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Calorimetric investigation of the antimicrobial action and insight into the chemical properties of "angelita" honey—a product of the stingless bee *Tetragonisca angustula* from Colombia

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Abstract

Honey of the stingless bee *Tetragonisca angustula* Illiger, called "angelita" in Colombia, is known to be a valuable folk medicine against different ailments. Scientific data on this honey are rare in the literature. Due to the very low amount of honey produced by a colony and the presumed reputation of healing power, the honey price is high.

The antimicrobial activity of angelita honey obtained from Colombia was investigated by means of flow calorimetry and Petridish bioassay on six bacterial and three fungal species. The results were compared with that of *Apis mellifera* honey from Berlin. The test on the fungal species was done only with the Petridish method.

Fungi were not sensitive to the treatments with honey. The bacteria reacted differently with the response not related to their Gram reaction. Honey treated with catalase lost its antimicrobial activity, indicating hydrogen peroxide's remarkable role. Honey concentrations below a critical value resulted in a dense microbial growth around the inhibition zone.

Treatments with 20, 10, and 5% dilutions of angelita honey caused inhibition of bacterial growth in the calorimetric experiments. The percentage drop of the power–time (p–t) curve after honey application was dependent on the bacterial species and the concentration of honey used, the higher the concentration the larger the drop.

The less sensitivity or even insensitivity of fungi and the promotion of growth by weak honey concentrations should be born in mind while using this honey as an apitherapeutic agent.

Keywords: Honey; Angelita; *Tetragonisca angustula*; Antimicrobial activity; Microcalorimetry

1. Introduction

Honey is a natural food, rich in essential nutrients, produced by different species of bees. The medicinal properties of honey have been known since ancient times. There is a renewed interest in honey treatment as evidenced by the number of reports appearing in the scientific literature [1]. Due to its quality, it is used by human beings as food and as medicine for the treatment of various diseases.

Honey of the usual honeybee *Apis* spp. has been investigated as a therapeutic agent by several researchers and its physicochemical properties are well known. But honey from stingless bees that are distributed in the tropics and subtropics is less studied, even though it is locally collected and used against different ailments and supposed to be superior to *Apis* honey.

Stingless bees (family: Meliponinae), like honeybees (family: Apinae), are eusocial insects. They construct small nests in tree cavities or on branches, in termite and ant nests, under ground, or in artificial cavities, like walls and tombs in cemeteries [2,3]. The nest entrance is a clear, porous, soft wax tube about 8 mm in diameter (in *Tetragonisca angustula*) and of variable length, impregnated by resin. The nest consists of a brood area made of several horizontal disc

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shaped combs and a storage region that is located outside the brood area and is made of ovoid dark wax pots where the bees store nectar and pollen [4].

T. angustula, known in Colombia as "angelita", is one of the most common stingless bee species in South America and is distributed from Argentina up to Mexico at altitudes from 1000 to 1500 m above sea level. Honey from this bee is highly appreciated for its pleasant flavor and is used for the treatment of respiratory and eye infections (anti cataract properties) [4].

The objectives of the present work were to elucidate the antimicrobial activities of honey of *T. angustula* collected in Colombia against different bacterial and fungal strains. The antimicrobial activity of this honey were compared with that of *Apis mellifera* honey from Berlin.

2. Experimental

2.1. Materials

2.1.1. Honey source

The sample of *T. angustula* honey was bought from honey collectors at Pitalito (Huila) (01°52′03″N and 76°03′23″W) in Southwest Colombia, at an altitude of 1271 m above sea level.

2.1.2. Biological material

Bioassays of the antimicrobial activities of the different honey samples were performed using three filamentous fungi, *Aspergillus niger* (DSM 737), *Penicillium chrysogenum* (DSM 844), *Trichoderma viride* (DSM 63065); four species of Gram positive bacteria: *Bacillus subtilis* (DSM 347), *Micrococcus luteus* (DSM 348), *Bacillus megaterium* (DSM 90), *Bacillus brevis* (DSM 5609); and two species of Gram negative bacteria: *Escherichia coli* (DSM 31), *Pseudomonas syringae* (DSM 5176). The bacterial and fungal strains were bought from the German collection for microorganisms and cell cultures (DMSZ Braunschweig, Germany).

2.1.3. Growth media

All bacterial strains were grown in Standard I nutrient broth (Merck Lot VL 630582) and/or on Standard I nutrient agar (Merck, Lot VL 694681).

T. viride was cultivated on malt extract peptone agar (MEPA) composed of 30 g malt extract (Sigma, Lot 41k0181), 3 g soya peptone (Hy soy T, Sigma, Lot 128 H0184), 15 g Oxoid agar (Agar Bacteriological No.1, Lot 817706-2) in 1 L distilled water. *A. niger* and *P. chrysogenum* were cultivated on potato dextrose agar (PDA) composed of 20 g glucose (Merck), 15 g Oxoid agar (Agar Bacteriological No.1, Lot 817706-2), in 1 l potato infusion obtained by boiling 200 g scrubbed and sliced potato in 1 l water for 1 h and passed through a fine sieve.

All types of media were sterilized by autoclaving at 121 °C for 15 min.

2.2. Methods

2.2.1. Bioassay

2.2.1.1. Preparation of the honey samples. The honey samples were serially diluted with distilled water to 1:1 (50%), 1:5 (20%), 1:10 (10%), 1:20 (5%), and 1:50 (2%) and used for the determination of total antimicrobial activities. The letter T is assigned to the total antimicrobial activity followed by a subscript of the letter for the name of the corresponding honey sample: C or D for honeys from Colombia and Germany, respectively. Therefore, the total antimicrobial activity of these honey samples is hereafter referred to as T_C and T_D. By analogy the corresponding non-peroxide (NP) antimicrobial activities of the two honey samples are referred to as NP_C and NP_D.

In order to remove H_2O_2 and elucidate the non-peroxide antimicrobial activity, the honey samples were treated by diluting them 1:1 with a 20,000 U ml^{-1} catalase solution modified slightly after Molan and Russell [5]. The latter solution was prepared by dissolving a 13,300 U mg^{-1} solid catalase from bovine liver (Sigma, Lot 32k 7031, E.C. 1. 11.1.6) in distilled water. The catalase treated honey, which was already diluted to 50% due to the treatment, was diluted further to 20, 10, and 5%, and used in the Petridish bioassay.

2.2.2. Petridish bioassay

2.2.2.1. Bacteria. An isolated pure colony of an overnight grown strain was picked carefully using a sterile transfer loop, inoculated to a nutrient broth in an Erlenmeyer flask and grown overnight at 30 °C. About 50 μl of the overnight culture were inoculated to a 20 ml solution of nutrient broth and grown further for about 3–5 h until an o.d. of 0.6 (546 nm) was achieved (method slightly modified after Faye and Wyatt [6]). The suspension was then diluted 1:50 with the corresponding nutrient broth in order to prepare the standard inoculum.

The sterilized nutrient agar was cooled to 48 °C, 5 ml of the standard inoculum were mixed with the 1 l nutrient agar and poured into plastic Petridishes of ∅ = 85 mm diameter, 10 ml in each. When the agar was solidified three holes were bored per Petridish using a cork borer of ∅ = 9 mm. Each hole was then filled with 50 μl honey of a certain dilution and the Petridishes were placed in a refrigerator for 24 h, giving the honey enough time to diffuse. Finally, the plates were removed from the refrigerator, incubated at 30 °C for 24–48 h and the inhibition zones measured. Each concentration of both honey samples was investigated in three Petridishes with three holes per Petridish, i.e. $n = 9$.

2.2.2.2. Filamentous fungi. An isolated pure colony of a fungal culture which was grown for 72 h on solid medium and started to sporulate was scrubbed up with a sterile transfer loop and placed in 5 ml sterile distilled water in a test tube. The hyphae were disintegrated by adding sterile glass

Table 1
Average composition of *Tetragonisca angustula* and *Apis* honey samples

Component	Method of analysis	Angelita honey	*Apis* honey
Water (%)	Refractometer	20.5	16.4
Proline (mg kg^{-1})	FT-IR	770	347
Total acidity (meq. kg^{-1})	Titration/FT-IR	24	14.7
Invertase activity (U s)	Enzyme test (DIN)	115	16.3
HMF (mg kg^{-1})	Winkler method	10.3	8.6
Fructose (g%)	HPLC/FT-IR	36.1/37.6	36.6
Glucose (g%)	HPLC/FT-IR	29.8/31.8	28.9
pH	pH-meter/FT-IR	4.2/4.1	4.9
Sucrose (g%)	HPLC/FT-IR	n.d.	3.29
Total sugars (g%)	HPLC/FT-IR	73.9	74.1
Reducing sugars (g%)	HPLC/FT-IR	65.9	65.5
Electrical conductivity (mS cm^{-1})	Conductometer	n.d.	n.d.

n.d., not detectable.

Table 2
Antimicrobial activity of 50% dilution of *Trigona* (*Tetragonisca*) *angustula* and *Apis* honey samples elucidated by the inhibition zone diameter (mm, mean ± S.D.)

Organism	T_D	T_C	NP_D	NP_C
B. brevis	7.3 ± 2.3	18 ± 3.6	10.3 ± 2.1	n.d.
B. megaterium	7.0 ± 1.0	2.0 ± 0.0	6.0 ± 1.0	n.d.
B. subtilis	42.3 ± 9.3	13 ± 1.0	46.7 ± 4.2	n.d.
M. luteus	42.0 ± 2.0	3.1 ± 0.7	41.7 ± 2.1	n.d.
E. coli	21.7 ± 2.9	2.8 ± 0.0	20.3 ± 0.6	n.d.
P. syringae	7.2 ± 0.8	10.3 ± 0.6	10.0 ± 2.0	n.d.
A. niger	n.d.	n.d.	n.d.	n.d.
P. chrysogenum	n.d.	n.d.	n.d.	n.d.
T. viride	n.d.	n.d.	n.d.	n.d.

First letters denote the total activity (*T*) or non-peroxide activity (NP) and the subscripts indicate the origin of each sample; C, Colombia D, Germany; n.d., not detectable.

beads and shaking vigorously for 1–2 min, in order to get a uniform suspension. The 5 ml suspension was added to a 1 l agar solution at 48 °C and the procedure mentioned above followed further. The plates were taken out of the refrigerator, left at room temperature (ca. 25 °C) for 72 h and the inhibition zones measured.

2.2.3. Microcalorimetric experiments

Calorimetric experiments were performed at a temperature of 30 °C using a flow calorimeter with a vessel volume of 0.587 ml (type 10700-1, LKB, Bromma, Sweden). The calorimeter was connected to an external fermenter, a 50 ml reaction vessel with 20 ml nutrient broth, by a Teflon tube of $\varnothing = 0.1$ cm inner diameter. The fermenter was placed in a water bath at a temperature of 30 °C. The bacterial culture circulated from the fermenter to the calorimeter and back to the fermenter by means of a peristaltic pump (LKB Pharmacia, Bromma, Sweden) at the outlet of the calorimeter in a sucking mode. Settling of cells and depletion of oxygen in the fermenter was avoided by vigorously stirring the culture with a magnetic stirrer. Pumping rate was 56 ml h^{-1}.

2.2.4. Chemical composition

Analysis of the chemical composition of the honey samples was performed in the bee research institute "Länderinstitut für Bienenkunde Hohen Neuendorf" Germany following the methods described in [7]. The different methods used in the chemical analysis of the honey samples and the corresponding results are tabulated in Table 1.

3. Results

Comparison of the chemical composition of honey from *T. angustula* and *A. mellifera* shows differences in most aspects. Humidity, electrical conductivity, free acids, pH, proline, and HMF (hydroxymethylfurfural) values were higher in *T. angustula* than in *A. mellifera* honey. Total and reducing sugars were almost the same and invertase and sucrose content were higher in *A. mellifera* honey (see Table 1).

The experiments with *T. angustula* honey did not show inhibitory non-peroxide action on any bacterial or fungal species tested. As an example, the activities of 50% concentrations of both honey samples are displayed in Table 2. None of the investigated strains showed any differences between T_D and NP_D of *Apis* honey.

The total activity T_C was highest against *B. brevis* and lowest against *B. megaterium*. In case of *B. megaterium* and *M. luteus* there were dense bacterial growth zones around the inhibition zones with *T. angustula* honey at a concentration of 20, 10, and 5%. Fig. 1 compares the effects of a high honey concentration (left) and a lower concentration (right). Both plates exhibit three different levels of microbial growth: an undisturbed mean "background" growth throughout the plate, the clear halos around the holes due to growth inhibition, and ring-like zones of stimulated growth around the inhibition halos. These stimulation zones would form concentric rings around the bore-holes in case of no honey inhibition. The halos with undiluted angelita honey (left) show diameters of about 3.5 cm, the right ones with a 50% honey dilution of about 1.7 cm. The o.d. of the stimulated-growth zones amount to about 4.4 (left) and 3.1 cm (right). Assuming honey concentration decreasing by diffusion after a 1/(radius)^2-rule, all four values had corresponding minimum figure of honey concentration provoking growth inhibition or growth stimulation. Such effects as seen with angelita honey were never observed with *Apis* honey. The two holes at the lower part of the dish present no stimulating or inhibiting action of the control liquid (sugar syrup).

Fungi were insensitive to both honey types. The response of the different bacterial species to the treatment with a certain honey sample is not dependent on the Gram reaction of the bacteria, both Gram positive and Gram negative bacteria responding to the treatments. The reaction of the bacterial species are rather dependent on the type of honey and the individual organisms within a category (Table 2).

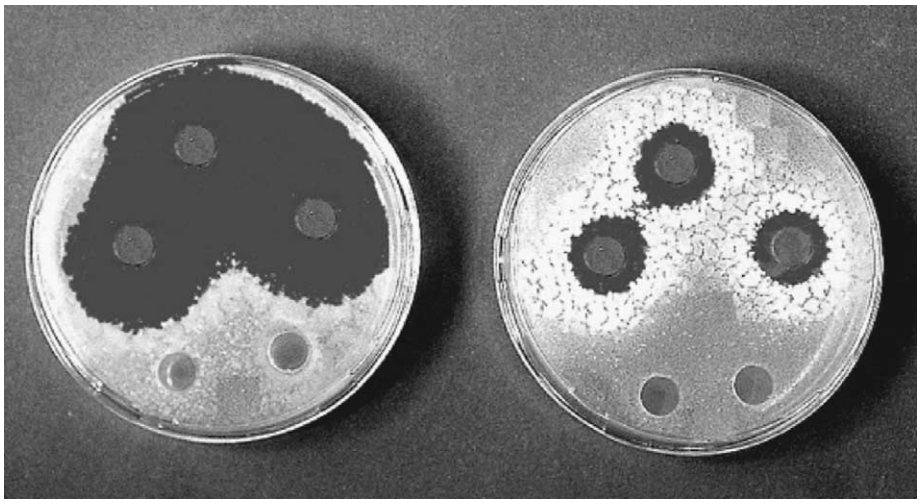

Fig. 1. Petridish investigations with different concentrations of *T. angustula* (angelita) honey and *B. megaterium*. The larger halos at high concentrations (left) and the smaller ones at low ones (right) are evident, also the rings of stimulated growth due to hormesis. Corresponding sugar concentrations produced no halos hormesis (lower part of the dish).

Microcalorimetric experiments render instantaneous, kinetic information about all processes in the calorimeter while Petridish investigations are integrating over time periods of one or more days. The power–time (*p*–*t*) curves of a growing microbial culture show the typical three main growth phases: lag, log or exponential and finally stationary phase. The log phase is the period of most intensive growth with the steepest increase in heat dissipation. This time was used for the honey application. Fig. 2 exhibits this situation for a growing culture of *B. subtilis* without and with honey injection. The exponential increase in power output is clearly seen, while the following steep descend is due to an instrumental artefact. During the flow from the well-aerated fermenter to the calorimeter oxygen becomes limiting leading to a strong reduction of metabolic activity. But as the honey samples are added about 1 h before the maximum, this reduction is without influence in the present investigations.

When concentrations of 20, 10, and 5% of *T. angustula* honey are added to the culture in the logarithmic phase, an immediate drop in the heat production rate is observed, usually followed by a new increase (Fig. 2). This behavior is due to a dilution effect by the honey and not to an inhibition of metabolism. But the further increase in heat output is less steep than before the honey injection indicating a reduced growth rate of the bacterial cells and thus an inhibition by the honey. This effect is most pronounced in curve “d”. Similar effects are also seen for *B. megaterium* (Fig. 3).

The situation is somewhat different with the facultatively anaerobic bacterium *E. coli*: after the steep drop of the heat production rate due to the oxygen depletion in the flow line the microbes switch over to an anaerobic metabolism after a

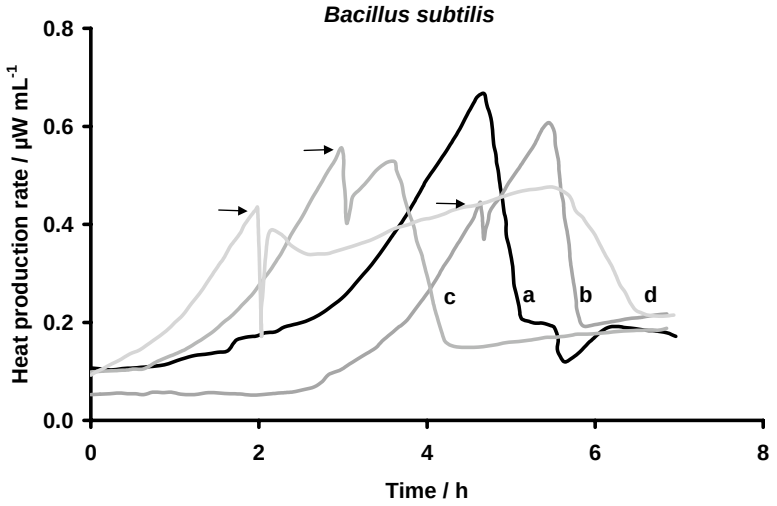

Fig. 2. Power–time curves of growth for the Gram positive bacterium *B. subtilis* and the effect of angelita honey (arrows) on their structure. (a) Control curve without honey, and (b) with 5%, (c) with 10% and (d) with 20% angelita honey.

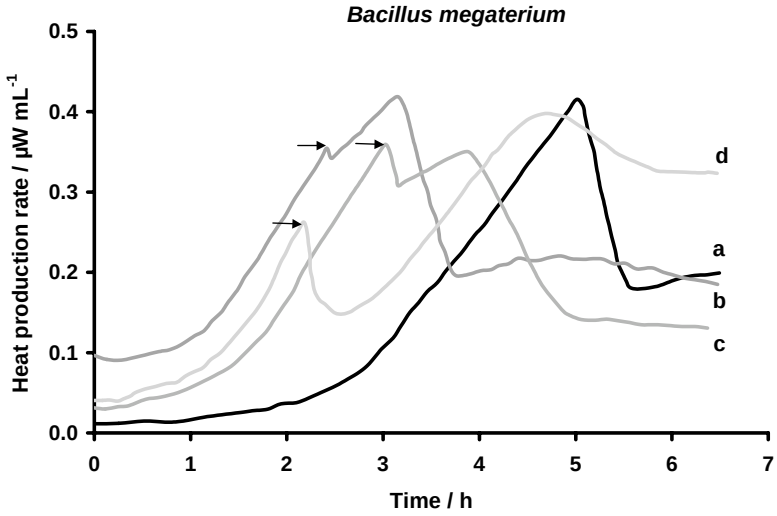

Fig. 3. Power–time curves of growth for the Gram positive bacterium *B. megaterium* and the effect of angelita honey (arrows) on their structure. (a) Control curve without honey, and (b) with 5%, (c) with 10%, and (d) with 20% angelita honey.

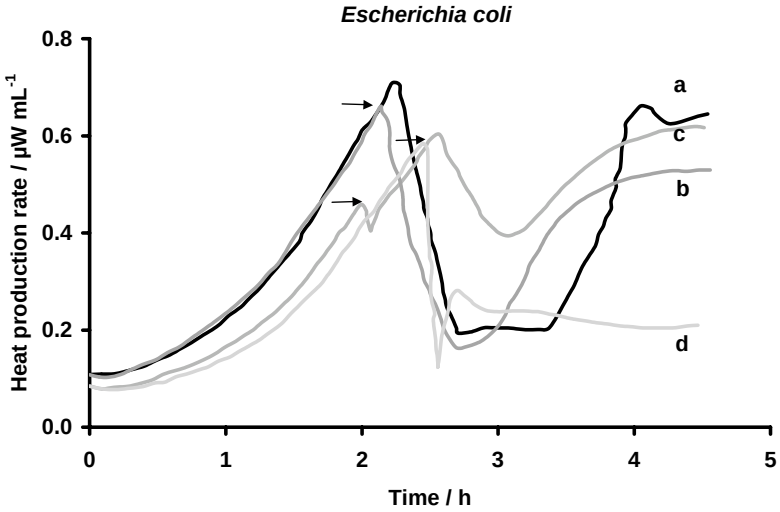

Fig. 4. Power–time curves of growth for the Gram negative and facultatively anaerobic bacterium *E. coli* and the effect of angelita honey (arrows) on their structure. (a) Control curve without honey, and (b) with 5%, (c) with 10%, and (d) with 20% angelita honey.

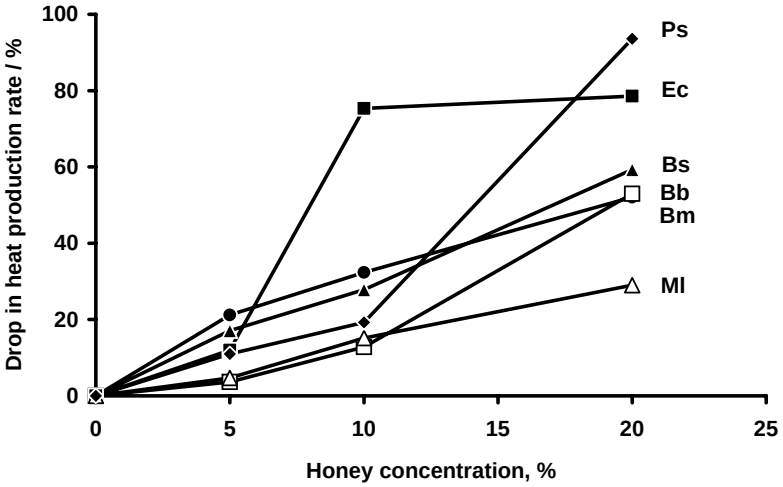

Fig. 5. Percentage drop in the heat production rate after the addition of different amounts of angelita honey for growing cultures of (Bs) *B. subtilis*, (Ml) *M. luteus*, (Bm) *B. megaterium*, (Bb) *B. brevis*, (Ec) *E. coli* and (Ps) *P. syringae*.

transition period and the heat production increases again to a later plateau (Fig. 4 curve "a"). Only at the highest honey concentration of 20% the growth of the cells is stopped and many are killed, so that the heat output rests on a very low level (curve "d"). The calorimetric curves for all investigated strains show that kinetic data about the action of honey can be obtained already after 1–2 h so that a quick screening is possible with this method.

The calorimetric results point to the fact that the dose response curves for the different bacterial species are similar though the degree of sensitivity differs highly (Fig. 5). After the treatment with 20% angelita honey *E. coli* showed the largest sensitivity with the drop in heat production rate approaching 100%. But the same treatment reduced the heat production rate of *M. luteus* by about 30% only.

4. Discussion

The precise composition of honey varies according to the plant species on which the bee forages, but the main constituents are the same in all honeys. More than 95% of the solids in honey are carbohydrates, and sensitive analytical and separation techniques have revealed that honey is a highly complex mixture of sugars, most of which are in an immediately digestible form for the small intestine.

The predominant acid found in honey is gluconic acid. It originates largely from the activity of glucose oxidase, which the bees add at ripening the honey [8] and to a lesser extent from the bacterial action [9]. The main enzymes found in honey, which are derived from the hypopharyngeal glands of worker honeybees, are invertase (which inverts sucrose to glucose and fructose); glucose oxidase (which oxidizes glucose to gluconic acid and hydrogen peroxide in the presence of water); and amylase (diastase) which breaks down starch.

The enzyme glucose oxidase is of considerable interest because its activity causes the production of hydrogen peroxide, which has a microbicidal action against potential spoilage bacteria.

The water content is an important criterion for evaluating the grade of ripeness and the shelf-life of honey [10]. The water content of angelita honey was higher in comparison with *Apis* honey. This is mainly because stingless bees collect small amounts of honey and use it faster than ripening and storing it. But in case of *Apis* spp., since they collect large amount of honey which they can not utilize immediately, they ripen (remove water) and store it in the hive for long periods. In spite of the high moisture content, angelita honey does not ferment in the nest or the shelf due to the abundance of hydrogen peroxide and to possible antimicrobial resins that impart dark color to this honey.

Besides moisture, the content of proline is also used as an appropriate parameter for identifying whether honey is ripe. According to a previous investigation [11], there should be at least 200 mg proline kg^{-1} ripe *Apis* honey. Our results show that both honeys are ripe though to different extents. In addition to that, angelita honey has a two fold proline content compared to *Apis* honey presumably due to the more intensive labor of the bees on the collected nectar by adding gland secretions [12].

Combination of HMF (maximum 40 mg kg^{-1}) content and diastase activity (<40 °G) is widely used for evaluating the freshness of honey [13,14]. Both samples can be classified as fresh pure honeys.

As stated by Auerbach and Bodlander [15], honey samples with a fructose/glucose ratio of less than 1.0 should be classified as adulterated honeys. According to that, the fructose/glucose ratio for angelita (1.21) and *Apis* honey (1.27) show that both were not adulterated.

The activity of *T. angustula* honey against the chosen bacterial species was mainly due to the presence of hydrogen peroxide, whose removal resulted in the normal growth of all bacteria without any detectable inhibition. This indicates that hydrogen peroxide plays a significant role in the bactericidal/bacteriostatic action. Our results point to the fact that honey from stingless bees possesses considerable amounts of hydrogen peroxide that could be responsible for preventing the fermentation of this honey which otherwise would have taken place due to its higher amount of water (20.5%). The *Apis* honey tested did not show a recognizable hydrogen peroxide based activity. One possible reason could be that the honey was very ripe because it was stored for 1 year.

White and Subers [16] reported that hydrogen peroxide produced by the glucose oxidase of honey could be the inhibitory substance against bacteria. However, it is known that honey itself as well as bacteria produce a catalase which eliminates hydrogen peroxide. Although catalase is active at high hydrogen peroxide concentrations, it is of low activity at physiological levels. The amount of catalase necessary to destroy the antibacterial activity of hydrogen peroxide was found to be unexpectedly high [8,17]. The hydrogen peroxide concentration remains stable under a given set of conditions of temperature and sugar concentration and is sufficiently high to give good protection against some harmful microorganisms by a biochemical mechanism that disrupts their metabolism. The same system is thought to operate when honey is diluted with water and for this reason honey has been successfully used as a microbicidal wound dressing. The lack of hydrogen peroxide activity of *Apis* honey demonstrates that the antibacterial activity of this honey is mainly due to the phytochemical components or to lysozyme. The acidity of this honey (pH = 4.06) is not strong enough to inhibit the growth of most bacteria as it is further diluted and weakened in the growth medium.

Phenomena like the rings of stimulated growth around distinct inhibition halos are well known in biology and called hormesis [18]. Sub harmful doses of some harmful agents may it be ionizing radiation or a chemical NOx, e.g.—can provoke stimulatory responses in a living organism. In the present work, the angelita honey inhibiting growth at high concentrations supports development at lower ones.

Kinetics of the *p–t* curves before and after treatment with honey displays the extent of antimicrobial action of concentrations that do not have bactericidal or total bacteristatic activities and hence could not be detected by other methods such as the Petridish bioassay. As most non-calorimetric methods display the effect of honey after an incubation for a day or more, antimicrobial activities can be detected if and only if the antimicrobial agent provokes complete inhibition. For this reason, the microcalorimetric experiments performed on bacteria underline that this method is highly suited for quick screening of antimicrobial honey activities and renders insight into the kinetics and the way of action on metabolism. The percentage drop in the heat production rate of the different bacterial strains shows a correlation with the concentration of *T. angustula* honey, the drop in the heat production rate increasing with ascending concentration.

In general the response of the various bacterial species to the treatments with lower honey concentrations was independent of the Gram reaction of the bacteria. But the treatment with the highest concentration of angelita honey (20%) displayed that the Gram negative bacteria were more sensitive than the others (Fig. 5). One possible explanation could be that some antimicrobial components in the honey act on the outer membrane of the Gram negative bacteria, making it easily permeable and thus open the access to the cell membrane, the most sensitive cell protecting structures. Such components may occur at lower concentrations in honey and may be negligible in highly diluted honeys.

5. Conclusions

The differences in chemical composition of *T. angustula* and *Apis* honey are probably due to the visited sources and the specific ways in the processes of transforming and storing honey.

The traditional use of *T. angustula* honey against different infections is plausible if they are caused by bacteria and not by fungi, as the later are insensitive to treatments with this honey.

The enhancement of growth of *B. megaterium* and *M. luteus* by lower concentrations of honey indicates that the use of honey at very low concentrations could be dangerous as a means of apitherapy. However, if used undiluted, *T. angustula* honey offers many possibilities as a broad-spectrum healing agent against both Gram positive and Gram negative bacteria. Especially, the use of this honey as a wound dressing agent may be effective since its dilution by body fluids is less intensive than for internal administration.

The strength of the antibacterial activity of *T. angustula* honey depends on the presence of hydrogen peroxide.

The percentage drop in the heat production rate of Gram negative bacteria after treatment with 20% *T. angustula* honey is higher than that of Gram positive bacteria.

Though it is premature to conclude at this stage that stingless bee honey can be used as a multifaceted drug, as claimed by the local people, it can be ascertained that it has a broad spectrum of antimicrobial actions inhibiting the growth of both Gram positive and Gram negative bacteria, but not of fungi.

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