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

Objective: There is an increasing search for antibiofilm agents that either have specific activity against biofilms or may act in synergy with antimicrobials. Our objective is to examine the the antibiofilm properties of stingless bee honeys.
Method: Meliponini honeys from Costa Rica were examined along with Medihoney as a reference. All honeys were submitted to a screening composed of minimum inhibitory concentration, inhibition of biofilm formation and biofilm destruction microplate-based assays against a *Staphylococcus aureus* biofilm forming strain. Dialysis led to the isolation of an antibiofilm fraction in *Tetragonisca angustula* honeys. The honey antibiofilm fraction was evaluated for protease activity and for any synergistic effect with antibiotics on a *Staphylococcus aureus* biofilm. The active fraction was then separated through activity guided isolation techniques involving SDS-PAGEs, anion exchange and size exclusion fast protein liquid chromatographies. The fractions obtained and the isolated antibiofilm constituents were tested for amylase and DNase activity.
Results: 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. Costa Rican *Tetragonisca angustula* honeys can inhibit the planktonic growth, biofilm formation, and are capable of destroying a *Staphylococcus aureus* biofilm. The antibiofilm effect was observed in the protein fraction of *Tetragonisca angustula* honeys. The biofilm destruction proteins allowed ampicillin and vancomycin to recover their antimicrobial activity over a *Staphylococcus aureus* biofilm. The antibiofilm proteins are of bee origin, and their activity was not due to serine, cysteine or metalloproteases. There were 2 proteins causing the antibiofilm action; these were named the *Tetragonisca angustula* biofilm destruction factors (TABDFs). TABDF-1 is a monomeric protein of approximately 50kDa that is responsible of the amylase activity of *Tetragonisca angustula* honeys. TABDF-2 is a protein monomer of approximately 75kDa.
Conclusion: *Tetragonisca 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 *Staphylococcus aureus* biofilm wound infections.
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Tetragonisca angustula ● **antibiofilm** ● ***Staphylococcus aureus*** ● ***Melipona beecheii*** ● **stingless bee honey**

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

Biofilms are composed of 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] and 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 recognises 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 neighbouring 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, biofilms can be 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 biofilms to survive. Moreover, cells in a latent state can regain metabolic activity once in more favourable conditions and quickly reestablish biofilm populations.[2,5,7] Hence, biofilms are considered the main reason for antimicrobial resistance in the clinical setting.[3,11]

Biofilms can act as reservoir for infection,[1,3,8,16,17]

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and an estimated 60% of hospital-acquired infections are biofilm related.[7,16,18] In addition, biofilms are associated to chronic wound infections and their inability to heal.[2,4,11,19–21] Hence, there is an increasing search for antibiofilm agents that either have specific activity against biofilms or may act in synergy with antimicrobials.[6,7,12–14]

Honey, a more than four millennia-old therapy for wound healing has had resurgence of interest.[22–27] 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.[28–30] Medihoney (DermaSciences, Inc.), a manuka honey-based wound treatment has proven clinical efficacy against antibiotic resistant microorganisms,[29,31] and in 2008 Medihoney received the US Food and Drug Administration (FDA) approval for use 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.[33–36]

Mesoamerican stingless bees (family Apidae, tribe Meliponini) produce honeys that have a long dated ethnopharmacological history.[38–40] 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 on the medicinal properties of Costa Rican stingless bee honeys have revealed that these honeys possess high microbiological quality. The later 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 proceeding 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.[23,44–46] Finally, Costa Rican Meliponini honeys reported inhibitory action over antibiotic-resistant isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* obtained from infected hospital patients.[47]

To our knowledge, this is the first article to examine the antibiofilm properties of stingless bee honeys using *Staphylococcus aureus* biofilm microplate-based assays and activity guided isolation techniques.

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Materials and methods

Sample collection

Meliponini honeys, from the *Tetragonisca angustula* and *Melipona beecheii* species, were bought directly from keepers in Costa Rica, from areas where meliponiculture is practiced. These samples have been assessed previously for density, percentage of humidity, microbiological safety, botanical origin, antimicrobial activity, antioxidant capacity, and immunomodulatory activity.[9,23,44–47]

A manuka honey-based wound dressing (Medihoney) was used as reference standard. The manufacturer states this dressing consists of 100% active *Leptospermum* honey (medical grade honey).

Biofilm forming bacteria

A *Staphylococcus aureus* biofilm forming strain (BMA/FR/0.32/0074) was used. The bacteria were isolated from cow mastitis. These grow as biofilm in polystyrene microplates when cultivated at 35°C in tryptic soya broth (TSB) (Oxoid, UK) enriched with 0.25% (mass/volume) glucose (TSBG).[48]

Preparation of honey solutions

All the honey solutions for the minimum inhibitory concentration (MIC) and biofilm tests were prepared in TSBG broth. For the preparation of the test solutions of each sample and reference, the individual density of every honey was taken into account, as a means of attaining comparable results since Meliponini honeys present higher water content values than *Apis mellifera* honeys.[23]

Minimum inhibitory concentration (MIC) assays

The antimicrobial activity of the Meliponini honeys and Medihoney against a *Staphylococcus aureus* biofilm was determined with a microplate MIC assay performed as previously described.[23] In final volume of 200µl per well, 1.0×10^6 colony forming units (CFU)/well *Staphylococcus aureus* were added to the TSBG broth. Aseptic technique was maintained during all the steps of the assays.

Inhibition of biofilm formation

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

Serial dilution of three aliquots (200µl) of honey test solutions were made in TSBG into a sterile round bottom microplate (Corning Inc., US). We used 100µl of TSBG as a blank. *Staphylococcus aureus* suspension (1.0×10^6 CFU/well) in TSBG (100µl) was added to the test honey dilution series. Wells containing TSBG (100µl) and *Staphylococcus aureus* culture (100µl) were used as control for biofilm formation (100% biofilm). The microplate was covered and incubated (35°C, 24 hours) in a gravimetric airflow incubator (Digisystem Laboratory Instruments Inc., Taiwan).

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After incubation, the broth and the planktonic bacteria were removed by inverting the microplate over a bucket with disinfectant and then placing the plate upside down on absorbent paper. The microplate wells were washed twice with demineralised water, the plate was then dried on absorbent paper. The biofilm was then fixed to the microplate using a solution of 0.1M HCl dispensed in each well and the plate was incubated at room temperature (23°C, 90 minutes). The HCl solution was removed and the plate was dried. The biofilm was stained by adding a solution of 0.1% (mass/volume) safranin to each well. The plate was incubated at room temperature (23°C, 60 minutes), after which, the excess of safranin was removed. The wells were washed four times (200µl demineralised water) and the plate was dried. Subsequently, 0.2M NaOH (125µl) was 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).

The content of each well was mixed with a micropipette and 100µl of every well were transferred to a flat bottom microplate (Greiner Bio-One GmbH, Germany). The absorbance of the test solution at 540nm 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 (biofilm inhibition IC_{50}). The inhibitory effect on biofilm was expressed in a concentration-dependent manner. All conditions were subject of three separate analyses.

Biofilm destruction assay

A biofilm culture was prepared in a sterile round bottom microplate. The wells of two columns were filled with *Staphylococcus aureus* suspension (100µl) 2.0 x 10^6 CFU/ml) in TSBG, in another column, TSBG broth (100µl) was dispensed in the wells as a blank. The lid and bottom borders of the microplate were sealed with parafilm, and the culture was incubated (35°C, 24 hours). In another sterile round bottom microplate, sample serial dilutions and blanks were prepared as previously described and 100µl of the sample dilutions and controls were added to the biofilm culture plate. The microplate assay was sealed with parafilm and incubated for 24 hours (35°C). After incubation, the broth and planktonic bacteria were removed and the plates were processed as described in for the biofilm formation assay.

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

The effects of ampicillin (5µg/ml) (Sigma A9393) and of vancomycin (500µg/ml) (Sigma V2002) on biofilm forming *Staphylococcus aureus* in TSBG were tested in the MIC, biofilm formation and biofilm destruction assays.

Activity guided isolation of organic fractions by Soxhlet extraction

Tetragonisca angustula honey (35g) was dissolved in demineralised water (35ml) and transferred into the extraction thimble of the Soxhlet extractor. Next, 100ml of the extraction solvent were added inside the thimble, and another 200ml 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, biofilm formation and biofilm destruction assays.

Activity guided isolation: concentration of the honey proteins fraction by dialysis

Tetragonisca angustula honey (50g) dissolved in demineralised water (100ml) was dispensed in three 50ml centrifuge tubes (Corning, US), centrifuged (2500rpm, 5 minutes), and the supernatant was removed and sterilised by filtration through a 0.220µm pore vacuum filter (Corning, US). The honey solution was kept in a sterile glass bottle before dialysis.

A dialysis membrane (Medicell International LTD., UK), with a 12–14kDa pore, a diameter of 1¼ inches and a width of 50–54nm, was filled with the sterile honey solution and placed inside a pitcher filled with demineralised water (5l). The water was changed 3 times a day, 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 1l sample of the dialysate and the content inside the membrane were freeze dried. A 1mg/ml solution of the honey proteins fraction (HPF>12kDa) and the dialysate were prepared in TSBG under sterile conditions and tested in the MIC, biofilm formation and biofilm destruction assays.

Separation of the honey protein antibiofilm constituents from HPF>12kDa: anion exchange chromatography

HPF>12kDa (200mg) was dissolved in 4ml of Tris-HCl 20mM buffer (pH 7.5), and passed through a 0.22µm syringe filter. This solution (2ml) was injected into an Äkta Fast Protein Chromatography System (FPLC) (GE Healthcare Bio-Sciences AB, Sweden) coupled to a HiTrap Q XL 5ml sepharose ion exchanger column (GE Healthcare Bio-Sciences AB, Sweden). The FPLC system was set to a flow rate of 5ml/min, Tris-HCl 20mM buffer (pH 7.5) was used as mobile phase (Buffer A), absorbance was monitored at 280nm, and proteins were eluted from the column using a linear NaCl gradient (0–1.0M) in Tris-HCl 20mM buffer (Buffer B). Fractions of 2ml were collected and grouped into pools according to the peaks obtained through the chromatography. The fraction pools were submitted to dialysis (as described previously)

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

Honey source	MIC	% active	Biofilm formation	% active	Biofilm destruction	% active
Tetragonisca angustula	98 mg/ml	100	22 mg/ml	100	32 mg/ml	94*
Melipona beecheii	96 mg/ml	100	52 mg/ml	100	28 mg/ml	29
Reference Medihoney	204 mg/ml	—	55 mg/ml	—	NE	—
Antibiotics	**MIC**		**Biofilm formation**		**Biofilm destruction**	
Ampicillin	125 ng/ml		6 ng/ml		NE	
Vancomycin	63 μg/ml		400 ng/ml		NE	

MIC–minimum inhibitory concentration; NE–no effect over the *Staphylococcus aureus* biofilm under the conditions tested; **Tetragonisca angustula* versus *Melipona beecheii* p<0.001

for 24 hours, and later freeze dried. Solutions of every pool (100μg/ml) were prepared in TSBG under sterile conditions and tested in the biofilm formation and destruction assays. Each sample was subjected to three individual tests per assay. Finally, the fractions active in both biofilm formation and destruction assays (Fplc 1, Fplc 3) were subjected to the next purification steps.

Separation of Fplc 1 by size exclusion chromatography

The fraction Fplc 1 (17.7mg) was dissolved in sodium and magnesium PBS (500μl; DPBS), and filtered through a 0.22μm SpinX centrifuge tube filter (Corning). The Fplc 1 solution (400μl) was injected into an Äkta FPLC System coupled to a Superdex HiLoad 75 26/60 preparative grade size exclusion chromatography column (GE Healthcare). The FPLC system was set to a flow rate of 2.5ml/minute, DPBS was used as a mobile phase, absorbance was monitored at 280nm, and 2ml fractions were collected. Before gathering the fractions into pools, 15μl of the fractions that contained the tip of each peak of the chromatogram were put through SDS-PAGEs. The fractions were grouped into pools according to the peaks obtained through the chromatography. The pools were dialysed for 24 hours and freeze dried. All samples were tested in the biofilm formation and destruction assay. Each sample was subject of three individual tests per assay.

Table 2. MIC, biofilm formation (BF) and biofilm destruction (BD) results obtained for dialysis fractions from a *Tetragonisca angustula* honey

	MIC	BF	BD
Tetragonisca angustula honey			
Sample #29	98mg/ml	21mg/ml	34mg/ml
Dialysate	NI	NI	NE
HPF>12 kDa	NI	18 μg/ml	10 μg/ml

HPF>12kDa–honey protein fraction >12kDa; MIC–minimum inhibitory concentration; NI–no inhibition under the conditions tested; NE–no effect over the *Staphylococcus aureus* biofilm under the conditions tested

Separation of Fplc 3 by size exclusion chromatography

Fplc 3 (50mg) of fraction were dissolved in 1.250ml of DPBS and filtered through a 0.22μm 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. 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, 15μl of each were put through SDS-PAGEs. In addition, 50μl of each fraction were tested in the biofilm destruction assay. Likewise, 50μl of DPBS were tested as a 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 biofilm formation and destruction assay. Each sample was subject of three individual tests per assay.

SDS-PAGE

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

Characterisation of HPF>12kDa and active fractions: effect of protease inhibitors

BF and BD assays were prepared with HPF>12kDa in the presence of protease inhibitors to test if the HPF>12kDa contained protease activity. Phenylmethanesulfonyl fluoride (PMSF) (Sigma, US), a serine protease inhibitor, was dissolved in TSBG (final concentration: 10mM), one tablet of cOmplete Mini protease inhibitor cocktail (Roche, Germany) was dissolved in TSBG (10ml).

A solution of the HPF>12kDa (2mg/ml) in TSBG was serially diluted in a sterile round bottom microplate. The protease inhibitor was added to the dilution series. For the biofilm formation tests *Staphylococcus aureus* suspension (100μl in TSBG, $1.0x10^6$ CFU/well) was added to the test dilution series. For the biofilm destruction assay 100μl of each of the dilutions were dispensed over a biofilm microplate culture. The effect of the protease inhibitors alone on the biofilm was used as a control.

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Effect of HPF>12kDa and antibiotics over a *Staphylococcus aureus* biofilm

Ampicillin (1 mg/ml) and vancomycin (1 mg/ml) were prepared in TSBG. The antibiotic solutions were serially diluted in a sterile round bottom microplate with 100 µl of TSBG enriched with HPF>12kDa (200µg/ml). Immediately, 100 µl of every dilution was dispensed over a biofilm microplate culture, including three controls. 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 for 24 hours (35°C). The lowest antibiotic concentration that produced absence of *Staphylococcus aureus* growth was considered the MIC value. In addition, the microplate assay was processed as described in order to confirm biofilm destruction and the performance of controls. Each antibiotic was subject of three individual tests.

DNAse assay

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

Amylase test

HPF>12kDa 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 et al.[51] A solution of 1 mg/ml of α-amylase (Sigma A3176, US) in demineralised water was prepared as reference. We dispensed 100 µl of the sample or reference in two wells of a flat bottom microplate and serially diluted with 50 µl of demineralised water. Next, 50 µl of the starch solution were delivered in all the wells, the assay was incubated for 30 minutes (40°C). Then, 50 µl of the iodine solution were added to the test wells and absorbance was determined at 660nm in a Multiskan Spectrum microplate reader. The blank absorbance was subtracted of every test readings, and a graph of absorbance versus concentration of sample was prepared using Microsoft Excel (Microsoft, US).

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 biofilm destruction active samples per bee species were done with InfoStat Software (InfoStat Group, Universidad Nacional de Córdoba, Argentina).

Results

A total of 57 Meliponini honeys were bought directly from keepers in Costa Rica, these belonged to the *Tetragonisca angustula* (n=36) and *Melipona beecheii* (n=21) species. The MIC, biofilm formation, and biofilm destruction screening results are presented in Table 1.

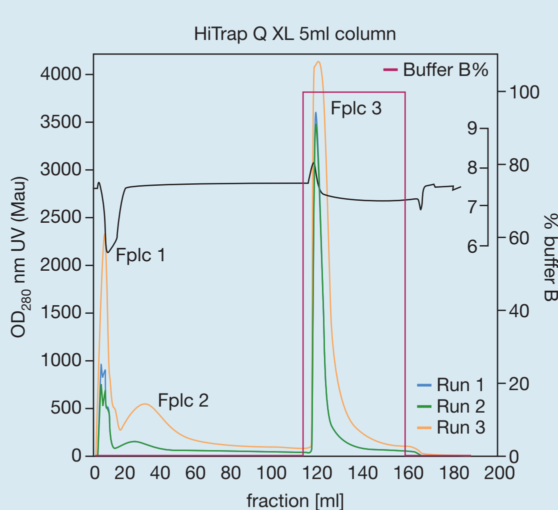
Based on the previous results, we decided to carry on activity-guided isolation studies with *Tetragonisca angustula* honeys. Sample #29 was selected to carry on activity-guided isolation studies since it presented

Table 3. Biofilm formation (BF) and biofilm destruction (BD) results for the honey protein fraction>12kDa in presence of protease inhibitors

Sample	BF	BD
HPF>12kDa	23 µg/ml	17 µg/ml
HPF>12kDa + 10mM PMSF	29 µg/ml	18 µg/ml
HPF>12kDa + cOmplete protease mix	16 µg/ml	16 µg/ml
10mM PMSF	NI	NE
cOmplete protease mix	NI	NE

HPF>12kDa–honey protein fraction >12kDa; NI–no inhibition under the conditions tested; NE–no effect over the *Staphylococcus aureus* biofilm under the conditions tested; PMSF– phenylmethane sulfonyl fluoride

Fig 1. FPLC anion exchange chromatography of HPF>12kDa. Biofilm formation (BF) and biofilm destruction (BD) results for the fractions obtained



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

NI–no inhibition under the conditions tested; NE–no effect over the *Staphylococcus aureus* biofilm under the conditions tested

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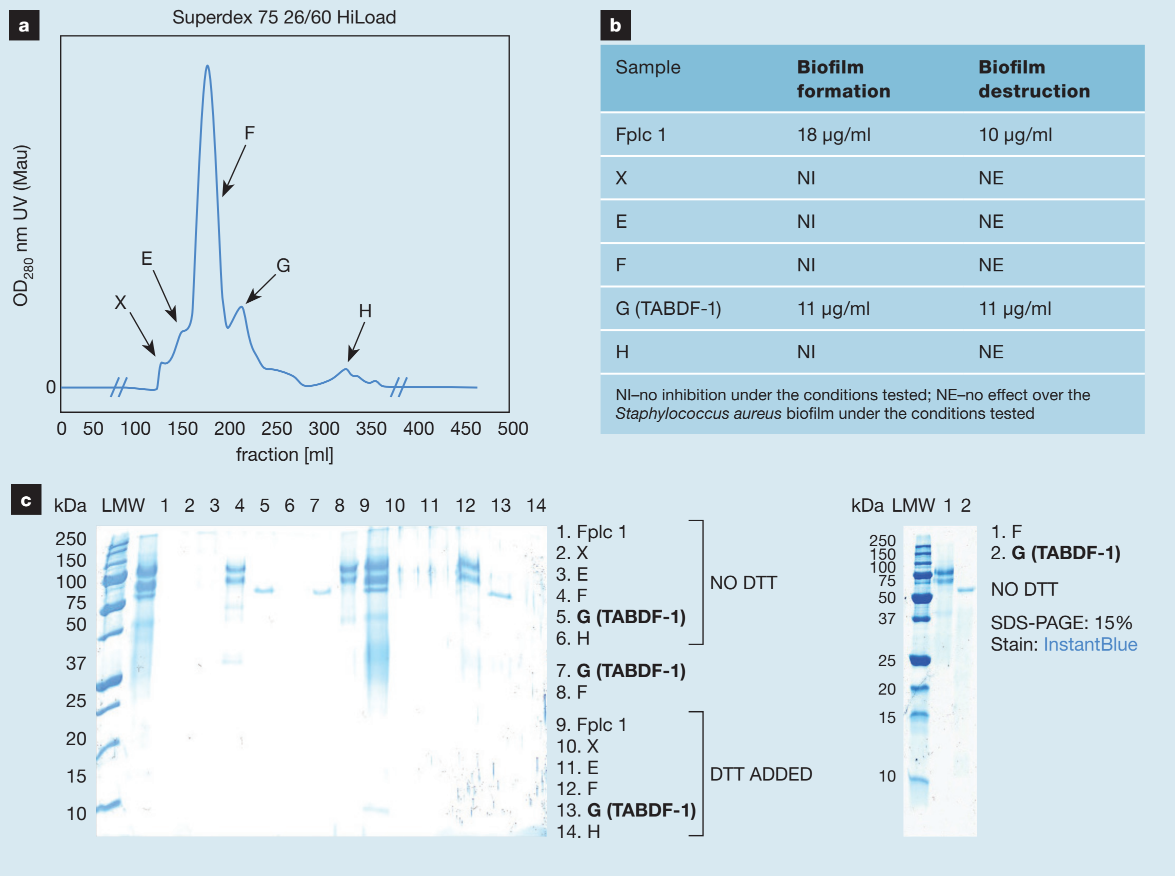

Sample	Biofilm formation	Biofilm destruction
Fplc 1	18 µg/ml	10 µg/ml
X	NI	NE
E	NI	NE
F	NI	NE
G (TABDF-1)	11 µg/ml	11 µg/ml
H	NI	NE

NI–no inhibition under the conditions tested; NE–no effect over the *Staphylococcus aureus* biofilm under the conditions tested

Fig 2. Size exclusion chromatography of Fplc 1 **(a)**. Biofilm formation and biofilm destruction results for the five identified fractions **(b)**. SDS-PAGE results for the fractions obtained **(c)**

MIC, and biofilm formation and destruction results that were similar to the median values obtained for *Tetragonisca angustula* honeys. The first step we took was to concentrate organic compounds through Soxhlet extraction. None of the fractions obtained by this method rendered inhibition over *Staphylococcus aureus* nor had any effect over the biofilm. Consequently, we concentrated the honey proteins through dialysis and tested this fraction in the MIC, biofilm formation, and biofilm destruction assays (Table 2).

It can be inferred that HPF>12kDa had no antimicrobial activity over *Staphylococcus aureus*. Notwithstanding, HPF>12kDa was the source of the inhibition of *Staphylococcus aureus* biofilm formation and the causing agent of the destruction of a formed *Staphylococcus aureus* biofilm by *Tetragonisca angustula* honeys. These antibiofilm features were not due to serine, cysteine or metalloproteases since the protease inhibitors did not alter the effect of HPF>12kDa (Table 3).

We performed the MIC test for antibiotics in the presence of HPF>12kDa. The concentration of HPF>12kDa was 100µg/ml since this concentration is close to the IC_{100} of HPF>12kDa in the biofilm destruction test (75µg/ml, data not shown). All controls behaved as expected. Under the conditions tested the biofilm was destroyed, leaving *Staphylococcus aureus* susceptible to the bactericidal activity of the antibiotics. The biofilm destruction effect of HPF>12kDa enabled ampicillin and vancomycin to regain antimicrobial activity over *Staphylococcus aureus*, the MIC being 4µg/ml and 63µg/ml respectively.

Isolation of HPF>12kDa antibiofilm constituents

Using FPLC anion exchange chromatography we obtained two fractions (Fplc 1 and Fplc 3) which displayed the antibiofilm activity (Fig 1). Fraction Fplc 1 was separated by size exclusion chromatography. According to SDS-PAGE, biofilm formation and biofilm destruction tests, this fraction included a protein with a molecular weight of approximately 50kDa as the main cause of the antibiofilm effect. This protein was named the *Tetragonisca angustula* biofilm destruction factor one (TABDF-1) (Fig 2).

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

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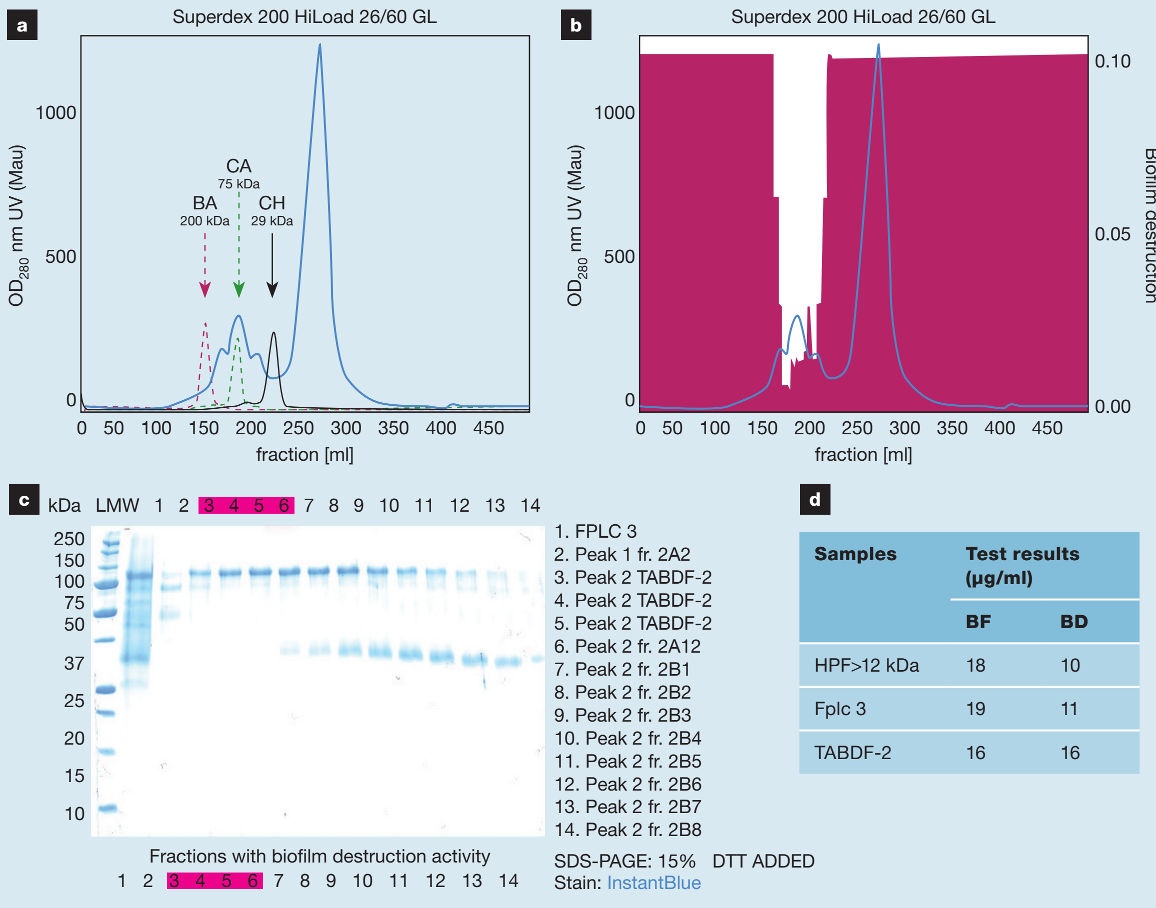
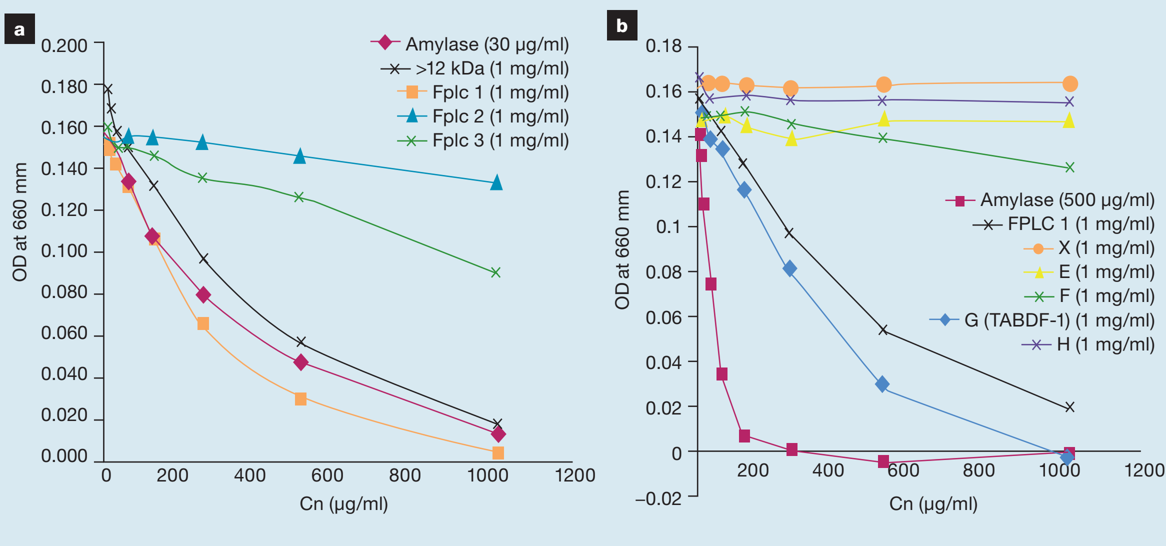
Fig 3. Activity guided isolation of TABDF-2. Size exclusion chromatography of Fplc 3. Beta amylase (BA), conalbumin (CA), and carbonic anhydrase (CH) as molecular weight markers **(a)**. Biofilm destruction tests performed in every FPLC fraction obtained. The reduction in biofilm absorbance indicates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



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

Fig 4. Amylase activity test results. Amylase activity tests for HPF>12kDa and the fractions obtained by anion exchange chromatography **(a)**. Amylase activity tests for Fplc 1 and the fractions obtained by size exclusion chromatography **(b)**



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Enzyme activity

The majority of amylase activity of *Tetragonisca angustula* honey was caused by TABDF-1 (Fig 4). DNase activity was only seen at very high concentrations for the fractions that contained the TABDFs (up to 1:1 dilution for TABDF-1 and up to 1:10 dilution for TABDF-2). Whereas DNase type 1 remained active in a 1:100000 dilution. In the presence of EDTA, the honey antibiofilm fractions could not degrade DNA. This indicates that all DNase activity present in the TABDFs was metal ion dependent.

Discussion

Several authors, by different methods, have demonstrated *in vitro* antibiofilm activity of Medihoney against *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains.[13,14,33–37] Under our testing conditions, Medihoney achieved a MIC and a biofilm formation IC_{50}, but could not cause any disruption to the *Staphylococcus aureus* biofilm in our biofilm destruction assay. A similar finding was reported by Maddoks et al.[34] where Medihoney caused extensive death in *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa* biofilms; however, the honey-based wound dressing did not completely remove these established biofilms. As a further matter, our biofilm destruction test was designed to expose complete removal of a biofilm, and to report if such effect is present in a dose dependent fashion by means of an IC_{50} determination.

The antibiotics tested behaved as has been extensively reported, they were effective against planktonic cells (as shown by our MIC and biofilm formation results), but incapable of causing any inhibitory effect over a *Staphylococcus aureus* biofilm.[1,7,19,52] Biofilm constitutes a major obstacle for wound healing.[4,7,12,19] As long as there is biofilm present, the host's immunity will delay healing, and the biofilm will recover via 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 biofilms.[6]

We found that *Tetragonisca angustula* honeys from Costa Rica can inhibit the planktonic growth, the biofilm formation, and are capable of destroying a *Staphylococcus aureus* biofilm. All of these effects are performed in a concentration-dependent manner. The antibiofilm effect is present in the protein fraction of *Tetragonisca angustula* honeys. The same set of Meliponini honey samples that were used in the present investigation had their botanical origin determined (up to species level) in a previous study by Zamora et al.[23] There are seven botanical species which comprised the main nectar sources of the *Tetragonisca angustula* honeys; five of them are shared with *Melipona beecheii* honeys.[23] The protein nature of the antibiofilm factors, and the melissopalynology results imply that the antibiofilm properties of *Tetragonisca angustula* honeys are of bee origin.

The protein fraction of *Tetragonisca angustula* honeys (HPF>12kDa) has no antimicrobial activity over *Staphylococcus aureus* planktonic cells. Nevertheless, its antibiofilm action allowed ampicillin to regain antimicrobial activity and vancomycin to recover its MIC value over a *Staphylococcus aureus* biofilm. This result suggests that the *Tetragonisca angustula* biofilm destruction factors disrupt the *Staphylococcus 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 *Tetragonisca angustula* honeys[9,23,44–46] demonstrate that this type of honeys could act along with antibiotics in antibiofilm wound healing therapies.

The antibiofilm proteins of *Tetragonisca angustula* do not have serine, cysteine or metalloprotease activity, and could only cleave DNA at high concentrations compared with DNase I. We hypothesise that the TABDFs have as targets polymer constituents of the EPS matrix of the *Staphylococcus 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 50kDa that is responsible of the amylase activity of *Tetragonisca angustula* honeys. In the biofilm destruction assay we tested five *Apis mellifera* honeys that expressed higher diastase (bee amylase) activity than the *Tetragonisca 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 75kDa. Although its target in the *Staphylococcus aureus* EPS biofilm matrix remains unknown, our data suggests that TABDF-2 should possess a mechanism of action over the *Staphylococcus aureus* biofilm different to TABDF-1.

Conclusion

Costa Rican *Tetragonisca angustula* honeys are capable of destroying an *Staphylococcus aureus* biofilm, via two proteins with the antibiofilm properties, TABDF-1 and TABDF-2. These proteins, most probably are of bee origin. The biofilm destruction factors of *Tetragonisca angustula* honey allowed ampicillin and vancomycin to recover their antimicrobial activity over a *Staphylococcus aureus* biofilm. Investigations of the sequence of the TABDFs and the proper identification of their targets inside the *Staphylococcus aureus* biofilm matrix are needed.

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

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