecheii* have received the most commercial interest in Costa Rica. It is common to find Meliponini honey bottled in small dropper containers in local natural medicine stores, sold at a substantially higher price than *A. mellifera* honey[19,20]. Stingless bee honeys in Costa Rica have the folk medicine granted feature of having better medicinal properties as a burn and wound dressing than *A. mellifera* honey[21,22].

The ideal antimicrobial topical agent, whose final goal is being the active constituent of a burn and wound dressing should render inhibitory activity against common agents of infection among other qualities[23]. In order to determine if the traditional value given to Meliponini honey from Costa Rica over *A. mellifera* honey is valid, an evaluation over the antimicrobial activity of honey samples of *T. angustula*, *M. beecheii*, and *A. mellifera* was performed.

Due to the growing problem of antimicrobial resistance, it is of vital importance to count with an innovative topical treatment for infected burns and wounds. This chapter provides updated information on progress that has been made in Costa Rica on the antibacterial activity of the honey produced by several of our stingless bee species and new data on *Melipona beecheii* and *Tetragonisca angustula* honeys compared to Costa Rican *Apis mellifera* honey.

Materials and methods

Honey collection

A total of 56 honey samples (500 g to 1 kg) proceeding from Costa Rica and collected by *A. mellifera* (N = 34), *T. angustula* (N = 14) and *M. beecheii* (N = 8) were directly obtained from producers. The honeys under study belonged to geographical locations were the keeping of the mentioned bees is practiced (see Table 1). All samples were kept in storage at 23 °C, in a cool and dry place, away from light.

Table 1. Geographical origin of Costa Rican honey samples

Region	Bee species		
	A. mellifera	*T. angustula*	*M. beecheii*
Central Valley	8	7	1
Mountain South	12	-	-
Central Pacific	2	-	-
North Pacific	12	3	7
South Pacific	-	4	-
Total	34	14	8

Evaluation of antibacterial activity

Honey solutions with final concentrations of 75 %, 50 %, 25 % and 12.5 % (w / v) were prepared in sterile peptone water 0.1 %, pH 7.2. These solutions and pure honey (100 %) as well were subjected to an antibacterial activity test following a Mueller-Hinton agar well diffusion assay as described by Mitscher et al. (1972)[24]. A test solution was qualitatively considered antimicrobial if a clear zone without microbial growth was present surrounding the well after the incubation. The analysis was conducted three times for all honey samples against the following American Type Culture Collection (ATCC) strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 19166) and *Pseudomonas aeruginosa* (ATCC 9027). In addition, a clinical isolate of *Staphylococcus epidermidis* (UCR 2902) was included in the present trial.

Statistical analysis

All descriptive statistics and statistical inference analyses between results obtained per bee species were carried on with InfoStat Software (InfoStat Group, Universidad Nacional de Córdoba, Argentina).

Results

The results of the antimicrobial activity evaluation are presented in Table 2.

Table 2. Antibacterial activity of stingless bee honeys from Costa Rica. Results are expressed as percentage of honeys per bee species successful to inhibit bacterial growth at a given concentration.

	Honey concentrations grouped by bee species														
Bacterial strains	**100 %**			**75 %**			**50 %**			**25 %**			**12.5 %**		
	Am	**Ta**	**Mb**	**Am**	**Ta**	**Mb**	**Am**	**Ta**	**Mb**	**Am**	**Ta**	**Mb**	**Am**	**Ta**	**Mb**
Staphylococcus aureus	82	100	100	79	100	100	71	100	100	21	64	100	0	7	78
Staphylococcus epidermidis	85	100	100	76	100	100	38	93	100	6	21	78	0	0	0
Escherichia coli	97	100	100	85	86	89	74	7	67	3	0	0	0	0	0
Salmonella enteritidis	94	100	100	88	100	100	85	7	56	18	0	0	0	0	0
Listeria monocytogenes	79	100	100	47	100	89	9	50	67	3	0	22	0	0	0
Pseudomonas aeruginosa	9	93	100	0	86	100	0	21	78	0	0	33	0	0	0

Am: Apis mellifera, Ta: Tetragonisca angustula, Mb: Melipona beecheii

Discussion

A previous study performed by DeMera and Angert (2004)[21] compared the antimicrobial activity of honey produced by *T. angustula* and *A. mellifera* from Costa Rica. In their evaluation, *S. aureus* showed no susceptibility to any of the samples analyzed. In contrast, Estrada et al. (2005)[25] reported 80 % of *A. mellifera* honeys to be active against *S. aureus.* By means of the same method, in our trial, all *T. angustula, M. beecheii* and 82 % of *A. mellifera* honeys exerted antibacterial activity against *S. aureus*. The present study shows no statistical differences ($p > 0.05$) with the results presented by Estrada et al. (2005)[25] for the inhibitory activity over *S. aureus* by *A. mellifera* honeys.

At a honey concentration of 25 %, the differences observed in the percentages of honeys able to inhibit *S. aureus* are statistically significant between *A. mellifera* and *T. angustula* honeys ($p < 0.05$) and highly significant in honeys collected by *A. mellifera* and *M. beecheii* ($p < 0.001$). Hence, at the given concentration, the stingless bee honey samples were more active against *S. aureus*. Moreover, at the lowest concentration tested, *M. beecheii* honeys were the most active ($p < 0.001$).

The results obtained for the *A. mellifera*, *T. angustula* and *M. beecheii* honeys that were inhibitory against *S. epidermidis* and *L. monocytogenes* at a concentration of 50 % were statistically significant ($p < 0.05$, $p < 0.001$ respectively). With regard to the results obtained by the 50 % honey solutions, *E. coli* and *S. enteritidis* were the only cases of the study were *A. mellifera* honeys were more active than *T. angustula* samples ($p < 0.001$). Nevertheless there was no statistical difference between the *A. mellifera* and *M. beecheii* data ($p > 0.05$).

Finally, the inhibitory effect of the honey under study towards *P. aeruginosa* unveils a statistically significant difference in the results. The samples collected by both stingless bee species are more active than *A. mellifera* honeys ($p < 0.001$, for 100 % and 75 % solutions).

Conclusions

The actual medical panorama reflects an increasing number of antibiotic resistant microorganisms causing resilient disease[3,26,27]. Considering this, innovative therapies towards wound healing are urgent[23].

The antibacterial effects presented herein are the first steps towards further studies on the nature of the medicinal activity exerted by Costa Rican Meliponini honeys. In general, these results exemplify the broad-spectrum antimicrobial activity of stingless bee honey from Costa Rica. Finally, it is worth to mention that the antibacterial activity towards *S. aureus* and *P. aeruginosa* was higher in *T. angustula* and *M. beecheii* honeys than in *A. mellifera* honey.

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Chapter 3

The antimicrobial activity and microbiological safety of stingless bee honeys from Costa Rica

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Abstract

Stingless bee honeys in Costa Rica possess etnopharmacological value mainly as wound dressings. A microbiological study using APHA methods reported that 83 % of the honeys analyzed had microbial counts that comply with the European Pharmacopoeia's acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use. All samples reported absence of *Clostridium botulinum* spores by PCR. Over 90 % of *Tetragonisca angustula* and *Melipona beecheii* honeys inhibited *Pseudomonas aeruginosa* and *Staphylococcus aureus* at minimum inhibitory concentrations lower than Medihoney™. Under the conditions tested, Medihoney™ was not active against *Candida albicans*, whereas 53 % of *T. angustula* honeys rendered inhibition. The melissopalynological analyses reported a homogeneous (monofloral) botanical composition for the Meliponini honeys, which emphasizes the contribution of nectar constituents to the antimicrobial activity and provides a foundation for the standardization of a desired inhibitory effect. The traditional use of Costa Rican stingless bee honey as a dressing for burns and wounds reveals the application of a proficient antiseptic agent with low health associated risks.

Introduction

Honey is a natural product that has more than 4000 years of pharmaceutical history[1,2,3,4]. Despite having a long established usage as a wound dressing, its application in medicine was progressively set-aside in mid 1900s due to the advent of antibiotic therapy[2,5].

During the last two decades, the antimicrobial resistance phenomenon has boosted research and development of alternative therapies for wound and burn healing[6,7,8,9]. These circumstances led to a resurgence of interest in the application of honey for medicinal purposes[4,10,11,12]. The best-known example of the rediscovery of honey as a wound dressing is provided by the studies of the honey produced by the honey bee (*Apis mellifera*) from the nectar foraged from the inflorescences of the manuka tree (*Leptospermum scoparium*) found in Australia and New Zealand[10,13,14,15,16].

The concept of "Active Manuka Honey" was developed as a means to standardize its antimicrobial activity. Manuka honey owes its effects to an unidentified constituent that allows honey to exert antimicrobial activity beyond the inhibitory action of osmolarity, pH, or hydrogen peroxide generated by the enzyme glucose oxidase present in *Apis mellifera* honey. A phenol calibration curve was used for the standardization of the UMF ("unique manuka factor")[16,17]. Recently, the identity of the UMF has been attributed to methylglyoxal[18,19,20,21,22]. However, this finding is still a point of discussion[23,24,25]. Active Manuka Honey has been subjected to clinical trials, were it has demonstrated its efficacy as a wound-dressing agent. In 2008 the Food and

Drug Administration (FDA) gave approval for marketing of Medihoney™ (Dermasciences); a manuka honey-based wound dressing[2,11,26,27].

Bees (Hymenoptera, Apidae) in the Meliponini tribe are commonly known as stingless bees. These social insects are found in the tropical regions of the world. In the neotropics, stingless bees have an important role as native pollinators responsible for the reproduction and conservation of indigenous flora[28,29,30,31].

The practice of keeping stingless bees (meliponiculture) was developed by the ancient Maya culture. Records of stingless beekeeping and its meaning to Maya society can be found in the Madrid Codex[32]. Meliponini bees constituted an important part of their cultural life. The Maya honoured the bee gods and considered the honey sacred. Honey was involved in their religious rituals, used as a sweetener and had medicinal applications[28,33,34,35].

Meliponiculture nowadays is a practice present in all tropical America. Stingless bee honey is still kept in high regard. The use of these honeys in traditional medicine as a treatment of infected wounds, burns, digestive disorders, respiratory tract infections and eye illnesses like cataracts and conjunctivitis is still common practice[28,31,33,34,35]. In Costa Rica, *Melipona beecheii* and *Tetragonisca angustula* are the stingless bee species of most commercial interest due to the unique taste and medicinal value of their honey[28,31,34,36,37]. According to traditions, the application of Meliponini honeys in wound healing is feasible. Nevertheless, there are few studies that report the antimicrobial activity of these products[38,39,40,41,42,43,44,45] and none that evaluate their microbial content and therefore, the health risk associated to the practice of using stingless bee honey as a wound dressing agent.

To our knowledge, this paper is the first report on microbiological safety and antimicrobial activity in comparison to Medihoney™ and the botanical origin of *Tetragonisca angustula*, *Melipona beecheii*, *Melipona costaricensis*, *Scaptotrigona pectoralis*, *Cephalotrigona capitata* and *Tetragona perangulata* honeys from Costa Rica.

Materials and Methods

Sample collection

A total of 65 honeys were directly bought from keepers of Meliponini bees. The samples were collected during the harvest season (April, 2008), a period of approximately two to three weeks right after Easter holidays. Every sample consisted of 500 g to 1 kg of honey. The association of each sample to a particular bee species was verified. When necessary, the species origin was confirmed by comparison of bee specimens collected from the hives with Ayala´s taxonomic classification key[46].

The honeys were harvested from hives of the following Meliponini species: *Tetragonisca angustula* (n = 36), *Melipona beecheii* (n = 21), *Melipona costaricensis* (n = 4), *Scaptotrigona pectoralis* (n = 2), *Cephalotrigona capitata* (n = 1) and *Tetragona perangulata* (n = 1). The sample collection was in areas where meliponiculture is traditionally practiced[28,34,37].

Commercially available Medihoney™ hydrocolloid wound paste (Dermasciences) was used as a reference for antimicrobial activity.

Microbial content

The total aerobic count, yeast and molds count and most probable number (MPN) of total coliforms, faecal coliforms (*Escherichia coli* as indicator) followed American Public Health Association (APHA) methods in accordance with Pouch-Downes and Ito (2001)[47]. The microbial counts are reported through descriptive statistics.

Determination of *Clostridium botulinum* by polymerase chain reaction (PCR)

The evaluation of the presence of *C. botulinum* types A, B, E and F was performed following the method developed by Lindstrom et al. (2001)[48], as described by Fournier et al. (2006)[49].

Water content and total solids percentage

The water content of the reference and samples was performed by refractive index determination in an ABBE-3L refractometer (Milton Roy Company, U.S.A) and expressed as percentage of humidity following the Harmonized Methods of the European Honey Commission as described by Bogdanov et al. (2002)[50].

Total solids percentage was calculated as the subtraction of the percentage of water content to the total composition of honey (100 %). This value was used in the MIC test interpretation as a correction factor due to the differences in water content that honeys presented.

Density

The density of honey was calculated by means of specific gravity calculations at 25.0 +/- 0.1 °C. This physical parameter was determined with an 11.00 mL picnometer (Cole-Parmer, U.S.A.). In brief, the picnometer is filled with distilled water, the temperature of the device brought to 25.0 +/- 0.1 °C in a water bath and its weight determined in an analytical balance (precision +/- 0.0001 g). The device was cleaned and dried, then filled with honey and its weight was measured under the conditions previously described.

The density was calculated at 25.0 +/- 0.1 °C according to the following equation:

δ honey = (mass of honey / mass of water) x δ H_20

Where δ honey is the density of honey and δ H_20 is the density of water at 25.0 +/- 0.1 °C (0.997 g / cm^3).

Antimicrobial activity
The evaluation of the antimicrobial activity of the samples and Medihoney™ was developed in a 96-well microtiter plate-based minimum inhibitory concentration (MIC) assay.

The tests were performed against American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19116), *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 9027), a clinical isolate of *Staphylococcus epidermidis* (UCR 2902) part of the collection of the Faculty of Microbiology of the Universidad de Costa Rica and *Candida albicans* (ATCC 10231).

Every test consisted of eleven serial dilutions (1:1) in Mueller-Hinton Broth (MHB) (Oxoid, U.K.) of a 500 mg / mL honey test solution in duplicate against the culture tested. Under the template proposed, four tests can be performed per microplate. Three replicates were done for every sample and reference. The final volume contained in every well of the plate was 200 µL. Aseptic technique was maintained during all steps of the assay. The MIC test was carried out in a biosafety level II laminar airflow chamber (Labconco, U.S.A.).

Sample preparation
An aliquot of 0.900 g to 1.000 g of the sample or Medihoney™ was weighed on an analytical balance (Ohaus, U.S.A.) and added to a 1.5 mL microcentrifuge tube. A start solution of 1000 mg / mL of honey in MHB was prepared for each test. The latter took in regard the individual density of each sample and reference. Finally, a test solution with a concentration of 500 mg / mL in MHB and a volume of 1 mL was prepared out of every start solution. Both preparations were fully dissolved using a vortex mixer (Cole-Parmer, U.S.A.).

Culture solution
A fresh culture of the microorganism to test was previously prepared in Brain Hearth Infusion Agar (BHIA) (Difco, U.S.A.) and subjected to incubation for 24 h at 35 °C (48 h at 35 °C for *C. albicans*). Colonies were withdrawn of the culture and dissolved in a tube that contained 5 mL of sterile peptone water (peptone, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, pH 7.2 +/- 0.2). The turbidity of this solution was calibrated to a concentration of 1.0×10^8 cfu / mL with a 0.5 Mac Farland nephelometric standard and later an aliquot of 150 µL was dissolved in 14850 µL of sterile MHB. The final concentration in the micro plate was 5.0×10^5 cfu / mL.

MIC test preparation
Once the dilutions of the samples or Medihoney™ were prepared, 100 µL of the culture solution were dispensed in all the wells of the microplate with the exception of the negative control. The negative control of the test consisted of four wells containing 200 µL of MHB only (0 % growth). Another four wells containing 100 µL of MHB and 100 µL of the culture solution performed as the positive control (100 % growth).

After the plates were prepared, the lid and bottom borders of the plates were sealed with parafilm (Pechiney, U.S.A.) to prevent evaporation during the incubation period (24 h at 35 °C for bacteria, 48 h at 35 °C for *C. albicans*) in a gravimetric airflow incubator (Digisystem Laboratory Instruments Inc., Taiwan).

MIC test interpretation
After incubation, the test plates were opened in the laminar airflow chamber and visually inspected over a dark background. Microbial growth inhibition was evidenced as absence of turbidity as established by the negative control. The MIC was determined as the lowest concentration that inhibited the growth of the culture being tested. As a confirmatory phase, a loopful of the MIC value well of every sample was seeded in a BHIA plate. Absence of growth after incubation for 24 h (48 h for *C. albicans*) at 35 °C was considered confirmation of results.

The minimum inhibitory concentration results presented herein are expressed in milligrams of total solids of honey per milliliter of solution. The MIC of every sample was multiplied by its percentage of total solids. The values obtained were subjected to this correction in order to achieve a quantitative result that allows a direct comparison between the antimicrobial activity of stingless bee honeys and Medihoney™ despite the differences of water content and density.

Botanical origin

The botanical origin of the stingless bee honey samples was determined through a melissopalynological analysis. Pollen grains were concentrated from samples, mounted on microscope slides and quantified (between 200 to 300 grains). Relative amounts were expressed in percentages, and classified according to frequency in the following classes: predominant pollen (> 45 %), secondary pollen (16 %-45 %), minor secondary pollen (3-15 %) and minor pollen (< 3 %) as described by Hodges (1984)[54].

The pollen grains were taxonomically identified to species level by means of capturing digital images from the slides and comparisons to pollen grain identification keys[51,52,53].

Statistical analyses

Normal distribution evaluation, statistical inference and all descriptive statistics were executed with the InfoStat software (InfoStat Group, Universidad Nacional de Córdoba, Argentina).

Results

All Meliponini samples presented a low total aerobic count (< 1.0×10^1 cfu / g; EST of honey), absence of total coliforms, faecal coliforms and *E. coli* (< 3 MPN / g of honey) in 25 grams. Concerning the total yeast and molds counts, 83 % of samples reported a count lower than 1.0×10^1 cfu / g of honey. The remaining 17 % reported count values in the range of 9.0×10^2 to 9.0×10^6 cfu / g.

The *C. botulinum* types A, B, E and F used as references produced by PCR the expected amplification products. On the contrary, none of the samples gave positive results for any serotype under examination.

The density, water content and MIC results of the *T. angustula* and *M. beecheii* samples were analyzed through a normality test. A modified Shapiro-Wilks test was performed for each parameter. The *M. costaricensis*, *S. pectoralis*, *C. capitata* and *T. perangulata* honeys were scarce in number, therefore not considered for this test.

The density of *M. beecheii* honeys ($p = 0.8684$) and the humidity of *T. angustula* samples ($p = 0.1669$) were normally distributed. The remaining parameters, including the MIC results did not follow a normal distribution ($p < 0.0001$). These conditions exclude the possibility of an analysis of variance (ANOVA) and consequently all the mentioned results were evaluated through descriptive statistics. The water content and density results are described in table 1. The mean water content of all Meliponini honeys (overall) was 25 % higher than the value obtained for Medihoney™. Likewise, the mean value reported per bee species was higher.

Table 1. Water content and density of stingless bee honeys and Medihoney™. Data are expressed as mean values and range per bee species.

Bee species	Water content (%) +/- 0.1	Density (g / cm³) +/- 0.001
***Apis mellifera* (Medihoney™)**	18.5	1.416
Overall Stingless bee honeys	23.2 (19.8-31.8)	1.378 (1.310-1.397)
Tetragonisca angustula	22.1 (19.8-24.8)	1.381 (1.334-1.397)
Melipona beecheii	23.6 (21.6-25.4)	1.380 (1.363-1.394)
Melipona costaricensis	26.7 (25.2-27.8)	1.357 (1.352-1.369)
Scaptotrigona pectoralis	25.1 (24.2-26.0)	1.373 (1.367-1.378)
Cephalotrigona capitata	27.4	1.359
Tetragona perangulata	31.8	1.310

A correlation analysis was performed between the density and the water content parameters of *T. angustula* and *M. beecheii* honeys. The statistical inference returned a Pearson coefficient of -0.62 for *T. angustula* and -0.84 for *M. beecheii* honeys respectively. Both are negative correlations.

All the Meliponini honeys under scope presented inhibitory activity at least against one of the microorganisms tested and 100 % accomplished an MIC lower than the reference against one of the type culture strains assayed as a minimum. The results comprised in table 2 and table 3 disclose the broad-spectrum antimicrobial activity of the Meliponini honey under trial against culture strains of medical importance.

Table 2. Percentage of *T. angustula*, *M. beecheii*, and overall stingless bee honeys that obtained a MIC under the conditions tested, and percentage of samples that achieved a MIC lower than Medihoney™ (< MDHY).

Microorganisms tested	Stingless bee species				Overall stingless bee honeys (n = 65)	
	Tetragonisca angustula (n = 36)		*Melipona beecheii* (n = 21)			
	Total (%)	< MDHY (%)	Total (%)	< MDHY (%)	Total (%)	< MDHY (%)
S. aureus	94	92	95	90	95	91
S. epidermidis	94	92	100	95	97	91
L. monocytogenes	78	78	100	100	88	88
E. coli	83	47	100	57	91	52
S. enteritidis	50	50	100	100	71	71
P. aeruginosa	97	97	100	100	98	98
C. albicans	53	53	5	5	33	33

Table 3. Minimum inhibitory concentrations of honeys against the microorganisms tested. Data are expressed as median values and range per bee species (mg / mL) and for stingless bees as a group (overall).

S.B.	Microorganisms tested						
	S. aureus	*S. epidermidis*	*L. monocytogenes*	*E. coli*	*S. enteritidis*	*P. aeruginosa*	*C. albicans*
T. a	49.2 (23.5-195.5)	96.6 (23.5-200.5)	97.3 (47.0-200.5)	99.0 (47.0-199.3)	196.0 (47.4-200.5)	97.8 (47.3-200.5)	195.0 (189.5-200.5)
M. b	24.5 (23.4-192.0)	24.2 (11.8-191.5)	93.8 (23.4-98.0)	97.5 (93.3-196.0)	188.5 (46.9-196.0)	48.6 (46.8-194.5)	188.5*
M. c	103.8 (45.1-187.0)	126.5 (45.1-187.0)	126.5 (45.1-187.0)	160.3 (92.3-187.0)	123.2 (90.3-187.0)	137.8 (90.3-187.0)	>250 mg/ml
S. p	23.4 (23.1-23.7)	17.5 (11.8-23.1)	23.4 (23.1-23.7)	70.6 (46.3-94.8)	46.9 (46.3-47.4)	46.9 (46.3-47.4)	185*
C.c	45.4*	45.4*	45.4*	45.4*	45.4*	45.4*	>250 mg/ml
T. p	21.3*	10.6*	10.6*	42.6*	21.3*	42.6*	170.5*
Overall	48.4 (21.3-195.5)	48.6 (10.6-200.5)	94.4 (10.6-200.5)	98.3 (42.6-199.3)	188.5 (21.3-200.5)	95.8 (42.6-200.5)	194.5 (170.5-200.5)
MDHY	101.9	101.9	203.8	101.9	203.8	203.8	>250 mg/ml

*S B = stingless bee species, T. a = Tetragonisca angustula, M. b = Melipona beecheii, M. c = Melipona costaricensis, S. p = Scaptotrigona pectoralis, C. c = Cephalotrigona capitata, T. p = Tetragona perangulata, MDHY = Medihoney™: reference, >250 mg/mL = no inhibition under the conditions tested, * = only one sample caused inhibition*

In general, a honey is considered produced mainly from one floral resource (monofloral) if the pollen of the plant is predominant[54,55]. All the Meliponini honeys analyzed posses a predominant nectar source (pollen frequency > 45 %). The predominant botanical source for the *T. perangulata* sample corresponded to *Brosimum alicastrum* (pollen frequency 63 %). The *M. costaricensis*, *S. pectoralis* and *C. capitata* honeys shared the same major nectar resource: *Miconia argentea* (pollen frequency range 67 % to 79 %). Ten floral sources constituted the main nectar origin of the *T. angustula* and *M. beecheii* samples (pollen frequency range 45 % to 94 %) (see figure 1).

The major botanical resource of 52 % of the *T. angustula* honeys is concentrated in *Spondias purpurea* and *Gliricidia sepium* flora. *M. beecheii* samples presented as predominant nectar source *Tabebuia ochracea* and *Andira inermis* species in 52 % of the analyzed cases.

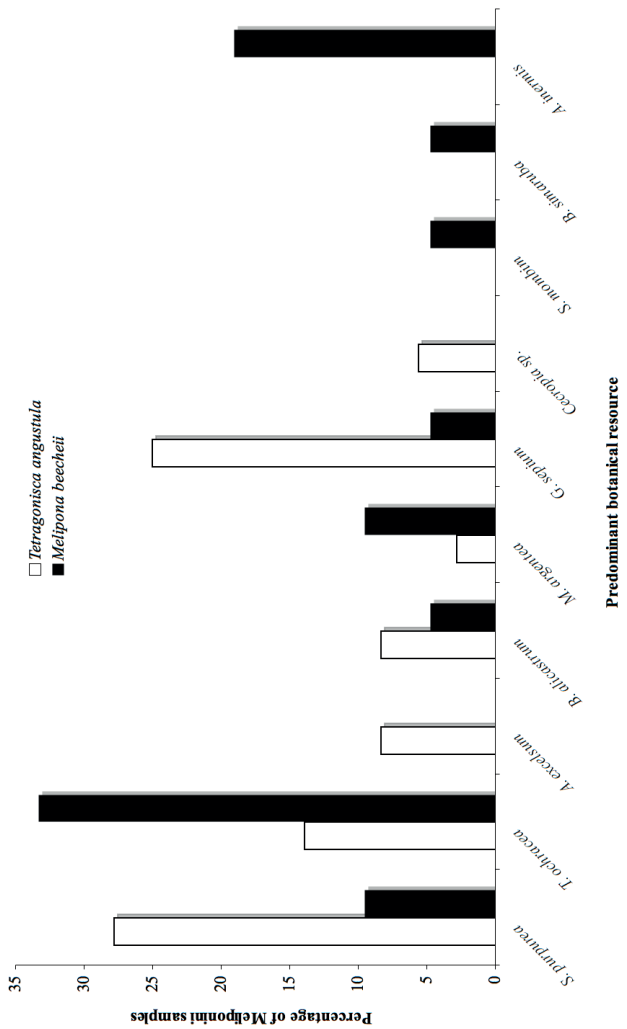

Figure 1. Predominant nectar resources of *M. beecheii* and *T. angustula* samples.
S. purpurea = Spondias purpurea, T. ochracea = Tabebuia ochracea, A. excelsum = Anacardium excelsum,
B. alicastrum = Brosimum alicastrum, M. argentea = Miconia argentea, G. sepium = Gliricidia sepium,
S. mombim = Spondias mombim, B. simaruba = Bursera simaruba, A. inermis = Andria inermis

Discussion

The harvest of stingless bee honey is still rudimentary[28,31]. Although all the samples were collected in meliponaries that use wooden boxes for housing the bee colonies, the lack of innovative tools for honey extraction means a slow and manual process outdoors or inside the keepers' house. With the aid of suction devices, honey is deposited in pitchers or jars during harvest and later dispensed in bottles or small dropper containers for commerce. The absence of adequate extraction facilities and tools could lead to spoilage of honey due to pathogen vectors like flies or excessive manipulation. Therefore, the microbiological quality and safety of the product was analyzed.

It can be hypothesized that the absence of *C. botulinum* spores reflects the scarcity of these sporulated anaerobe in the neotropical environment where the indigenous bees collected the nectar to produce honey. The biodiversity present in Mesoamerica and in particular in Costa Rica, might create a highly competitive microbial niche that in addition to the presence of antimicrobial agents in honey, do not offer suitable conditions for *C. botulinum* survival.

At present there are no microbiological quality standards specified for stingless bee honeys. Nevertheless, as stressed by the Codex Alimentarius Commission, the absence of food borne pathogens is mandatory[56,57]. In addition, for wound healing applications the product must be free of viable *C. botulinum* spores[11,58,59,60]. Our results show that 83 % of the honeys analyzed had microbial counts that comply with the European Pharmacopoeia's acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use[61]. The absence of *C. botulinum* spores suggests that the prevalence of this microorganism in Meliponini honeys proceeding from Costa Rica is low. Both conditions indicate that the health risk associated with the practice of using Costa Rican stingless bee honey as a wound-dressing agent is low.

Several authors have reported the high water content of stingless bee honeys compared to that of *A. mellifera*[38,62,63]. In addition, the percentage of humidity of Meliponini honey can vary according to the species and the geographical area where it is produced[35,64].

The statistical inference performed between the density and the water content parameters returned negative correlations, thus the density value decreases along with an increase in the percentage of humidity. The latter justifies the determination of density and water content parameters of every honey sample and their suggested role in achieving quantitative and comparable minimum inhibitory concentrations.

Burn and wound patients are high-risk groups for infection. Strains of *S. aureus*, *P. aeruginosa* and *C. albicans* are the most common agents that severely undermine the healing process[8,65]. In addition, infected burns and wounds can play a role as reservoir and source of dissemination of antibiotic resistant microorganisms[8,15,66].

The broad-spectrum antimicrobial activity[10,13,67], clinical efficacy[27] and subsequent Federal Drug Administration (FDA) approval of the use of Medihoney™ as a dressing[11,26] set a milestone in burn and wound healing therapy. To our knowledge, this is the first report of the antimicrobial activity of stingless bee honeys directly compared to Medihoney™ by means of a minimum inhibitory concentration assay.

The commerce of stingless bee honey in Costa Rica has a blooming market. This trade is mainly sustained by *T. angustula* and *M. beecheii* honeys owing to their use as a traditional medicine[28,34]. A high percentage of the samples produced by these two bee species inhibited *S. aureus* and *P. aeruginosa*; the two major causes of morbidity and mortality due to burn and wound infection[8] at MICs lower than Medihoney™. Under the conditions tested, the reference was not active against *C. albicans*; the dominant cause of opportunistic mycoses[68,69], whereas 19 *T. angustula* honeys (n = 36) rendered inhibition. *Candida* infections in burn and wound patients represent a major risk for invasive candidiasis[66,68,70]. Therefore, in accordance with the results, *T. angustula* honey from Costa Rica represents a suitable subject for further research oriented towards the development of a burn and wound dressing comprising selected broad-spectrum antibacterial and anti-*Candida* honey.

The microbiological safety and inhibitory activity of the stingless bee honey samples reported here, justify their traditional use as a dressing for infected burns and wounds. Furthermore, the determination of the antimicrobial activity is the cornerstone of upcoming research and development[71].

There are only three previous reports of the inhibitory activity of Meliponini honey from Costa Rica. De Bruijn and Sommeijer (1997)[38] analyzed through an agar diffusion assay the antimicrobial activity of stingless bee honeys against strains of *S. aureus*, *Bacillus cereus*, *E. coli* and *P. aeruginosa*. The honeys under trial included an undisclosed number of *M. beecheii* honeys from Costa Rica. The authors solely reported that the growth of most bacteria was inhibited. De Mera and Angert (2004)[47] by a method similar to the described by de Bruijn and Sommeijer (1997)[38] reported inhibition of *P. aeruginosa*, *B. cereus*, *Candida albicans* and *Saccharomyces cerevisiae* by *A. mellifera* and *T. angustula* honeys from Costa Rica. None of the samples under study inhibited *S. aureus*. Contrary to this report, Estrada et al. (2005)[72] determined that 80 % of the *A. mellifera* samples studied caused inhibition of *S. aureus*. In addition, 94 % of the *T. angustula* samples, subject of the

present trial, had antimicrobial activity against the mentioned gram-positive cocci. The inhibitory effects of honey from the two mentioned bee species against *S. aureus* was also reported for samples derived from Brazil[42]. Up to the present, the most recent studies of antimicrobial activity of honey produced by Mesoamerican stingless bees are the manuscripts published by Dardón et al. (2013)[40] and Zamora et al. (2013)[45]. Dardón et al. (2013)[40] reported the inhibitory activity of honey produced by eight bee species from Guatemala against eight pathogen microorganisms. Zamora et al. (2013)[45] compared the antimicrobial activity of *Apis mellifera* honey and stingless bee honey from Costa Rica. The authors reported a higher biological activity in the honey produced by Meliponini bees.

Temaru et al. (2007)[44], Dardón and Enríquez (2008)[39], Sgariglia et al. (2010)[43] and recently Zamora et al. (2013)[45] reported inhibition by *T. angustula* and *M. beecheii* honeys against the same type culture strains of *S. aureus*, *E. coli* and *P. aeruginosa* subject of the present study. Unfortunately, with the exception of Miorin et al. (2003)[42] and Sgariglia et al. (2010)[43], all the studies mentioned performed antimicrobial activity determinations by means of agar diffusion. These methods are low in sensitivity, do not determine MIC's and since non-polar constituents may not diffuse through agar, do not represent the total antimicrobial activity of honey[73]. Moreover, none took into account the differences of water content and the density of the samples and also different methodologies were used. Additionally, they did not use a proper reference (medical grade honey) or determined up to species level the botanical origin of the honey studied. Under these conditions, a proper results comparison between studies is not possible. We suggest the minimum inhibitory concentration assay described herein as a standard to achieve comparable results between Meliponini and *Apis mellifera* honeys. A quantitative measure of the antimicrobial activity is fundamental in setting quality parameters for future research and wound dressing developments.

The proposed method rules out the osmotic effect of honey. As a matter of example, the first concentration in the serial dilution of the MIC test is of 250 mg / mL of honey (volume 200 µL). In the case of the Medihoney™ used as a reference (density 1.416 g / cm^3) this would represent a solution of 18 % (v / v). As reported by Cooper et al. (1999)[67] a 22 % (v / v) sugar solution is the minimum concentration capable to inhibit microbial growth due to an osmotic shock. Consequently, the antimicrobial activity reported for the reference and the stingless bee honey samples under study cannot be explained by the extent of the microbial inhibition attributable to sugar content. Similar findings were reported for *Apis mellifera* honey by Molan (1992)[74] and Snow et al. (2004)[23].

Several authors have hypothesized on the nature of the antimicrobial activity caused by stingless bee honeys. Vit et al. (1994)[63] stated that the stability to fermentation despite high water content might be related to the inhibitory activity. Moreover, the mentioned effect was suggested to find a partial explanation through diffusion of secondary plant metabolites from the cerumen walls of the storage pots to the honey, symbiotic bacteria, enzymes and other non identified constituents that bees may add during the process of transforming nectar into honey[38,39,44,75].

Although the cited explanations may play a role in the nature of the antimicrobial activity of Meliponini honeys, all the mentioned articles on the subject share the same weak point: absence of a proper identification of the botanical origin of honey to species level; which at the same time disregards the contribution of nectar sources to the effect caused by stingless bee honeys. Only the melissopalynological examination can reveal the true nature of the bee forage in an area and its contribution to the honey[55].

The botanical resources influence the antimicrobial activity of honey[76,77,78]. Nectar is the prime material of honey production and its composition depends on the plant species. Among nectar constituents, secondary plant metabolites like flavonoids and terpenoids play important roles as signaling molecules for pollinators. These compounds control microbial contaminants due to their antimicrobial activity and are part of the metabolic offering for flower visitors[79]. The identification of the nectar resources foraged by stingless bees to produce honey is a key point towards antimicrobial activity standardization. The latter since a determined botanical origin may define the presence and concentration of specific plant derived antimicrobials.

The melissopalynology results reflect the optimum forage behavior pattern of stingless bees. A reduced number of botanical species constitute the bulk of the diet resources[80,81]. This behavior is strengthened by the massive blooming syndrome that numerous flora exhibit in the neotropics. An abundant supply of resources that are eagerly collected by bees and generate homogenous and convergent dieting strategies that focuses on more profitable sources in close proximity[82,83,84,85]. A defined and monofloral botanical composition, as the one reported herein, emphasizes the contribution of nectar constituents to the antimicrobial activity.

Conclusions

The stingless bee honeys studied are predominantly of high microbiological quality, are free of *C. botulinum* spores and exhibit antimicrobial efficacy comparable to Medihoney™. Hence, the traditional use of Costa Rican Meliponini honey as a dressing for burns and wounds reveals the application of a proficient antiseptic agent with low health associated risks.

Finally, the monofloral botanical composition reported herein provides a foundation for further research on the antimicrobial activity against antimicrobial-resistant clinical isolates, the standardization of a desired inhibitory effect and sets the cornerstone for upcoming studies on other beneficial bioactivities that stingless bee honeys could render to wound care.

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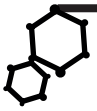
Chapter 4



The antioxidant capacity and immunomodulatory activity of stingless bee honeys proceeding from Costa Rica

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Abstract

Medihoney™ is considered currently the only antioxidant therapy specifically approved by the United States Food and Drug Administration for wound healing. This is the first study that evaluates the antioxidant capacity and immunomodulatory activity of stingless bee honeys in comparison to Medihoney™. The goal of this study was to evaluate in vitro the potential of Meliponini honey from Costa Rica as an antioxidant wound dressing agent. A total of 57 honeys belonging to the *Tetragonisca angustula* (n = 36) and *Melipona beecheii* (n = 21) species were studied. The following tests were carried out: screening of radical scavenging activity by the 2,2-diphenyl-1-picryl hydrazyl assay (DPPH), superoxide anion radical scavenging assay, inhibition of xanthine oxidase (XO), total phenolic (TP) content by the Folin-Ciocalteu reagent, and the oxygen radical absorbance capacity (ORAC) assay. Furthermore, we evaluated the effect of honey over reactive oxygen species (ROS) produced by human polymorphonuclear leucocytes (PMNL) using luminol and a cypridina luciferin analog as luminescence probes. The positive correlation between the TP content and the ORAC value reported herein, suggests that the antioxidant capacities of the stingless bee honeys examined are to a large extent attributed to their phenolic constituents. According to the present results, the in vitro immunomodulatory activities are explained mainly by the radical scavenging effect and the XO inhibition. Both mechanisms may play a role in the impairment of the inflammatory process and the promotion of redox homeostasis. The *T. angustula* honeys showed antioxidant capacities not statistically different from Medihoney™. In addition, their radical scavenging activity over ROS produced by human PMNLs and inhibition of XO justify their ethnopharmacological use as a wound dressing and further research towards novel wound-healing developments.

Introduction

The medical applications of *Apis mellifera* (honeybee) honey are ancient. The use of honey as a medicine is present in a myriad of cultures and this practice possesses over four millennia of pharmaceutical history[1]. The advent of the antibiotic era of the 20th century set the medical use of honey aside[2,3]. The subsequent onset of antimicrobial resistance led to the search of alternative treatments for wound healing[4,5,6], which promoted the resurge of honey as a medicine[7,8,9]. In recent years, honey-based wound dressings made a breakthrough as validated treatments for wounds and burns. In 2008, supported on its proven clinical efficacy[10], Medihoney™ (Derma Sciences) received the Food and Drug Administration (FDA) approval for its application as a wound dressing[11]. Medihoney™ is today a treatment for burns and wounds accepted by the medical community and considered up to now as the only antioxidant therapy that has been specifically approved for wound healing[12].

The bees (Hymenoptera, Apidae) of the Meliponini tribe are eusocial insects present in the tropical regions of America, Africa and Australia. Although taxonomically and morphologically diverse, all share the same feature: the absence of a functional sting; hence they are known as stingless bees[13]. The ancient Maya and Aztec Mesoamerican cultures were the first to practice the keeping of stingless bees or meliponiculture as it is commonly known. They considered the Meliponini bees sacred and used the stingless bee honey in their religious rituals and as a medicine[14,15]. Among the ancient medical applications of the Meliponini honey, its use as a wound dressing and as a treatment for cataracts and conjunctivitis are still common practice in tropical America today[16,17].

The stingless bee honey of Mesoamerica has mostly been the subject of study regarding its antimicrobial activity, physical and chemical quality in comparison to *A. mellifera* honey standards[17,18]. To our knowledge, this is the first study that evaluates the antioxidant capacity and immunomodulatory activity of stingless bee honeys related to their wound healing properties in comparison to Medihoney™. Our goal was to evaluate in vitro the potential of Meliponini honey from Costa Rica as an antioxidant wound dressing agent.

Materials and Methods

Sample collection

A total of 57 stingless bee honeys took part in the present study. The samples were bought directly from keepers. The hives were located in regions of Costa Rica where meliponiculture is practiced[19]. The same set of samples and reference were the subject of study of a previous investigation by Zamora et al (2014)[20]. The honeys under study belonged to the following stingless bee species: *Tetragonisca angustula* (n = 36) and *Melipona beecheii* (n = 21).

Medihoney™ hydrocolloid wound paste (Derma Sciences) was used as a reference for antioxidant capacity.

Reagents and consumables

All reagents were purchased from Sigma-Aldrich (U. S. A). Greiner Bio-One GmbH (Germany) provided the microplates. Sanquin Bloedbank Noordwest (The Netherlands) provided the human polymorphonuclear leukocytes (PMNL), which were isolated from blood donated by healthy volunteers. The blood was processed in accordance to local guidelines; every donor signed an informed consent stating that their blood could be used for more than patient care only.

Sample preparation
An aliquot of 0.900 g to 1.000 g of the Meliponini honey or Medihoney™ was weighed on an analytical balance (Ohaus, U.S.A.). A start solution of 1000 mg / mL of the honey or the reference was prepared for each analysis. The preparation of the solution took into consideration the individual density of the sample or the reference in order to achieve comparable results, as reported previously[20]. Every solution was prepared in the appropriate solvent for each test.

Screening of radical scavenging activity by the 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay
The radical scavenging activity of the stingless bee honeys and Medihoney™ over DPPH was executed by means of a microplate method based on the procedure described by Ancerewicz et al (1998)[21].

Test solutions of 500 mg/mL of the sample or the reference were prepared in 75 % ethanol. Three aliquots of 150 µL were serially diluted with 100 µL of 75 % ethanol in a flat-bottomed microplate. Later, 100 µL of a solution of 0.2 mg/mL of DPPH were added to two of the dilution series. 100 µL of 75 % ethanol were added to the third series, which was used as a blank. The final volume per well was 200 µL. The test included a calibration curve composed of three concentrations of DPPH: 0 % DPPH (200 µL of 75 % ethanol), 50 % DPPH (150 µL of 75 % ethanol and 50 µL of the DPPH solution), and 100 % DPPH (100 µL of 75% ethanol and 100 µL of the DPPH solution). Once the DPPH solution was added to the serial dilutions and calibration curve, the microplate assay was incubated for 15 minutes at room temperature (23 °C) before measuring absorbance at 550 nm in a MultiSkan Spectrum microplate reader with SkanIt® DDE software (Thermo Scientific, Finland). The scavenging activity was calculated as the concentration (mg / mL) capable of achieving a 50 % inhibition of DPPH (IC_{50}). Every sample and the reference were subject of three separate analyses.

Superoxide anion ($O_2^{\cdot -}$) radical scavenging assay
The scavenging activity of the test samples over the superoxide anion was determined in a cell-free system as described by Beukelman et al (2008)[22]. Every sample and the reference were subject of two separate analyses. The scavenging activity was calculated as the IC_{50}.

Inhibition of xanthine oxidase
The inhibitory effect of honeys on xanthine oxidase was analyzed by means of a kinetic microplate assay.

Three aliquots of 100 µL of honey test solutions of a concentration of 200 mg / mL in phosphate buffered saline (PBS, pH 7.4) were serially diluted in an ultraviolet (UV) transparent microplate with 50 µL of PBS. Subsequently, 50 µL of hypoxanthine (0.27 mg / mL in PBS) were added to all the dilution series.

Later, 50 µL of PBS and finally 50 µL of xanthine oxidase (2.5 mU / per well, in PBS) were added. The assay included a control per serial dilution. The control consisted of 100 µL of PBS, 50 µL of hypoxanthine and 50 µL of xanthine oxidase. The final volume per well was 200 µL. Immediately, uric acid production was monitored by a kinetic measurement of absorbance at 290 nm for 30 minutes at 23 °C (7 absorbance readings, with an interval of 5 minutes). The average kinetic rate (normal rate) of every well was used for calculating the inhibition of xanthine oxidase as an IC_{50}. The kinetic readings and the average kinetic rate calculations were performed in a MultiSkan Spectrum microplate reader with SkanIt® DDE software (Thermo Scientific, Finland). Every sample and the reference were subject of three separate analyses.

The effect of honeys over xanthine oxidase was analyzed as a verification of the results obtained in the superoxide radical scavenging assay. Superoxide is generated by means of the reaction of the enzyme xanthine oxidase on hypoxanthine. The goal of this test was to distinguish between superoxide radical scavenging and inhibition of xanthine oxidase.

Total phenolic content by the Folin-Ciocalteu reagent

The total phenolic content of the honeys in study was determined by a microplate method based on the publication by Singleton, Orthofer and Lamuela-Raventós (1999)[23].

Three aliquots of 320 µL of honey test solutions with a concentration of 20 mg / mL in demineralized water were serially diluted in a microplate with 160 µL of demineralized water. Two dilution series took part of the test reaction and one series was used as blank. For this purpose, 40 µL of demineralized water were added to the blank. The microplate assay included a calibration curve of eleven concentrations of gallic acid (0-12.5 µg / mL). 160 µL of every gallic acid solution were distributed in duplicate. 200 µL of demineralized water were dispensed in two wells as blank of the calibration curve. Subsequently, 10 µL of the Folin-Ciocalteu reagent were added to the test dilution series and the calibration curve. After 5 minutes, 30 µL of a solution of 200 mg / mL of Na_2CO_3 were added to the dilution series and calibration curve. The final volume per well was 200 µL. Later, the microplate was incubated for 2 hours at room temperature (23 °C) in a microplate shaker (Barnstead, Finland). Finally, the absorbance at 765 nm of the assay was measured in a MultiSkan Spectrum microplate reader with SkanIT® DDE software (Thermo Scientific, Finland). In the present investigation, the total phenolic content values are presented as micrograms of gallic acid equivalents (GAE) per milligram of honey (µg GAE / mg). Every sample and the reference were subject of three separate analyses.

Oxygen Radical Absorbance Capacity (ORAC) Assay
The ORAC values of the honeys in study were quantified using a microplate assay based on the method described by Huang, Ou and Prior (2005)[24].

Two aliquots of 50 µL of a honey test solution with a concentration of 10 mg / mL in 75 mM sodium phosphate buffer (PBS, pH 7.4), were serially diluted in a black microplate with 25 µL of PBS. The microplate assay included a calibration curve of six concentrations (6,25-50 µM) of 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (trolox). 25 µL of every solution of trolox were dispensed in triplicate. Subsequently, 150 µL of a 80 nM fluorescein sodium salt solution were added to the test dilution series and the calibration curve. The test included a blank composed of 50 µL of PBS and 150 µL of the fluorescein solution. Following, the assay was incubated for 10 minutes at 37 °C. Afterwards, 25 µL of a solution of 150 mM of 2,2-azobis (2-methylpropionamidine-dihydrochloride) (AAPH) in PBS were dispensed to every well with exception of the blank. The test included a control for the AAPH activity. This control consisted of 25 µL of PBS, 150 µL of the fluorescein solution and 25 µL of the AAPH solution. Right after the addition of the AAPH solution, fluorescence intensity was measured [485 nm (excitation) / 525 nm (emission)] at 37 °C for 60 minutes, with an interval between readings of 1 minute and under constant stirring in a Fluoroskan FL fluorometer with Ascent Software (Thermo Scientific, Finland). The ORAC values were calculated as µmol of trolox equivalents per 100 g of honey (µmol TE / 100 g). Every sample and the reference were subject of three separate analyses.

Effect over reactive oxygen species produced by human polymorphonuclear leucocytes
The radical scavenging activity of the test samples over human polymorphonuclear leukocytes (PMNLs) stimulated with opsonised zymosan was performed in microplate chemiluminescence assays. 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and 2-methyl-6-phenyl-3,7-dihydroimidazo [1, 2-a] pyrazin-3-one (cypridina luciferin analog) were used as luminescence probes. Every sample and the reference were subject of two separate analyses with each probe. This method was described previously[22].

Statistical analyses
All descriptive statistics, normal distribution evaluation, Pearson correlation tests, one sample sign non-parametric test on median values and multivariate analysis were performed using MiniTab® software (Minitab Inc., U. S. A.).

Results
The test results did not follow a normal distribution. We performed a multivariate analysis for the *M. beecheii* and *T. angustula* honeys taking in regard the results of the following parameters: ORAC value (ORAC), total phenolic content (TP), the effect over reactive oxygen species produced by stimulated polymorphonuclear leukocytes using luminol (PMNs-LUM) and cypridina luciferin analog (PMNs-CLA) as luminescence probes, 2,2-diphenyl-1-picryl hydrazyl and xanthine oxidase inhibition (DPPH and XO, respectively). See figure 1.

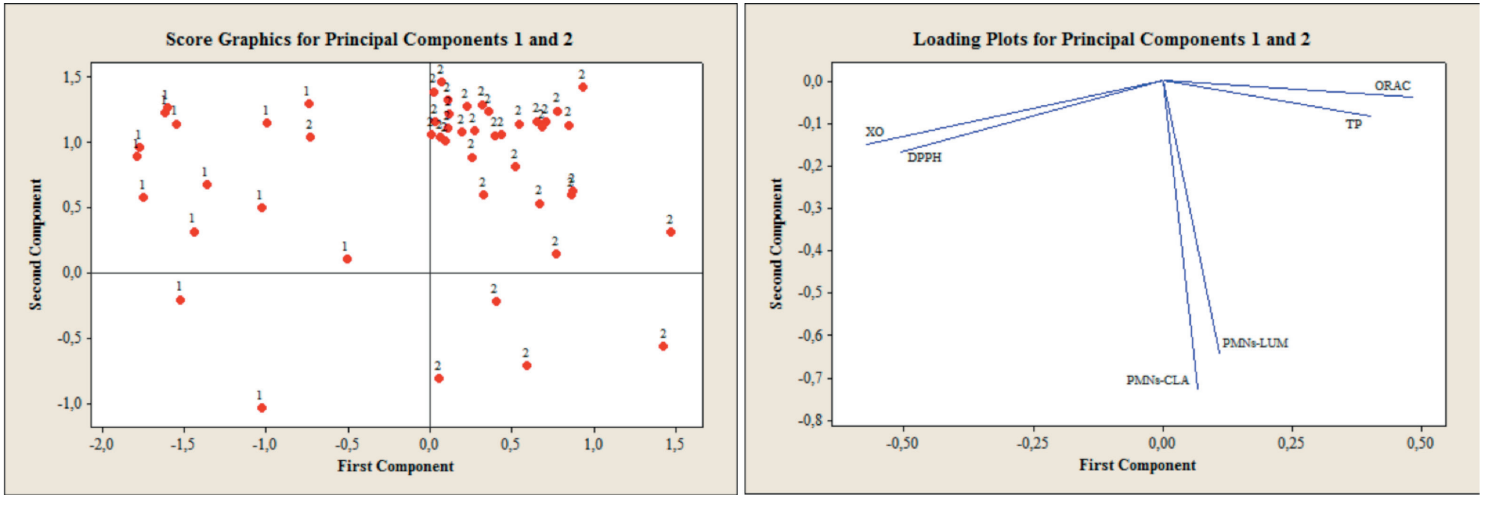

Figure 1. Principal components analysis for stingless bee honeys.
(1) = *M. beecheii*, (2) = *T. angustula*

The principal components analysis (PCA) unveiled that the first component was capable to separate 8,2 % of the Meliponini honeys according to species and explains 49,5 % of the variance between the data. In addition, both components together explain 74,4 % of the variance between the data. High TP content and high ORAC values characterize *T. angustula* samples. Likewise, high IC_{50} values in DPPH and XO inhibition categorize *M. beecheii* honeys.

The Pearson correlation analyses returned three high significant correlations ($p < 0,001$) between the assays performed on the stingless bee honeys. A positive correlation between DPPH and XO inhibition (Pearson coefficient = 0,695); likewise, a positive correlation between TP and ORAC (Pearson coefficient = 0,848); and finally, a negative correlation between ORAC and XO inhibition (Pearson coefficient = - 0,728) was present.

All *T. angustula* samples and Medihoney™ possessed efficacy in every test performed. In contrast, 33,3 % of *M. beecheii* honeys did not reach an IC_{50} in the DPPH inhibition assay. Figure 2 comprises the results for the antioxidant capacity tests, the immunomodulatory assays and the one sample sign non-parametric tests on median values.

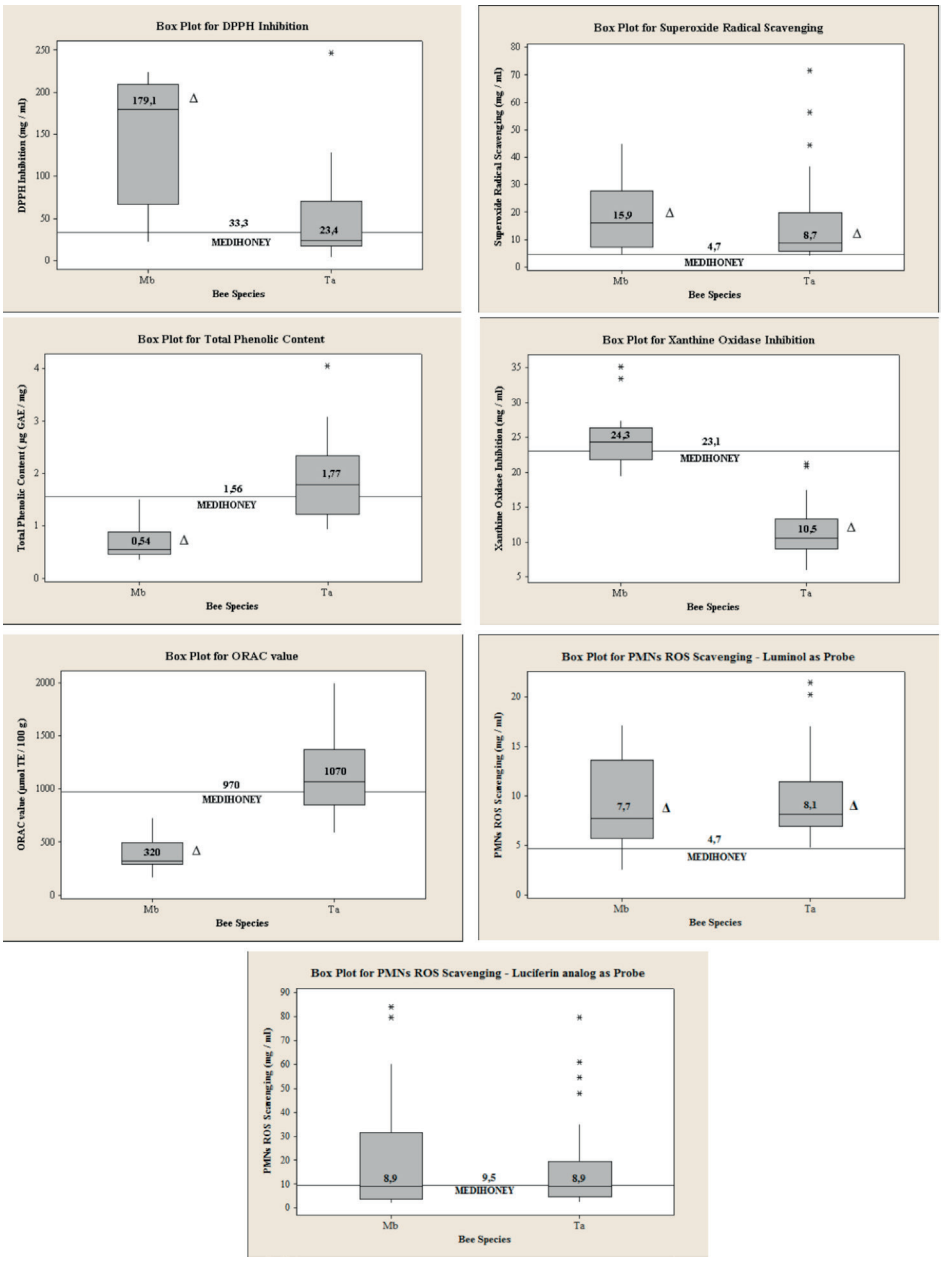

Figure 2. Box-plots for the results of the antioxidant capacity and immunomodulatory assays. The data are expressed as median values per bee species. The horizontal line in each graph indicates the results for Medihoney™.
* = atypical value
Δ = the difference between the median value and the reference result is statistically significant ($p < 0,0001$)

Discussion

In Costa Rica, *T. angustula* followed by *M. beecheii* are the stingless bee species of greatest commercial interest. Their honey is the main product sold. Meliponini honey presents a blooming market due to an increased interest in its folk-attributed medicinal properties; among which, its use as a wound dressing [19,25]. A recent publication by Zamora et al. (2014)[20] reported that Costa Rican stingless bee honeys presented values of microbiological quality in accordance to European Pharmacopoeia's standards for non-sterile substances for pharmaceutical use and a broad-spectrum antimicrobial activity comparable to Medihoney™. In the present study, we decided to use Medihoney™ as a reference since it is an FDA approved honey-based wound dressing and the only antioxidant therapy specifically approved for wound healing[11,12]. To our knowledge, this is the first study that compares Medihoney™ and stingless bee honeys for their in vitro antioxidant capacities and immunomodulatory activities.

An infected wound or a burn follows a Th2 type immune response, which is characterized by innate immunity[26,27,28]. The activation of the complement, coagulation and the arachidonic acid cascades, in addition to the liberation of Th2 type cytokines promote the transendothelial migration of PMNLs to the affected tissue[29]. PMNLs will then recognize microorganisms via opsonins or lectins. Subsequently, the microorganisms will be engulfed in vacuoles, where, by means of the oxidative burst, the radical oxygen species (ROS) produced will kill the microorganisms, to finally, be digested by proteolytic enzymes[30]. Hence, PMNLs are considered the first line of cell defense of innate immunity[31].

PMNLs and the ROS they produce, play vital roles in keeping homeostasis. They seek and destroy damaged cells and there is evidence that involves them with the facilitation of angiogenesis[32,33]. The ROS generated by PMNLs can act as signaling molecules that modulate crucial events like phagocytosis, gene expression, apoptosis, and epidermal growth factor-stimulated cell adhesion[34,35]. Although of utmost importance, excessive priming of PMNLs and the following liberation of ROS in the extracellular medium can severely damage healthy tissues[29,36]. ROS can damage macromolecules like proteins, DNA and can destroy cell membranes by lipid peroxidation[37,38]. Moreover, this oxidative stress delivers more free radicals like reactive nitrogen species and the activation of more inflammatory cells that perpetuate the inflammatory process (which is already a pro-oxidant condition); ironically complicates microbial infection and therefore, delays healing[26,28,34,36,39].

The basis of antioxidant therapy for wound healing is the interference of the signal transduction pathways of inflammation. A milestone that can be achieved by the administration of dressing agents with an antioxidant capacity that scavenge ROS, restore redox homeostasis by antagonizing oxidative stress, and consequently enhance wound healing[33,38,40].

We used the DPPH inhibition test for screening the radical quenching potential of stingless bee honeys and Medihoney™. Our results suggest that special care should be taken while reaching a conclusion on the antioxidant capacity of honeys if such values are determined solely by the DPPH assay. Notwithstanding that most honeys inhibited DPPH in a dose-dependent fashion and the evident differences in the results according to bee species; in the present investigation the results of this assay did not correlate with the total phenolic content and ORAC value. In addition, the positive correlation between the total phenolic content and ORAC value reported herein, suggests that the antioxidant capacity of the stingless bee honeys analyzed is attributed at a large extent to their phenolic constituents.

The foremost ROS produced by PMNLs is the superoxide anion ($O_2\cdot^-$). In order to determine the scavenging activity of honeys specifically of $O_2\cdot^-$, we performed a cell-free assay. Although the tests reported inhibition in a dose-dependent manner for all samples and the reference, these results were unreliable since all honeys and Medihoney™ inhibited the enzyme xanthine oxidase. In the cell-free $O_2\cdot^-$ scavenging assay, the superoxide anion is generated as a product of the activity of xanthine oxidase on hypoxanthine[27,36].

In the wound environment the main producers of $O_2\cdot^-$ are PMNLs. Although the $O_2\cdot^-$ generation by xanthine oxidase is considered by some authors redundant; this enzyme is expressed in the outer membrane of endothelial cells where its production of $O_2\cdot^-$ contributes to neutrophil recruitment and the generation of other more potent ROS via Fenton or Haber-Weiss reactions; all of which enable a microvascular inflammatory response[34,36,41,42,43]. The inhibition of xanthine oxidase may not directly inhibit the generation of ROS by PMNLs[43], but a dose-dependent inhibitory activity on this enzyme may modulate the inflammatory response by affecting the ROS cascades and the neutrophil recruitment. The *T. angustula* honeys under study presented the highest activity for xanthine oxidase inhibition; a feature that correlates to its antioxidant capacity and unveils an adjuvant immunomodulatory action by means of inhibition of xanthine oxidase-mediated inflammatory responses.

The PMNL tests are antioxidant cell-based assays[22,44]. The chemiluminescence probe luminol detects the intra-and extracellular ROS, whereas the cypridina luciferin analog solely detects ROS in the extracellular medium[39,46]. Medihoney™ obtained the highest activity in the PMNLs test using luminol as luminescence probe, while the Meliponini honeys accomplished IC_{50} values in a close concentration range. Our results reported no statistical differences between Medihoney™ and the stingless bee honey's performance in the PMNLs test using cypridina luciferin analog as probe. The results delivered by both PMNLs experiments allow stating that the Meliponini honeys and the reference analyzed perform the function of radical scavengers in a similar fashion. The capability of all the honeys studied to render scavenging activity over ROS produced by activated human PMNLs evidences an immunomodulatory activity. The later by disruption of the ROS cascades; which impairs the inflammatory response and leads to redox homeostasis[33,38,39,40,45].

Conclusions

This is the first comparison of Meliponini honey and Medihoney™ in their antioxidant capacities and immunomodulatory activities. Our study allows concluding that the in vitro immunomodulatory activities of the honeys under study can be attributed to a large extent to radical scavenging and xanthine oxidase inhibition. Both mechanisms may play a role in the impairment of the inflammatory process and the promotion of redox homeostasis.

The *T. angustula* honeys proceeding from Costa Rica presented antioxidant capacities not statistically different from Medihoney™. In addition, their radical scavenging activity of reactive oxygen species produced by human polymorphonuclear leukocytes and the inhibition of xanthine oxidase justify their ethnopharmacological use as a wound dressing and further research towards novel wound-healing developments.

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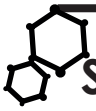
Chapter 5



Stingless bee honeys from Costa Rica exhibit antimicrobial activity against antibiotic-resistant clinical isolates

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Abstract

The antimicrobial resistance boosted the development of alternative wound healing therapies. A successful example is Medihoney™. To our knowledge, this is the first report of minimum inhibitory concentration (MIC) assays of Meliponini honeys against antibiotic-resistant isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* coming from infected hospital patients in comparison to Medihoney™. The samples under study belonged to the *Tetragonisca angustula* (n = 36) and *Melipona beecheii* (n = 21) species. 64 % of the *T. angustula* and 10 % of the *M. beecheii* honeys inhibited all the antibiotic-resistant bacteria. Medihoney™ was active against every strain tested with the exception of a *P. aeruginosa* isolate. Costa Rican *T. angustula* honeys can be selected according to their broad-spectrum antimicrobial activity. Moreover, such selection can render MICs similar to Medihoney™ in order to carry on further research and development towards a novel antimicrobial topical therapy for burn and wound healing.

Introduction

The rise of antimicrobial resistance is a natural cause of antibiotic use[1]. Currently, infections caused by antibiotic-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* hold the utmost clinical importance. These types of infections lead to longer periods of antibiotic therapy, longer hospital stays, and in critically ill patients to a compromised outcome[1,2,3]. Furthermore, the prevalence, morbidity and mortality attributed to antibiotic-resistant *S. aureus* and *P. aeruginosa* infections are increasing[1,4,5].

Antimicrobial resistance has boosted research towards the development of novel therapies for wound healing[6,7,8,9]. A successful example is Medihoney™, an FDA approved honey-based wound dressing[10,11,12].

The honeys produced by *Melipona beecheii* and *Tetragonisca angustula* are the stingless bee honeys of dominant commercial interest in Costa Rica. The use of Meliponini honey as a wound and burn dressing is part of Costa Rica's ethnopharmacology[13,14,15]. In a recent evaluation, stingless bee honey from Costa Rica reported antimicrobial activity comparable to Medihoney™ against type culture microorganisms of clinical relevance for the wound healing practice[16]. To the best of our knowledge, the current study is the first report for minimum inhibitory concentration (MIC) assays of Meliponini honeys against antibiotic-resistant bacteria coming from infected hospital patients in comparison to Medihoney™.

Materials and methods

Stingless bee honeys and Medihoney™

A total of 57 Meliponini honey samples were bought directly from keepers. The honeys collected came from regions of Costa Rica where the keeping of stingless bees (meliponiculture) is practiced[17]. The honeys were purchased during the month of April, harvest season of 2008. The samples under study belonged to the following bee species: *T. angustula* (n = 36) and *M. beecheii* (n = 21). The same set of samples took part of a previous study on the antimicrobial activity of stingless bee honeys against type culture microorganisms[16]. Prior to analysis, the honeys and the reference were stored in a dry place at room temperature (23 °C) and away from light.

Commercially available Medihoney™ (Dermasciences Inc., Canada) hydrocolloid wound paste was used as reference. According to the manufacturer, this is a moist dressing composed of 100 % active Leptospermum honey (medical grade honey).

Antibiotic-resistant bacteria

Strains of antibiotic-resistant *S. aureus* (n = 8) and *P. aeruginosa* (n = 2) were isolated from infected hospital patients of the National Children's Hospital of Costa Rica (HNN). The identification and antibiogram for every strain were performed by means of the Vitek® 2 System (BioMérieux, France). Table 1 lists the identity and antibiogram for every clinical isolate under trial.

Table 1. List of antibiotic-resistant clinical isolates of *S. aureus* and *P. aeruginosa*.

Strains of *S. aureus*		
Strain Code	**Antibiotic resistance according to antibiogram**	**Source**
ARSA-1	Oxacillin, Erythromycin	Abscess
ARSA-2	Oxacillin, Clindamycin, Ciprofloxacin, Erythromycin	Bronchoalveolar lavage
ARSA-3	Oxacillin, Erythromycin	Wound
ARSA-4	Oxacillin	Wound
ARSA-5	Oxacillin	Abscess
ARSA-6	Oxacillin	Wound
ARSA-7	Oxacillin	Articular
ARSA-8	Oxacillin, Clindamycin, Ciprofloxacin, Erythromycin	Blood
Strains of *P. aeruginosa*		
Strain Code	**Antibiotic resistance according to antibiogram**	**Source**
ARPA-1	Ceftazidime, Imipenem, Meropenem	Bronchoalveolar lavage
ARPA-2	Ceftazidime, Piperacillin, Tazobactam, Gentamycin, Erythromycin	Sputum

Minimum inhibitory concentration (MIC) assays

A test solution of 500 mg / mL of every stingless bee honey sample and Medihoney™ in Mueller-Hinton broth (Oxoid, U. K.) were prepared for each test. The preparation of these solutions took into account the individual density of every honey. All samples and the reference were subject of three individual tests against each microorganism. The MIC tests were performed in a 96-well microtiter plate-based assay as described previously[16]. The final volume contained in every well of the plate was 200 µL and the final concentration of antibiotic-resistant bacteria prior incubation was 5,0 X 10^5 cfu / mL.

Statistical analyses

All descriptive statistics (median values, percentages) and statistical inferences based on two samples (difference of proportions) between MIC performance results obtained per bee species were carried out with InfoStat Software (InfoStat Group, Universidad Nacional de Córdoba, Argentina).

Results

Of the 57 Meliponini honey samples under study, 25 (44%) stingless bee honeys were capable of inhibition of all the antibiotic-resistant clinical strains that took part in this trial. 64 % of the *T. angustula* honeys and 10 % of the *M. beecheii* samples presented this broad-spectrum antimicrobial activity. Medihoney™, the reference, presented antimicrobial activity against every antibiotic-resistant microorganism tested with exception of ARPA-2, a *P. aeruginosa* isolate. The antimicrobial activity performance of the honeys under study is presented in Table 2. The MIC results are shown in Table 3.

Table 2. Antimicrobial performance of stingless bee honeys as a whole (overall) and grouped per bee species against antibiotic-resistant clinical isolates.

Antibiotic resistant clinical isolate	Overall		Stingless bee species			
			M. beecheii		*T. angustula*	
	Active (%)	≤ MDHY (%)	Active (%)	≤ MDHY (%)	Active (%)	≤ MDHY (%)
ARSA-1	95	56	95	67	94	47
ARSA-2	98	98	95	95	100	100
ARSA-3	60	23	33	19	75 (Δ)	25
ARSA-4	89	39	76	19	97 (Δ)	50 (Δ)
ARSA-5	63	28	33	10	81 (ΔΔ)	39 (Δ)
ARSA-6	89	39	86	19	92	50 (Δ)
ARSA-7	89	26	90	10	89	36 (Δ)
ARSA-8	77	18	48	0	94 (ΔΔ)	28 (Δ)
ARPA-1	98	98	100	100	97	97
ARPA-2	93	93	90	90	100	100

≤ MDHY: percentage of honey samples that were capable of achieving a MIC in an equal or lower concentration than the reference, Medihoney®.
(Δ): the difference between the performance of the *T. angustula* and *M. beecheii* honeys is statistically significant ($p < 0,05$).
(ΔΔ): the difference between the performance of the *T. angustula* and *M. beecheii* honeys is highly statistically significant ($p < 0,001$).

Table 3. Minimum inhibitory concentrations (MIC) of Meliponini honeys and Medihoney™ against antibiotic-resistant bacteria of clinical origin. The MIC results are expressed as median values and range (mg / mL).

Antibiotic resistant clinical isolate	Stingless bee species		Reference Medihoney™
	M. beecheii	*T. angustula*	
ARSA-1	125 (62,5-250)	125 (62,5-250)	125
ARSA-2	125 (62,5-250)	125 (62,5-250)	250
ARSA-3	125 (125-250)	250 (125-250)	125
ARSA-4	250 (62,5-250)	125 (125-250)	125
ARSA-5	250 (125-250)	250 (125-250)	125
ARSA-6	250 (125-250)	125 (125-250)	125
ARSA-7	250 (125-250)	250 (62,5-250)	125
ARSA-8	250	250 (62,5-250)	125
ARPA-1	125 (62,5-250)	125 (62,5-250)	250
ARPA-2	125 (125-250)	125 (62,5-250)	N.I.

N.I.: The honey rendered no inhibition under the conditions tested.

Discussion

The ideal antimicrobial agent, whose goal is being the active constituent of a burn and wound dressing should render inhibitory activity against common agents of infection[18]. *S. aureus* and *P. aeruginosa* are among the most frequent microorganisms associated with burn and wound infections[8,19,20]. In clinical practice, infections with antibiotic-resistant strains are not uncommon to find and represent a major challenge to healing therapies; since these resilient infections impair wound healing and are strongly related to further life-threatening complications[20,21]. We determined the inhibitory effects of stingless bee honeys from Costa Rica against clinical isolates of *S. aureus* and *P. aeruginosa* that, apart from being casual agents of infection; present resistance to antibiotics.

Topical antimicrobial agents are currently few and there is an increasing need for innovative broad-spectrum antimicrobial agents for burn and wound healing[20,21]. The basis of such further developments is the determination of antibacterial activity[7]. The purpose of topical antimicrobial therapy is to enhance the normal healing process in an infected tissue by means of rendering a localized concentration of broad-spectrum antimicrobial agents that gain access to the affected area with low dependence of vascular transport, which, in an infected burn or wound is not optimal due to the inflammatory process[20]. In the present trial, we included an F.D.A. approved honey-based topical antimicrobial therapy as a reference. Our MIC method allows comparing the antimicrobial activity of the Meliponini honeys to Medihoney™.

The antimicrobial activity that the *T. angustula* honeys reported unveil a broad-spectrum inhibitory action against gram positive and gram negative microorganisms of clinical relevance; regardless of their antibiotic resistance. Such resistance may include resistance to penicillins, ß-lactamase resistant penicillins, macrolides, lincosamides, second and third generation quinolones, third generation cephalosporins, carbapenems, ß-lactamase inhibitors and aminoglycoside antibiotics.

It can be inferred from these results, that a selection of *T. angustula* honeys from Costa Rica according to its broad-spectrum antimicrobial activity is possible for further research and developments. Moreover, such a selection can render MICs similar to Medihoney™. To our knowledge, this is the first report of MIC assays of stingless bee honeys against clinical isolates of antibiotic-resistant bacteria coming from infected hospital patients.

Conclusions

Costa Rican Meliponini honeys exhibit antimicrobial properties against antibiotic-resistant bacteria isolated from wounds and respiratory infections. *T. angustula* honeys from Costa Rica can be selected according to their antimicrobial activity in order to carry on further research towards the development of a novel antimicrobial topical therapy for burn and wound healing.

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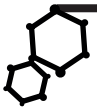
Chapter 6



An insight into the antibiofilm properties of Costa Rican stingless bee honeys

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Abstract

Biofilms constitute a major obstacle for wound healing. At the present time, there is an increasing search for antibiofilm agents that either have specific activity against biofilms or may act in synergy with antimicrobials. To the best of our knowledge, this is the first study that offers an insight into the antibiofilm properties of stingless bee honeys.

A total of 57 Meliponini honeys from Costa Rica were studied in this research. The honeys studied belonged to the *Tetragonisca angustula* (n = 36) and *Melipona beecheii* (n = 21) species. Medihoney™ was used as reference. All honeys were submitted to a screening composed of minimum inhibitory concentration (MIC) assay, an inhibition of biofilm formation and a biofilm destruction microplate based assays against a *S. aureus* biofilm forming strain. Dialysis led to the isolation of an antibiofilm fraction in *T. angustula* honeys. The honey antibiofilm fraction was evaluated for protease activity and for its synergistic effect with antibiotics over a *S. aureus* biofilm. Next, the active fraction was separated through activity guided isolation techniques involving SDS-PAGEs, anion exchange and size exclusion fast protein liquid chromatographies. The fractions obtained and the antibiofilm constituents isolated were tested for amylase and DNase activity.

Costa Rican *Tetragonisca angustula* honeys can inhibit planktonic growth, biofilm formation, and are capable of destroying a *S. aureus* biofilm. The antibiofilm effect is present in the protein fraction of *T. angustula* honeys. The biofilm destruction proteins allowed ampicillin and vancomycin to recover their antimicrobial activity against a *S. aureus* biofilm. The antibiofilm proteins are of bee origin, and their activity was not due to serine, cysteine or metalloproteases. Two proteins cause the antibiofilm action; these were named the *Tetragonisca angustula* biofilm destruction factors (TABDFs). TABDF-1 is a monomeric protein of approximately 50 kDa that is responsible for the amylase activity of *T. angustula* honeys. TABDF-2 is a protein monomer of approximately 75 kDa.

T. angustula honeys from Costa Rica are a promising candidate for research and development of novel wound dressings focused on the treatment of acute and chronic *S. aureus* biofilm wound infections.

Introduction

Biofilms constitute the predominant living strategy that bacteria adopt in order to withstand the most diverse and harsh environments[1]. It is estimated that 80 % of all microbial infections involve biofilms[2,3].

Biofilms are composed of two bacterial subpopulations: planktonic or free living, and sessile cells embedded in an extracellular polymeric substance (EPS) matrix[1,4,5]. Planktonic bacteria can be easily controlled by the host's immunity, antibiotics, and antiseptics[1,6,7]. The bacteria inside the biofilm matrix are the key to survival in adverse environments[1,7]. Biofilms in a wound are protected from the host's immune system. An acute wound infection follows a type Th2 immune response[7,8,9]. Although innate immunity avidly recognizes and reacts to the biofilm matrix constituents[8,10], polymorphonuclear leucocytes cannot eliminate a biofilm through phagocytosis[4,5,8]. This leads to phagocytic enzymes, and reactive oxygen species to be released in the intercellular medium. Consequently, the immune response damages healthy neighboring tissue, prolongs inflammation, and leads to a delayed healing or a chronic process[2,4,6,8,11].

Despite the fact that most antibiotics and antiseptics can diffuse inside the biofilm matrix channels, a biofilm wound infection can turn non-responsive to antibiotic therapy and antiseptic treatments[2,4,7,11,12,15]. The metabolic heterogeneity of the bacterial population inside the EPS matrix, along with the presence of persister cells allow a biofilm to prevail. Moreover, these cells in a latent state can regain metabolic activity once in lesser unfavorable conditions, and quickly reestablish the planktonic and sessile biofilm populations[2,5,7]. Hence, biofilms are nowadays considered the main reason why antimicrobial resistance is present in the clinical setting[3,11].

Biofilms can act as reservoir for infection[1,3,8,16,17]; an estimated 60 % of hospital-acquired infections are biofilm related[7,16,18]. In addition, biofilms are associated with chronic wound infections and their inability to heal[2,4,11,19,20,21]. At the present time, there is an increasing search for antibiofilm agents that either have specific activity against biofilms or may act in synergy with antimicrobials[6,7,12,13,14].

In recent years, honey, a more than four millennia-old therapy for wound healing has resurged[22,23,24,25,26,27]. A medicinal honey is produced in Australia and New Zealand. Honeybees (*Apis mellifera*) collect the nectar of the manuka tree (*Leptospermum scoparium*) to produce manuka honey[28,29,30]. Medihoney™ (DermaSciences, Inc.), a manuka honey-based wound treatment has proven its clinical efficacy against antibiotic resistant microorganisms[29,31]. Consequently, in 2008, Medihoney™ received the U. S. Federal Drug Administration approval as a wound dressing[26,32]. In addition, recent investigations report that Medihoney™ has in vitro inhibitory activity against *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus aureus* biofilms[33,34,35,36].

Mesoamerican stingless bees (family Apidae, tribe Meliponini) produce honeys that have a long dated ethnopharmacological history[38,39,40]. The ancient Maya and Aztec cultures started the keeping of stingless bees (meliponiculture) and used the honey for several medical applications; among them, wound healing[41,42]. Meliponiculture, and the use of Meliponini honeys as wound dressings are still part of Costa Rica's traditions and folk medicine[38,40,43,44].

Recently, investigations focused on the medicinal properties of Costa Rican stingless bee honeys have revealed that these honeys possess high microbiological quality. The latter was confirmed by the absence of pathogens, and low microbial counts that allow compliance with the European Pharmacopoeia's acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use[23]. In addition, these Meliponini honeys reported in vitro antioxidant capacities and immunomodulatory activities (that are relevant to wound healing) with no statistical significant differences to Medihoney™ [45]. Furthermore, stingless bee honeys originating from Costa Rica have broad-spectrum antimicrobial activity. These honeys were active against type culture microbial strains of clinical relevance for the wound healing practice[23,44,45,46]. Finally, Costa Rican Meliponini honeys reported inhibitory action over antibiotic-resistant isolates of *S. aureus* and *P. aeruginosa* obtained from infected hospital patients[47].

To our present knowledge, this is the first article that offers an insight into the antibiofilm properties of stingless bee honeys by means of *S. aureus* biofilm microplate-based assays and activity guided isolation techniques.

Materials and Methods

Sample collection

A total of 57 Meliponini honeys were bought directly from keepers in Costa Rica. The samples originated from areas where meliponiculture is practiced. The honeys studied belonged to the *Tetragonisca angustula* (n = 36) and *Melipona beecheii* (n = 21) species. The same set of samples were the subject of study of previous investigations focused on the density, percentage of humidity, microbiological safety, botanical origin, antimicrobial activity, antioxidant capacity, and immunomodulatory activity of stingless bee honeys from Costa Rica[9,23,44,45,46,47].

A manuka honey-based wound dressing (Medihoney™, DermaSciences Inc.) was used as reference. According to the manufacturer, this dressing consists of 100 % Active Leptospermum honey (medical grade honey).

Biofilm forming bacteria

A *S. aureus* biofilm forming strain (BMA / FR / 0.32 / 0074) was used in the present investigation. These bacteria were isolated from cow mastitis, and grow a biofilm in polystyrene microplates when cultivated at 35 °C in tryptic soya broth (Oxoid, U. K.) enriched with 0.25 % (m / v) glucose (TSB+G)[48].

Preparation of honey solutions for minimum inhibitory concentration (MIC) and biofilm assays

All the honey solutions for the MIC and biofilm tests were prepared in TSB+G broth. Test solutions of 500 mg/mL of every sample and reference were prepared for the MIC assays. For the preparation of these solutions, the individual density of every honey was taken into consideration. The latter, as means to attain comparable results since Meliponini honeys present higher water content values than *Apis mellifera* honeys [23]. The honey test solutions for the biofilm assays had a concentration of 200 mg / mL and were prepared following the same method.

Minimum inhibitory concentration (MIC) assays

The antimicrobial activity of the Meliponini honeys and Medihoney™ against a biofilm forming *S. aureus* was determined with a microplate MIC assay performed as described by Zamora et al. (2014)[23]. The MIC tests were prepared in TSB+G broth; the final volume per well was 200 µL, and the final concentration of bacteria prior incubation was 1.0 x 10^6 cfu / well. Aseptic techniques were maintained during all the steps of the assays. The MIC and biofilm tests were done in a biosafety level II laminar airflow chamber (Labconco, U. S. A.).

Inhibition of biofilm formation (BF)

The inhibitory action of honeys over the biofilm formation of *S. aureus* was determined by a microplate method based on the procedure described by Hensen[48]. An overnight culture (35 °C, 24 hours) of *S. aureus* was prepared on blood agar. The culture on agar was used to prepare an overnight culture in TSB+G broth. This *S. aureus* culture was then diluted 1 : 50 in TSB+G and used for the biofilm test.

Three aliquots of 200 µL of the honey test solutions were serially diluted in a sterile round bottom microplate (Corning Inc., U. S. A.) with 100 µL of TSB+G. Two dilution series were used for the test and one series was used as a blank. 100 µL of TSB+G were added to the blank series. Later, 100 µL of the *S. aureus* suspension in TSB+G (1.0×10^6 cfu / well) were added to the test dilution series. Two wells containing 100 µL of TSB+G and 100 µL of the *S. aureus* culture were used as control for biofilm formation (100 % biofilm). Immediately, the microplate was covered with a sterile lid and incubated (35 °C, 24 hours) in a gravimetric airflow incubator (Digisystem Laboratory Instruments Inc., Taiwan). The final volume per well was 200 µL. After incubation, the broth and the planktonic bacteria were removed. This was done by inverting the microplate over a bucket with disinfectant and then placing the plate upside down in sorbent paper sheets. Next, the microplate wells were washed twice with 200 µL of demineralized water. The water was removed after every wash in a bucket with disinfectant and the plate was then dried in sorbent paper as described previously. After this, the biofilm was fixed to the microplate. 200 µL of a solution of 0.1 M HCl were dispensed in each well and the plate was incubated at room temperature (23 °C, 90 minutes). Later, the HCl solution was removed and the plate was dried with sorbent paper. Next, the biofilm was stained by adding 200 µL of a solution of 0.1 % (m / v) safranin to the microplate wells. The plate was then incubated at room temperature (23 °C, 60 minutes). Afterwards, the excess of safranin was removed by inverting the microplate over a bucket with disinfectant. Then, the wells were washed four times with 200 µL of demineralized water and later the plate was dried with sorbent paper. Subsequently, 125 µL of a 0.2 M solution of NaOH were added to the wells. The lid and bottom borders of the plate were sealed with parafilm and the plate was then incubated (57 °C, 60 minutes). Afterwards, the content of each well was mixed with a micropipette and 100 µL of every well were transferred to a flat bottom microplate (Greiner Bio-One GmbH, Germany). Finally, the absorbance of the test at 540 nm was measured in a Multiskan Spectrum microplate reader with SkanIT DDE software (Thermo Scientific, Finland). Biofilm inhibition was calculated as the concentration capable of achieving a 50 % inhibition (BF IC_{50}). The latter, in order to evaluate if the inhibitory effects over biofilms were expressed in a dose-dependent fashion. Every sample and reference were subject of three separate analyses.

Biofilm destruction assay (BD)
First, a biofilm culture was prepared in a sterile round bottom microplate. For this purpose, the wells of two columns were filled each with 100 µL of a *S. aureus* suspension (2,0 x 10^6 cfu / ml) in TSB+G. In another column, 100 µL of TSB+G broth were dispensed in each well as blanks. Later, the lid and bottom borders of the microplate were sealed with parafilm, and the culture was incubated (35 °C, 24 hours). Next, in another sterile round bottom microplate, three aliquots of the sample solution were serially diluted as described for the BF assay. Blanks were prepared in the same fashion as well. Then, 100 µL of every well included in the honey dilution series and controls were dispensed over the biofilm culture plate. The final volume per well was 200 µL. Afterwards, the microplate assay was sealed with parafilm and incubated (35 °C, 24 hours). After incubation, the broth and planktonic bacteria were removed, and the plates were processed as described in the BF assay.

The ability of samples to disrupt a previously formed *S. aureus* biofilm was calculated as the concentration capable of destroying 50 % of the biofilm (BD IC_{50}). Every sample and the reference were subject of three separate analyses.

Effects of antibiotics over a biofilm forming *S. aureus*
5 µg / mL solution of ampicillin (Sigma A9393) and 500 µg / mL of vancomycin (Sigma V2002) in TSB+G were tested in the MIC, BF and BD assays.

Activity Guided Isolation

Isolation of organic fractions by Soxhlet extraction
35 g of *T. angustula* honey were dissolved in 35 mL of demineralized water. This solution was transferred into the extraction thimble of the Soxhlet extractor. Next, 100 mL of the solvent used for the extraction were added to the solution inside the thimble, and another 200 mL were dispensed in the Soxhlet apparatus flask. The extraction procedures were performed with solvents of increasing polarity (petroleum ether, diethyl ether, and ethyl acetate). Each solvent was refluxed for 8 hours, and the temperature of every extraction corresponded to the boiling point of each of the solvents applied. After every extraction, the solvent was removed from the fraction obtained with the aid of a rotary evaporator. At the end of the Soxhlet extractions, the remaining water-soluble fraction was freeze dried. All the fractions obtained were tested in the MIC, BF, and BD assays.

Concentration of the honey proteins fraction by dialysis
A dialysis membrane (Medicell International LTD., U.K.) with a 12-14 kDa pore, a diameter of 1 ¼ inches and a width of 50-54 nm was cleansed with 200 mL of a 2 % (m / v) $NaHCO_3$ and 1 mmol EDTA solution at 80 °C for 30 minutes. Next, the membrane was washed thoroughly with demineralized water and kept humid prior use.

50 g of a *T. angustula* honey were dissolved with 100 mL of demineralized water. This honey solution was dispensed in three 50 mL centrifuge tubes (Corning, U. S. A.). The tubes were centrifuged (2500 rpm, 5 minutes), and the supernatant was sterilized by filtration through a 0.220 µm pore vacuum filter (Corning, U. S. A.). The honey solution was kept in a sterile glass bottle prior dialysis.

The dialysis membrane was filled with the sterile honey solution and placed inside a 5 L pitcher filled with demineralized water. The water inside the pitcher was changed 3 times daily in periods of 4 hours. The dialysis was performed at 4 °C, under constant stirring, and for a period of 144 hours. During this process the dialysate was stored. Afterwards, a 1 L sample of the dialysate and the content inside the membrane were freeze dried. A 1 mg / mL solution of the honey proteins fraction (>12 kDa) and the dialysate were prepared in TSB+G under sterile conditions and tested in the MIC, BF, and BD assays.

Separation of the honey protein antibiofilm constituents from >12 kDa

Anion exchange chromatography

200 mg of the >12 kDa honey fraction were dissolved in 4 mL of Tris-HCl 20 mM buffer (pH 7.5), and later passed through a 0.22 µm syringe filter. 2 mL of this solution were injected into an Äkta Fast Protein Chromatography System (FPLC) (GE Healthcare Bio-Sciences AB, Sweden) coupled to a HiTrap Q XL 5 mL sepharose ion exchanger column (GE Healthcare Bio-Sciences AB, Sweden). The FPLC system was set to a flow rate of 5 mL / min, Tris-HCl 20 mM buffer (pH 7.5) was used as mobile phase (Buffer A), absorbance was monitored at 280 nm, and proteins were eluted from the column using a linear NaCl gradient (0 to 1.0 M) in Tris-HCl 20 mM buffer (Buffer B). A total of 3 FPLC anion exchange chromatographies were performed. Fractions of 2 mL were collected and grouped into pools according to the peaks obtained through the chromatography. The fraction pools were submitted to dialysis (as described previously) for 24 hours, and later freeze dried. 100 µg / mL solutions of every pool were prepared in TSB+G under sterile conditions and tested in the BF and BD assays. Each sample was subjected to 3 individual tests per assay. Finally, the fractions active in both BF and BD tests (Fplc 1, Fplc 3) were subjected to the next purification steps.

Separation of Fplc 1 by size exclusion chromatography

17.7 mg of fraction Fplc 1 were dissolved in 500 µL of low sodium and magnesium PBS (DPBS), and later filtered through a 0.22 µm SpinX centrifuge tube filter (Corning, U. S. A.). 400 µL of the Fplc 1 solution were injected into an Äkta FPLC System coupled to a Superdex HiLoad 75 26 / 60 preparative grade size exclusion chromatography column (GE Healthcare Bio-Sciences AB, Sweden). The FPLC system was set to a flow rate of 2.5 mL / min, DPBS was used as a mobile phase, absorbance was monitored at 280 nm, and 2 mL fractions were collected. Before gathering the fractions into pools, 15 µL of the

fractions that contained the tip of each peak of the chromatogram were put through SDS-PAGEs. Next, fractions were grouped into pools according to the peaks obtained through the chromatography. The pools were dialyzed for 24 hours and freeze dried. 100 µg / mL solutions of every pool were prepared in TSB+G under sterile conditions. All samples prepared were tested in the BF and BD tests. Each sample was subject of 3 individual tests per assay.

SDS-PAGE

SDS-PAGEs were performed in non-denaturing and denaturing conditions with dithiothreitol (DTT). InstantBlue (C. B. S. Scientific, U. S. A.) was used as SDS remover and stain. This method was described previously[49].

Separation of Fplc 3 by size exclusion chromatography

50 mg of fraction Fplc 3 were dissolved in 1.250 mL of DPBS and filtered through a 0.22 µm SpinX centrifuge tube filter. The Fplc 3 solution was injected into an Äkta FPLC System coupled to a Superdex HiLoad 200 26 / 60 GL column (GE Healthcare Bio-Sciences AB, Sweden). The FPLC chromatography, dialysis, and freeze-drying of pools were performed under the same conditions described for the Fplc 1 separation. Before gathering the fractions into pools, 15 µL of each were put through SDS-PAGEs. In addition, 50 µL of each fraction were tested in the BD assay. Likewise, 50 µL of DPBS were tested as control. In this particular case, besides the chromatogram, the fraction pools were prepared taking into consideration SDS-PAGE and BD test results. Finally, the fractions obtained were tested in the BF and BD tests. Each sample was subject of 3 individual tests per assay.

Characterization of >12 kDa and active fractions

Effect of protease inhibitors

BF and BD assays were prepared with >12 kDa in the presence of protease inhibitors as means to put to test if the >12 kDa honey fraction presented protease activity. Phenylmethanesulfonyl fluoride (PMSF) (Sigma, U. S. A.), a serine protease inhibitor, was dissolved in TSB+G to a final concentration of 10 mM. One tablet of cOmplete™ Mini protease inhibitor cocktail (Roche, Germany) was dissolved in 10 mL of TSB+G. According to the manufacturer, this protease inhibitor mix is active against cysteine, serine and metalloproteases.

100 µL of a 2 mg / mL >12 kDa solution in TSB+G were serially diluted with 50 µL of TSB+G in a sterile round bottom microplate. Next, 50 µL of the protease inhibitor to test were added to the test dilution series. For the BF tests, 100 µL of the *S. aureus* suspension in TSB+G (1.0×10^6 cfu / well) were added to the dilution series. For the BD tests, 100 µL of every well of the dilution series were dispensed over a previously prepared biofilm microplate culture. The BF and BD tests were prepared and executed as described previously. The effect of the protease inhibitors over the biofilm was assessed as control.

Effect of >12 kDa and antibiotics over a *S. aureus* biofilm

1 mg / mL solutions of ampicillin and vancomycin were prepared in TSB+G enriched with >12 kDa in a concentration of 200 µg / mL. Three aliquots of 200 µL of the antibiotic solutions were serially diluted in a sterile round bottom microplate with 100 µL of TSB+G enriched with >12 kDa. Immediately, 100 µL of every dilution series were dispensed over a biofilm microplate culture. This test included 3 controls. 100 % biofilm destruction: 100 µL of the TSB+G enriched with >12 kDa were added to six biofilm culture wells. 0 % biofilm destruction: 100 µL of TSB+G were added to six biofilm culture wells. Effect of the antibiotics over the biofilm: 100 µL of 1 mg / mL solutions of ampicillin and vancomycin in TSB+G were added each to three biofilm culture wells. The final volume per well was 200 µL. The assay was incubated at 35 °C for 24 hours. Next, 50 µL of every well of the dilution series were seeded on blood agar and incubated at 35 °C for 24 hours. The lowest antibiotic concentration that produced absence of *S. aureus* growth was considered the MIC value. In addition, the microplate assay was processed as described for the BD test in order to confirm biofilm destruction and the performance of controls. Each antibiotic was subject of 3 individual tests.

DNAse test

>12 kDa and fractions separated through activity-guided isolation were tested for DNase activity according to the method described by Nijland et al[50]. In brief, DNase activity was tested by incubating purified plasmid DNA with the fractions at 37 °C for 30 minutes. DNase type I was used as control. EDTA was added to another set of samples and DNase type I prior incubation with plasmids. EDTA inactivates metal ion dependent DNases like DNase type I. All the tests were run on a 1% agarose gel that contained ethidium bromide to visualize DNA degradation.

Amylase test

>12 kDa and fractions separated through anion exchange and size exclusion chromatography were tested for amylase activity. The starch and iodine solutions for this assay were prepared as described by Bogdanov, Martin and Lulmann (2002)[51]. 1 mg / mL solutions of every honey fraction to test were prepared in demineralized water. Likewise, a solution of 1 mg / mL of α-amylase (Sigma A3176, U. S. A.) in demineralized water was prepared as reference.

100 µL of the sample or reference were dispensed in two wells of a flat bottom microplate and serially diluted with 50 µL of demineralized water. Two microplate wells were filled with 150 µL of demineralized water as blank. Next, 50 µL of the starch solution were delivered in all the dilution series. Immediately, the assay was incubated at 40 °C for 30 minutes. Then, 50 µL of the iodine solution were added to the test wells and absorbance was determined at 660 nm in a Multiskan Spectrum microplate reader. The blank absorbance was subtracted of every test readings, and a graph of absorbance vs concentration of sample was prepared using Microsoft Excel® (Microsoft, U. S. A.).

Statistical analyses

The descriptive statistics (percentages, mean and median values) and the statistical inference based on two samples (difference of proportions) between the percentages of BD active samples per bee species were done with InfoStat Software (InfoStat Group, Universidad Nacional de Córdoba, Argentina).

Results

The minimum inhibitory concentration (MIC), inhibition of biofilm formation (BF), and biofilm destruction (BD) screening results are presented in Table 1.

Table 1. MIC, BF, and BD results for Meliponini honeys, Medihoney™ and antibiotics. Results are presented as median values and percentage of active samples per bee species.

Stingless Bee Honeys	Test Results (mg / mL)					
	MIC	% Active	BF	% Active	BD	% Active
T. angustula	98	100	22	100	32	94$^{\Delta}$
M. beecheii	96	100	52	100	28	29
Reference Medihoney™	204	---	55	---	NE	---
Antibiotics	**Test Results**					
	MIC		**BF**		**BD**	
Ampicillin	125 ng / mL		6 ng / mL		NE	
Vancomycin	63 µg / mL		400 ng / mL		NE	

$^{\Delta}$: the difference between the percentage of active *T. angustula* and *M. beecheii* honeys is highly statistically significant ($p<0.001$).
---: this calculation does not apply.
NE: no effect over the *S. aureus* biofilm under the conditions tested.

Based on the previous results, we decided to carry on activity-guided isolation studies with *T. angustula* honeys. Sample #29 was selected to carry on activity guided isolation studies since it presented MIC, BF, and BD results similar to the median values obtained for *T. angustula* honeys.

The first step we took towards activity-guided isolation was to concentrate organic compounds through Soxhlet extraction. None of the fractions obtained by this method rendered inhibition over *S. aureus* nor had any effect over biofilms. Consequently, we concentrated the honey proteins through dialysis and tested this fraction in the MIC, BF, and BD assays (see table 2).

Table 2. MIC, BF, and BD results obtained for dialysis fractions proceeding from a *T. angustula* honey.

Samples	Test Results		
	MIC	BF	BD
***T. angustula* honey Sample #29**	98 mg / mL	21 mg / mL	34 mg / mL
Dialysate	NI	NI	NE
>12 kDa	NI	18 µg / mL	10 µg / mL

NI: no inhibition under the conditions tested.
NE: no effect over the *S. aureus* biofilm under the conditions tested.

It can be inferred that >12 kDa had no antimicrobial activity over *S. aureus*. Notwithstanding, >12 kDa was the source of the inhibition of *S. aureus* biofilm formation and the causing agent of the destruction of a formed *S. aureus* biofilm by *T. angustula* honeys. These antibiofilm features were not due to serine, cysteine or metalloproteases (see table 3).

Table 3. BF and BD results for >12 kDa in presence of protease inhibitors.

Sample	Test Results (µg / mL)	
	BF	BD
>12 kDa	23	17
>12 kDa + 10 mM PMSF	29	18
>12 kDa + cOmplete™ protease mix	16	16
10 mM PMSF	NI	NE
cOmplete™ protease mix	NI	NE

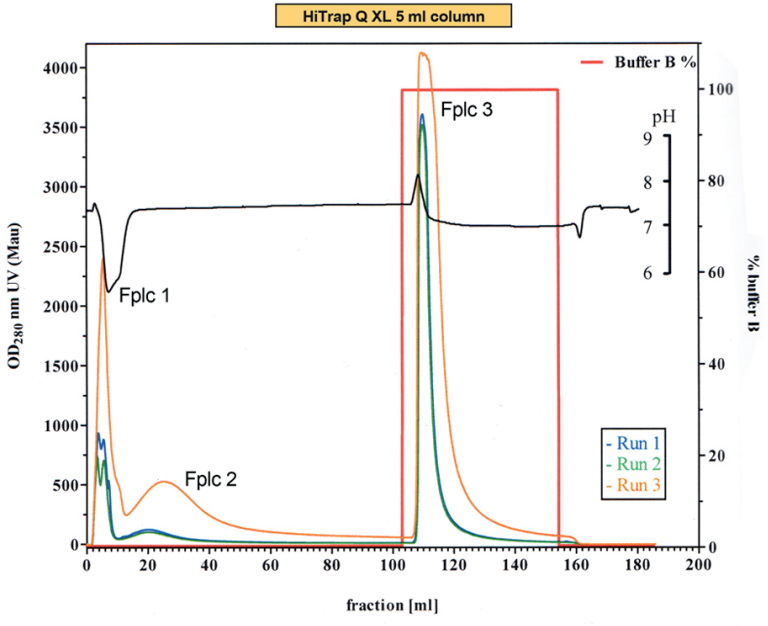
NI: no inhibition under the conditions tested.
NE: no effect over the *S. aureus* biofilm under the conditions tested.

We performed the MIC test for antibiotics in the presence of >12 kDa. The concentration of >12 kDa was 100 µg / mL since this concentration is close to the IC_{100} of >12 kDa in the BD test (75 µg / mL, data not shown). All controls behaved as expected. Under the conditions tested the biofilm was destroyed, leaving *S. aureus* susceptible to the bactericidal activity of the antibiotics. The biofilm destruction effect of >12 kDa enabled ampicillin and vancomycin to regain antimicrobial activity over *S. aureus* (see table 4).

Table 4. MIC results for antibiotics over a *S. aureus* biofilm in the presence of >12 kDa.

Antibiotics	MIC in the presence of 100 µg / mL of >12 kDa
Ampicillin	4 µg / mL
Vancomycin	63 µg / mL

The next step towards isolation of the antibiofilm constituents present in >12 kDa was FPLC anion exchange chromatography. Two of the fractions obtained (Fplc 1 and Fplc 3) displayed the antibiofilm activity (see figure 1).



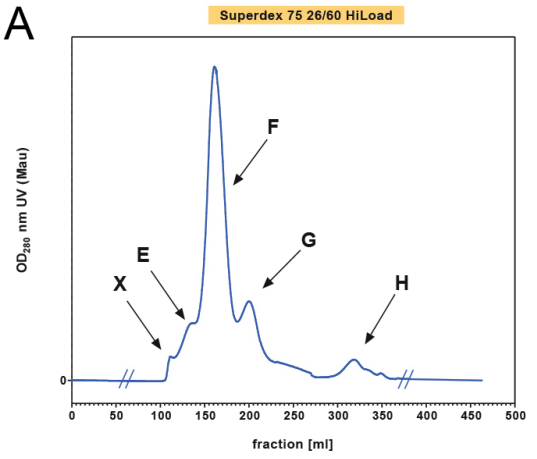
Samples	Test Results (µg/mL)	
	BF	BD
>12 kDa	18	10
Fplc 1	6	6
Fplc 2	NI	NE
Fplc 3	19	11

Figure 1. FPLC anion exchange chromatography of >12 kDa. BF and BD results for the fractions obtained.
NI: no inhibition under the conditions tested. NE: no effect over the *S. aureus* biofilm under the conditions tested.

Fraction Fplc 1 was separated by size exclusion chromatography. According to SDS-PAGE, BF, and BD tests: this fraction included a protein of approximately 50 kDa in molecular weight as the main agent of cause of the antibiofilm effect. This protein was named the *Tetragonisca angustula* biofilm destruction factor one (TABDF-1) (see figure 2).

The size exclusion chromatography of Fplc 3 and the subsequent activity guided isolation revealed a novel constituent with an approximate molecular weight of 75 kDa with antibiofilm properties. This protein was named the *Tetragonisca angustula* biofilm destruction factor two (TABDF-2) (see figure 3).

The amylase activity of *T. angustula* honey was caused to a large extent by TABDF-1 (see figure 4). DNase activity test results are presented in figure 5.



B

Samples	Test Results (µg/mL)	
	BF	BD
Fplc 1	18	10
X	NI	NE
E	NI	NE
F	NI	NE
G (TABDF-1)	11	11
H	NI	NE

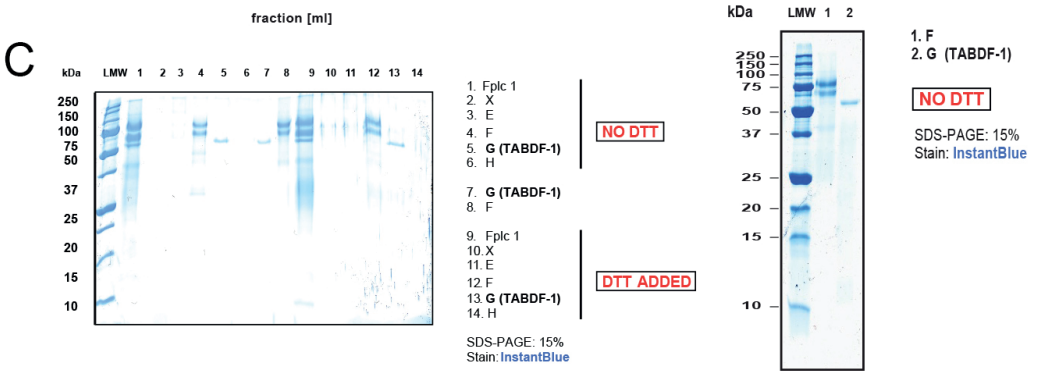
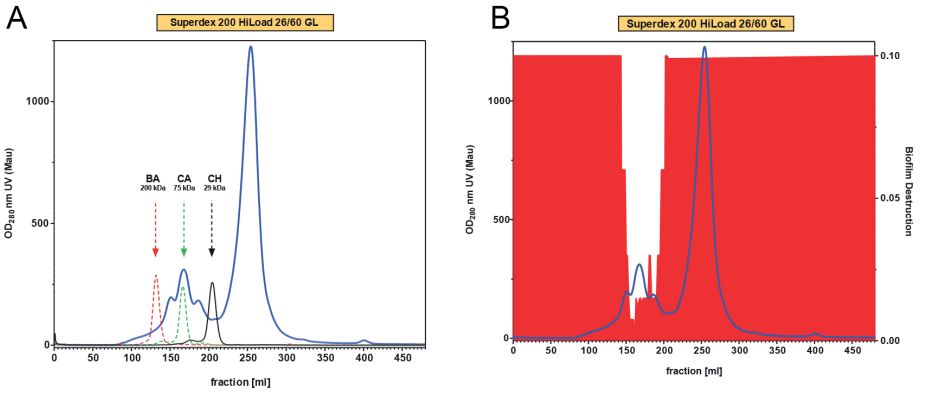


Figure 2. A) Size exclusion chromatography of Fplc 1. B) BF and BD results. C) SDS-PAGE results for the fractions obtained.
NI: no inhibition under the conditions tested. NE: no effect over the *S. aureus* biofilm under the conditions tested.



Samples	Test Results (µg/mL)	
	BF	BD
>12 kDa	18	10
Fplc 3	19	11
TABDF-2	16	16

Figure 3. Activity guided isolation of TABDF-2. A) Size exclusion chromatography of Fplc 3. Beta amylase (BA), conalbumin (CA), and carbonic anhydrase (CH) as molecular weight markers. B) Biofilm destruction tests performed in every FPLC fraction obtained. The reduction in biofilm absorbance indicates the antibiofilm fractions. C) SDS-PAGE (DDT added) results for the antibiofilm fractions. D) BF and BD results for TABDF-2.

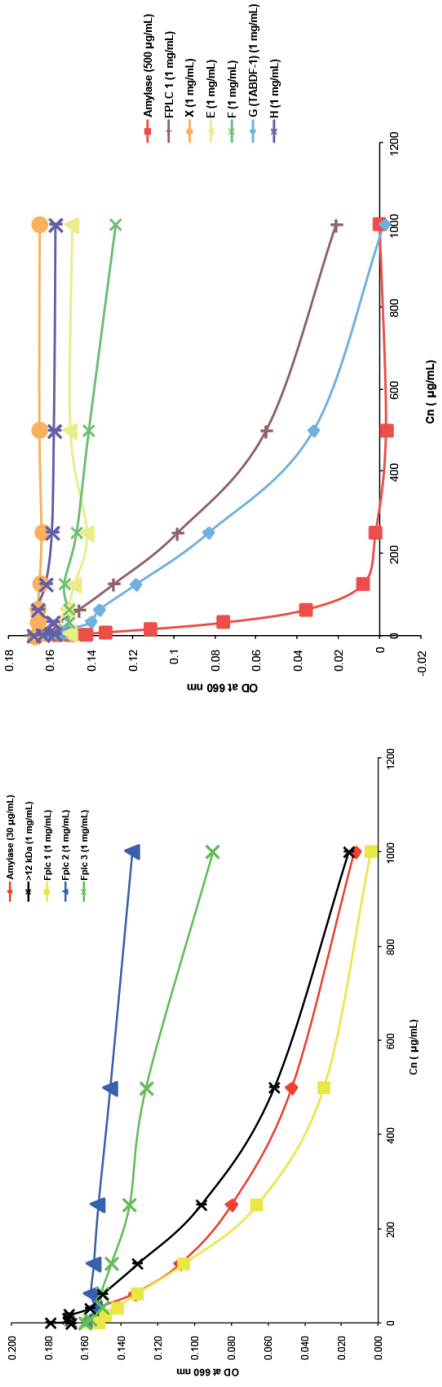


Figure 4. Amylase activity test results. Left: amylase activity tests for >12 kDa and its fractions obtained through anion exchange chromatography. Right: amylase activity tests for Fplc 1 and its fractions obtained through size exclusion chromatography.

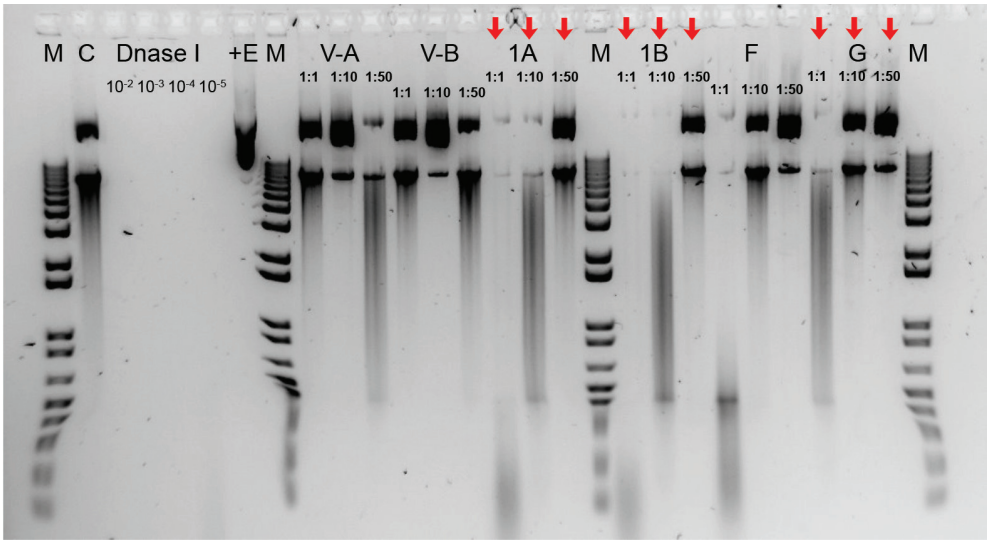


Figure 5. DNase activity test results for antibiofilm fractions separated from >12 kDa. Arrows in the figure highlight the fractions that contain TABDFs.
M: molecular marker.
E+: EDTA was added to DNase I. This inactivates metal ion dependent DNases like DNase type I.
V-A, V-B: fractions product of purification steps on Fplc 3.
1-A, 1-B: fractions product of purification steps that contain TABDF-2.
F: fraction product of size exclusion chromatography of Fplc 1.
G: fraction product of size exclusion chromatography of Fplc 1 that contains TABDF-1.

In the presence of EDTA, the honey antibiofilm fractions could not degrade DNA (not shown in figure 5). This indicates that all DNase activity present in TABDFs was metal ion dependent.

Discussion

Several authors, have previously shown the in vitro antibiofilm activity of Medihoney™ against *S. aureus* and *P. aeruginosa* strains[13,14,33,34,35,36,37] using various methods. In our hands, Medihoney™ showed antimicrobial activity (MIC), biofilm prevention activity (BF IC_{50}), but did not cause any disruption to an existing *S. aureus* biofilm in our BD assay. A similar finding was reported by Maddoks et al (2013)[34]; where Medihoney™ caused extensive cell death in *S. aureus*, *S. pyogenes*, and *P. aeruginosa* biofilms, but this honey-based wound dressing did not result in a complete clearance of the established biofilms. Furthermore, our BD test was designed to expose activity of complete removal of a biofilm, and to report if such an effect is present in a dose dependent manner by means of an IC_{50} determination.

The antibiotics tested behaved as was extensively reported: effective against planktonic cells (as shown by our MIC and BF results), but incapable of causing any inhibitory effect over an existing *S. aureus* biofilm[1,7,19,52].

Biofilms constitute a major obstacle for wound healing[4,7,12,19]. As long as the biofilm is present, the host's immunity will delay healing, and the biofilm bacteria will recover out from persisting cells in between wound treatments, and antibiotic therapy[2,7,15]. It would be beneficial to the wound treatment to use antibiofilm agents that can remove them[6].

We discovered that *T. angustula* honeys originating from Costa Rica can inhibit planktonic growth, biofilm formation, and are capable of destroying a *S. aureus* biofilm. All of these effects occur in a dose dependent manner. The antibiofilm effect is present in the protein fraction of *T. angustula* honeys. The same set of Meliponini honey samples that were used in the present investigation had their botanical origin determined (up to the species level) in a previous study by Zamora et al (2014)[23]. Seven botanical species comprised the main nectar resources of the *T. angustula* honeys; five of them are shared with *M. beecheii* honeys[23]. The protein nature of the antibiofilm factors, and the melissopalynology results imply that the antibiofilm properties of *T. angustula* honeys are of bee origin.

The protein fraction of *T. angustula* honeys (>12 kDa) has no antimicrobial activity over *S. aureus* planktonic cells. Nevertheless, its antibiofilm action allowed ampicillin to regain antimicrobial activity and vancomycin to recover its MIC value over a *S. aureus* biofilm. This result suggests that the *T. angustula* biofilm destruction factors disrupt the *S. aureus* biofilm EPS matrix in an extensive manner; thus allowing exposure of cells embedded in the biofilm matrix to antibiotics. This finding along with the antimicrobial activity, antioxidant capacity and immunomodulatory activities previously reported for *T. angustula* honeys[9,23,44,45,46] demonstrate that these type of honeys could act synergistically with antibiotics in antibiofilm wound healing therapies.

The antibiofilm proteins of *T. angustula* have no serine, cysteine or metalloprotease activity. When compared to DNase I, only at high concentrations is DNA cleavage ability observed. We hypothesize that the TABDFs target polymer constituents of the EPS matrix of the *S. aureus* biofilm; since the damage of the matrix polymers could disrupt the cohesiveness of the biofilm, and consequently destroy the biofilm structure[17].

According to our results, TABDF-1 is a monomeric protein of approximately 50 kDa that is responsible of the amylase activity of *T. angustula* honeys. We tested in the BD assay five *Apis mellifera* honeys that expressed higher diastase (bee amylase) activity than the *T. angustula* honeys of the present study. The *Apis mellifera* honeys yielded no biofilm destruction activity (data not shown). Our findings suggest that TABDF-1 may present structural differences to bee amylase that could explain its biofilm destruction activity. TABDF-2 is a protein monomer of approximately 75 kDa. Although its target in the *S. aureus* EPS biofilm matrix remains unknown, our data suggests that TABDF-2 should possess a mechanism of action over the *S. aureus* biofilm different from TABDF-1.

Conclusion

We discovered that Costa Rican *T. angustula* honeys possess the capability of destroying an *S. aureus* biofilm. Two proteins cause the antibiofilm properties: TABDF-1 and TABDF-2. These proteins, most probably are of bee origin. The biofilm destruction factors of *T. angustula* honey allowed ampicillin and vancomycin to recover their antimicrobial activity over a *S. aureus* biofilm.

New investigations concerning the sequence elucidation of the TABDFs and the proper identification of their targets inside the *S. aureus* biofilm matrix are needed.

The antibiofilm effect we are reporting herein, makes Costa Rican *T. angustula* honey a promising candidate for research and development of novel wound dressings focused on the treatment of acute and chronic *S. aureus* biofilm wound infections.

Acknowledgements

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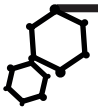
Chapter 7



Summary and Perspectives

Summary

Antimicrobial resistance created the need for innovations in topical treatments for wound healing[1,2]. This thesis comprises a series of investigations that allowed Costa Rican *Tetragonisca angustula* honeys to pass from being a highly regarded traditional medicine to becoming a novel candidate for wound dressing developments.

Chapter 2 reports the results of an antimicrobial activity screening of Costa Rican stingless bee honeys by means of agar well diffusion assays. In addition, a comparison between the inhibitory activity of Meliponini honeys and *Apis mellifera* honeys is presented. Stingless bee honeys reported higher antimicrobial activity effects than *Apis mellifera* honeys from the same region.

The health risk associated with the use of Meliponini honey as a wound dressing, and the determination of the honey's botanical origin are described in **chapter 3**. In addition, this chapter includes minimum inhibitory concentration (MIC) assays for Costa Rican stingless bee honeys against type culture collection microorganisms of relevance for the wound healing practice. For this analysis, Medihoney™, a medical grade honey-based wound dressing was used as reference.

The results presented in **chapter 3** demonstrate the low health risk associated with the practice of using Meliponini honey as a wound dressing agent; given the low microbiological counts, absence of *Clostridium botulinum* spores by polymerase chain reaction (PCR) tests, and consequently, the compliance of 83 % of the honey samples analyzed with the European Pharmacopoeia's acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use.

Over 90 % of *T. angustula* and *Melipona beecheii* honeys inhibited *Pseudomonas aeruginosa* and *Staphylococcus aureus* at MICs lower than Medihoney™. Under the conditions tested, Medihoney™ was not active against *Candida albicans*, whereas 53 % of *T. angustula* honeys rendered inhibition. Furthermore, all the stingless bee honeys under study reported a homogenous (monofloral) botanical composition, which provides foundation to the standardization of a desired antimicrobial effect.

This chapter provides evidence supporting the ethnopharmacological application of Costa Rican Meliponini honeys as a wound dressing. A practice that unveils the application of a proficient antiseptic agent with low health risks associated.

The interference of the signal transduction pathways of inflammation is the cornerstone of antioxidant therapy for wound healing[3,4]. **Chapter 4** describes an evaluation of the potential of Costa Rican stingless bee honeys as an antioxidant wound dressing agent.

Results suggest that the antioxidant capacities of Meliponini honeys are given in a major grade to their phenolic constituents. In addition, the in vitro immunomodulatory activities are explained mainly due to radical scavenging and xanthine oxidase inhibition. Both mechanisms may act in the impairment of the inflammatory process and the promotion of redox homeostasis.

Medihoney™ is up to the present, the only antioxidant therapy specifically approved by the United States Food and Drug Administration for wound healing[5]. *T. angustula* honeys reported antioxidant capacities not statistically different from Medihoney™.

Infections with antibiotic-resistant microorganisms are major obstacles for wound healing[6]. **Chapter 5** reports the results for MIC assays of stingless bee honeys against clinical isolates of antibiotic-resistant *S. aureus* and *P. aeruginosa*. 64 % of the *T. angustula* honeys inhibited all the antibiotic resistant strains. Costa Rican *T. angustula* honeys possess broad-spectrum inhibitory action against microorganisms of clinical relevance, regardless of their resistance to antibiotics.

Biofilms can defy a host's immune system, resist antibiotic therapy and antiseptic treatments[7]. Hence, biofilms compromise the process of wound healing[8]. **Chapter 6** presents an insight into the antibiofilm properties of Costa Rican Meliponini honeys.

T. angustula honeys can inhibit the planktonic growth, the biofilm formation, and are capable of destroying a *S. aureus* biofilm. All of these antibiofilm activities are exerted by honeys in a dose-dependent manner.

The antibiofilm effect is present in the protein fraction of *T. angustula* honeys. The biofilm destruction proteins allowed ampicillin and vancomycin to recover their antimicrobial activity over a *S. aureus* biofilm. These proteins are of bee origin, and their activity was not due to serine, cysteine or metalloproteases. Two proteins cause the antibiofilm action; this finding was named the *T. angustula* biofilm destruction factors (TABDFs). TABDF-1 is a monomeric protein of approximately 50 kDa that is responsible of the amylase activity of *T. angustula* honeys as well. TABDF-2 is a protein monomer of approximately 75 kDa.

Perspectives

The results gathered in this series of investigations justify the introduction of a novel candidate for innovative wound dressing developments: Costa Rican *Tetragonisca angustula* honey.

This type of honey could deliver the following benefits for wound healing therapies:

- Low microbiological risks to health associated to its use as a wound dressing.
- Broad-spectrum antimicrobial activity; effective against antibiotic-resistant bacteria.
- Antioxidant capacity and immunomodulatory activity similar to Medihoney™.
- Antibiofilm activities: these honeys can inhibit the *S. aureus* planktonic growth, biofilm formation, and moreover, are capable of destroying a previously formed *S. aureus* biofilm.

Furthermore, Costa Rican *T. angustula* honeys can be selected in order to deliver a standardization of the aforementioned biological activities in order to develop wound dressings that can be tested in clinical studies. These clinical evaluations could be focused on acute and chronic antibiotic-resistant wound infections, as well as acute and chronic *S. aureus* biofilm wound infections.

The antimicrobial, antioxidant, immunomodulatory and antibiofilm properties of Costa Rican *T. angustula* honeys can turn into an added commercial value. A quality that could make the keeping of *T. angustula* a practice to consider for sustainable development projects in Costa Rican rural communities.

Given the homogeneous (monofloral) composition of *T. angustula* honeys; this type of honeys could be used as source for the isolation of novel antimicrobial and antioxidant compounds with possible applications in the pharmaceutical, cosmetic and food industries.

Two bee proteins give the antibiofilm properties to *T. angustula* honeys: the TABDFs. New investigations concerning the sequence elucidation of the TABDFs and the proper identification of their targets inside the *S. aureus* biofilm matrix are needed.

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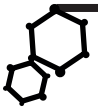
Appendices



Nederlandse Samenvatting
List of Abbreviations
Curriculum Vitae
List of Publications
Acknowledgements

Nederlandse Samenvatting

Toenemende bacteriële antibiotica resistentie maakt dat er behoefte is aan innovatie op het gebied van topicale wondbehandeling[1,2]. Dit proefschrift bevat een aantal onderzoeksresultaten waarmee honing van de angel-loze bij *Tetragonista angustula* zich kandidaat stelt voor nieuwe ontwikkelingen op het gebied van wonddressings.

In **hoofdstuk 2** wordt duidelijk dat angel-loze bijenhoning superieur is aan honing van de gewone honingbij (*Apis mellifera*) uit dezelfde regio's waar het antimicrobiële activiteit betreft.

In **hoofdstuk 3** werden de effecten van botanische herkomst bestudeerd. Van alle angel-loze bijenhoningsamples werd de minimale inhiberende concentratie (MIC) bepaald tegen een serie klinisch relevante bacteriestammen; Medihoney™, een medicinale honing, werd als referentie gebruikt. Het gezondheidsrisico voor gebruik van Meliponini honing in wonddressings werd als zeer laag beoordeeld omdat: de honing van zichzelf weinig bacteriën bevat, er middels PCR-analyse geen sporen van *Clostridium botulinum* werden gemeten, en omdat 83% van de honingsamples voldoet aan de microbiële criteria van de Europese farmacopee voor farmaceutisch gebruik van niet steriele substanties.

Meer dan 90% van *T. angustula* en *Melipona beecheii* honing remt *Pseudomonas aeruginosa* en *Staphylococcus aureus* beter (lagere MIC-waarden) dan Medihoney™. Verder bleek Medihoney™ niet in staat om *Candida albicans* te remmen waar dit bij 53% van de *T. angustula* honingsamples wel het geval was. Omdat alle angel-loze bijenhoningsamples een homogene mono-florale botanische samenstelling hebben behoort standaardisatie op gewenst antimicrobieel effect tot de mogelijkheden.

Dit hoofdstuk levert onderbouwing voor het ethnofarmacologische gebruik van Costa Ricaanse Meliponi honingsoorten voor wondbehandeling. Angel-loze bijenhoning blijkt een efficiënt antisepticum met een laag risicoprofiel.

Antioxidanttherapie bij wondheling is vooral gestoeld op interventie in de signaaltransductieroutes van de ontstekingsreactie[3,4]. In **hoofdstuk 4** wordt de antioxidantcapaciteit van Costa Ricaanse angel-loze bijenhoning bepaald. Het zijn met name de fenolische componenten die tekenen voor de antioxidant activiteit. In *in vitro* immunomodulatie-assays werd zowel scavenging-activiteit gemeten als het remmen van xanthine-oxidase-activiteit. Beide processen kunnen een rol spelen bij het remmen van de ontsteking en het in stand houden van de redoxhomeostase.

Tot op heden is Medihoney™ de enige FDA geregistreerde medicinale honing die gebruikt wordt in de antioxidanttherapie[5]. Uit deze studie blijkt *T. angustula* honing niet voor Medihoney™ onder te doen.

Infecties met antibioticaresistente bacteriën vormen een groot probleem bij de wondheling[6]. **Hoofdstuk 5** toont de MIC-waarden van angel-loze bijenhoning tegen klinische isolaten van antibioticaresistente *S. aureus* en *P. aerogunosa* stammen. 64% van de *T. angustula* honingsamples blijkt in staat om deze resistente stammen te remmen. Kennelijk remt Costa Ricaanse *T. angustula* honing klinisch relevante stammen in weerwil van hun antibioticaresistentie.

Veel bacteriën zijn in staat om een zogenaamde biofilm te vormen. Exopolysacchariden en of DNA fragmenten zijn de bouwstenen waarmee de bacteriën een welhaast onneembare vesting vormen waardoor het afweersysteem, antibiotica en antiseptica vele malen minder effectief zijn[7]. Biofilms maken daarmee wondheling bijzonder moeilijk[8]. **Hoofdstuk 6** gaat over anti-biofilm eigenschappen van Costa Ricaanse Meliponini honing.

Van *T. angustula* honing is aangetoond dat het dosisafhankelijk *S. aureus* in zijn planktonische groei remt, dat het de biofilmformatie voorkomt en dat het een door *S. aureus* gevormde biofilm afbreekt. Met name dit laatste is uniek en nog nooit eerder aangetoond.

De *T. angustula* anti-biofilm-activiteit bevindt zich in een eiwitfractie. Ampicilline en vancomycine bleken niet in staat om *S. aureus*, beschermd door een biofilm, te doden. Na destructie van de biofilm door anti-biofilm-eiwitten werd de bactericide activiteit van ampicilline en vancomycine weer hersteld. De anti-biofilm-eiwitten worden door de bij aangemaakt; het gaat hier niet om serine-, cysteine-, of om metalloproteases.

Middels activiteits-geleide isolatie en sub-fractionering kon worden aangetoond dat *T. angustula* twee biofilm-destructie-eiwitten maakt, TABDFs gedoopt (**T**. **A**ngustula **B**iofilm **D**estruction **F**actors). TABDF-1 is een monomeer eiwit van 50kD waarvan amylase activiteit kon worden aangetoond. TABDF-2 is een monomeer eiwit van ongeveer 75 kD.

Perspectieven

Met dit onderzoek is aangetoond dat honing van de Costa Ricaanse angelloze bij *Tetragonisca angustula* in potentie een innovatieve bijdrage kan leveren aan de ontwikkeling van nieuwe wonddressings.

Deze honingsoort heeft de volgende positieve eigenschappen die van groot nut kunnen zijn bij wondtherapie:

- Laag microbieel risico
- Breed spectrum antibiotisch actief, ook tegen resistente bacteriën
- Qua antioxidantactiviteit en immunomodulatie vergelijkbaar met Medihoney™ .
- Unieke anti-biofilm-activiteit waarbij zowel biofilmformatie geremd wordt alsook een bestaande biofilm wordt afgebroken. Daarnaast remt deze honing de planktonische groei van *S. aureus*.

Geselecteerde *T. angustula* honing kan op biologische activiteiten worden gestandaardiseerd en als basis dienen voor nieuw te ontwikkelen wonddressings die klinisch getest kunnen worden bij acute en chronische antibioticaresistente wondinfecties en bij chronische *S. aureus* biofilm wondinfecties.

De antimicrobiële, de antioxidantactiviteit, de immunomodulatoire, maar zeker de unieke antibiofilmactiviteit van Costa Ricaanse *T. angustula* honing vertegenwoordigen een commerciële toegevoegde waarde die van groot belang kan zijn als basis voor duurzame ontwikkelingen op het Costa Ricaanse platteland.

Vanwege de mono-florale, homogene samenstelling van de *T.angustula* honing is zij bij uitstek geschikt als bron voor nieuwe antimicrobiële componenten en antioxidanten met mogelijke toepassingen in de farmaceutische, de cosmetische en de voedingsmiddelen industrie.

Twee bijen-eiwitten zijn verantwoordelijk voor de anti-biofilm activiteit van *T. angustula* honing: TABDF-1 en TABDF-2. Het vraagt om extra onderzoek om de exacte samenstelling en structuur van deze eiwitten op te helderen. Uitzoeken waar deze beide TABDFs aangrijpen in de matrix van een *S.aureus* biofilm is ook een uitdaging.

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List of abbreviations

AAPH	2,2-azobis (2-methylpropionamidine-dihydrochloride)
ANOVA	Analysis of Variance
APHA	American Public Health Association
ARPA	Antibiotic-Resistant *Pseudomonas aeruginosa*
ARSA	Antibiotic-Resistant *Staphyloccus aureus*
ATCC	American Type Culture Collection
BD	Biofilm Destruction Assay
BD IC_{50}	Concentration that achieves a 50 % Biofilm Destruction
BF	Inhibition of Biofilm Formation Assay
BF IC_{50}	Concentration that achieves a 50 % Inhibition of Biofilm Formation
BHIA	Brain Heart Infusion Agar
Cypridina Luciferin Analog	2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazin-3-one
DDT	Dithiothreitol
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
DPPH	2,2-diphenyl-1-picryl hydrazyl
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substance
FDA	Food and Drug Administration
FPLC	Fast Protein Liquid Chromatography

FPLC 1	Fraction #1 product of anion exchange chromatography of *T. angustula* honey proteins fraction
FPLC 2	Fraction #2 product of anion exchange chromatography of *T. angustula* honey proteins fraction
FPLC 3	Fraction #3 product of anion exchange chromatography of *T. angustula* honey proteins fraction
GAE	Gallic Acid Equivalent
HCl	Hydrochloric Acid
HNN	National Children's Hospital of Costa Rica
H_20_2	Hydrogen Peroxide
IC_{50}	50 % Inhibitory Concentration
IC_{100}	100 % Inhibitory Concentration
>12 kDa	Honey Proteins Fraction
Luminol	5-amino-2,3-dihydro-1,4-phthalazinedione
MIC	Minimum Inhibitory Concentration
MHB	Mueller-Hinton Broth
MPN	Most Probable Number
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
NaOH	Sodium Hydroxide
$O_2\cdot^-$	Superoxide Anion
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffered Saline
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction

PMNL	Polymorphonuclear Leucocytes
PMNs-LUM	The effect over ROS produced by stimulated PMNLs using luminol
PMNs-CLA	The effect over ROS produced by stimulated PMNLs using a cypridina luciferin analog
PMSF	Phenylmethanesulfonyl Fluoride
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TABDF	*Tetragonisca angustula* Biofilm Destruction Factor
TE	Trolox Equivalents
TP	Total Phenolic Content
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
Trolox	6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid
TSB+G	Tryptic Soya Broth enriched with 0.25 % Glucose
UMF	Unique Manuka Factor
UV	Ultra Violet
XO	Xanthine Oxidase

Curriculum Vitae

Gabriel Zamora was born on November 2nd, 1976 in San José, Costa Rica. He attended the Liceo Mauro Fernández Acuña in Tibás, San José, were he got his high school diploma in 1994.

In 1995, he was admitted to the University of Costa Rica (UCR), where he studied microbiology and clinical chemistry. During his last two years of studies, he worked as a research assistant in the Molecular Microbiology and Genetic Bioprospecting Laboratory of the Center for Research in Cellular and Molecular Biology (Centro de Investigaciones en Biología Celular y Molecular, CIBCM) of the UCR. Here, from 2001 through 2002 he mastered skills in applied and environmental microbiology, phytopathology, polymerase chain reaction tests, and DNA sequencing. In addition, it was in this research center where he developed his thesis for opting for his degree in microbiology and clinical chemistry. In 2003, his thesis entitled "Determination of the etiology of a bacterial infection in the pejibaye palm (*Bactris gasipaes*) in Costa Rica" received a "with honors" mention from the Faculty of Microbiology of the UCR. Moreover, Gabriel Zamora received recognition from the Costa Rican Microbiology and Parasitology Association for the high quality of this work.

On November 2003, he was hired by the Tropical Beekeeping Research Center (Centro de Investigaciones Apícolas Tropicales, CINAT) of the National University (Universidad Nacional, UNA) of Costa Rica as coordinator of the Microbiology Research Program.

During October-December 2006 he visited the Medicinal Chemistry and Chemical Biology (Med Chem) Program of the Department of Pharmaceutical Sciences of Utrecht University (UU). This visit set the basis for a collaborative research between Med Chem and CINAT, and the opportunity to develop doctoral studies at the Med Chem Program.

In 2008, after acquiring funding through grants presented to the Special Funding for Higher Education (Fondos Especiales para la Educación Superior) of CONARE Costa Rica, he managed to provide CINAT's Microbiology Program with the reagents, consumables, and the necessary equipment that enabled him to start his Ph.D. studies. The series of investigations that comprise his doctoral thesis were performed partially at CINAT in Costa Rica and at Med Chem in The Netherlands.

In 2013, an investigation that took part in his doctoral studies was published as a Chapter for the book entitled "Pot-Honey: A Legacy of Stingless Bees" of Springer. Product of this publication, the author received the 2012-2013 UNA Award; a distinction given by UNA to academics that make a valuable contribution to society.

Since 2012, Gabriel holds the position of Coordinator of the Microbiology and Medicinal Chemistry Program of CINAT. Up to present, he has been author in more than 50 academic publications.

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