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INHIBITION OF PATHOGENS BY SPOROGENIC BACTERIA ISOLATED FROM HONEY OF *Melipona* sp. (APIDAE: APINAE: MELIPONINI)[1]

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ABSTRACT - The aim of this study was to isolate sporogenic bacteria from the honey of stingless bees *Melipona* sp., in dry forest, and to evaluate their antagonistic potential for medicinal employment purposes and animal production. The honey samples were collected in Serra Talhada-PE, where honey was taken from four different hives (in triplicate), totaling 12 samples. The samples were diluted and subjected to 80 ºC for 20 minutes to eliminate vegetative cells. The dilutions were plated onto nutrient agar and incubated at 30 ºC for 72 hours. Then the colony forming units (CFU) were quantified. The samples were also plated onto malt agar and Sabouraud agar, and incubated at 30 ºC for 14 days for the growth of yeast and molds. Total and fecal coliforms were quantified by the most probable number method (MPN). Seven isolates (I) of sporogenic bacteria (*Bacillus*) were obtained, however only four showed probiotic potential. Isolate I-5 showed the greatest probiotic potential and inhibited the growth of *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa, Salmonella* sp., and *Staphylococcus aureus*. The growth of the *Sarcina* sp. was not inhibited by any isolate. No yeast, molds or coliforms were found. The *Melipona* sp. honey is a source of spore-forming bacteria and is antagonistic to microorganisms that contaminate honey. It has good microbiological quality.

Keywords: Stingless bee. *Bacillus*. Caatinga.

INIBIÇÃO DE PATÓGENOS POR BACTEIAS ESPORULANTES ISOLADAS DE MEL DE *Melipona* sp. (APIDAE: APINAE: MELIPONINI)

RESUMO - Este trabalho teve como objetivo isolar bactérias esporulantes a partir do mel de *Melipona* sp. em ambiente de caatinga e avaliar o seu potencial antagonista com vistas à aplicação na medicina e produção animal. As amostras de méis foram coletadas no município de Serra Talhada-PE, onde o mel foi retirado de quatro colméias diferentes (em triplicata), totalizando 12 amostras que amostras foram diluídas (10^{-1} a 10^{-5}) e submetidas a 80 ºC por 20 minutos e resfriadas a 5 ºC para indução de esporos e eliminação de células vegetativas. Em seguida, as diluições foram semeadas em Agar Nutriente e incubadas a 30 º por 72 horas para contagem das unidades formadoras de colônias (UFC). Antes do tratamento térmico foram realizados testes para verificar a presença de fungos filamentosos, leveduras e coliformes totais e termotolerantes. As diluições foram semeadas em Agar Malte e Agar Sabouraud e incubadas a 30 ºC por 14 dias. Coliformes totais e termotolerantes foram quantificados pelo método do número mais provável (NMP). Foram obtidos sete isolados (I) de bactérias esporulantes (*Bacillus*), porém apenas quatro mostraram algum potencial antagonista. O isolado I-5 apresentou o maior potencial antagonista, inibindo o crescimento de *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa, Salmonella* sp. e *Staphylococcus aureus*. Apenas *Sarcina* sp. não foi inibida. Não foram encontrados fungos filamentosos, leveduras ou coliformes nas amostras. O mel de *Melipona* sp. é uma fonte de bactérias esporulantes resistentes a elevadas temperaturas e com potencial antagonista a micro-organismos contaminantes e apresenta boa qualidade microbiológica.

Palavras–chave: Abelhas sem ferrão. *Bacillus*. Caatinga.

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INTRODUCTION

In Brazil, honey from native bees has commonly been used to heal many bacterial and fungal diseases, as well as being a scar promoter and antioxidant (GONÇALVES et al., 2005). *In vitro* assays showed that honey from *Nannotrigona testaceicornis* and *Tetragonisca angustula* bees inhibited the development of *Bacillus* sp., *Escherichia coli*, *Proteus* spp., *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, coagulase-positive *Staphylococcus* spp. and yeasts (GONÇALVES; ALVES FILHO; MENEZES, 2005; BOBANY et al., 2010).

On the other hand, several microorganism species can be found in the nests of stingless bees, especially bacteria, such as *Bacillus*, and filamentous fungi and yeast, which might contaminate not only honey, but also pollen, larvae food, and the intestines of the worker bees. In pollen and larvae food, several microorganism species provide digestive enzymes, which participate in the pre-digestion of food stores, and also organic acids and antibiotics, which inhibit the development of competitor microorganisms (GILLIAM; ROUBIK; LORENZ, 1990).

The bee intestine can carry many microorganism species, of which 1% are yeast, 27% are Gram-positive bacteria, including *Bacillus, Bacteridium, Streptococcus,* and *Clostridium*, and 70% are Gram-negative bacteria, including *Escherichia*, *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Flavobacterium*, and *Proteus* (OLAITAN; ADELEKE; OLA, 2007).

Some bacteria and fungi species secrete substances that are antagonistic to other microorganisms and that have been used as probiotics against enteric diseases of man and animals (MELLO, 2012). The discovery of new natural sources of antibiotics is important, thus a great diversity of microorganisms living in many environments, especially those environments understudied, have been explored (GUIMARÃES; MOMESSO; PUPO, 2010). Due to the large number of bacteria that are resistant to commonly used antibiotics, particularly those responsible for hospital infections, many researchers are seeking new natural biologically active substances that play roles in the control of infectious diseases (GUIMARÃES; MOMESSO; PUPO, 2010).

The bacteria that are currently used as probiotic producers are *Bacillus*. Despite not being considered indigenous to the intestine, spore-producing bacilli are capable of growing and surviving in the environment and in the animal's intestine. The sporulation ability confers on them a greater resistance to digestive enzymes during intestinal transit (MELLO, 2012). Rocha et al. (2010) used *B. subtilis* and a commercial mixture of *Lactobacillus plantarum, L. bulgaricus, L. acidophilus, L. rhamnosus, Bifidobacterium bifidum, Streptococcus thermophilus*, and *Enterococcus faecium* as probiotics in broiler feed, and concluded that the birds showed better breast growth than those fed with additive-free feed.

Many organic acids produced by microorganisms, such as acetic (REZENDE et al, 2008), fumaric, and propionic (ROCHA et al., 2010) have been used as antibiotic substitutes in the feed of broilers and laying hens. Bassan et al (2008) controlled *Salmonella enteritidis* infection in broilers by adding formic and propionic acids, and a mannan oligosaccharide, to the feed of infected chicks.

The aim of the present study was to isolate sporogenic bacteria from *Melipona* sp. of the Caatinga (Brazilian dry forest) and to evaluate their antagonistic potential against other pathogenic bacteria, as well as their use in human health and animal production. The microbiological quality of honey was tested by determining the presence of filamentous fungi, yeast, and total and thermotolerant coliforms, to check whether it reached the safety requirements to be used as food for humans.

MATERIAL AND METHODS

Honey samples were collected from four *Melipona* sp. beehives in a natural environment of a Caatinga area, near to the PE 390 roadway in the Serra Talhada municipality (08º06.067 to 08º05.756 S and 30°20,844 W). Beehives were housed in trunks of "catingueira" (*Caesalpinia pyramidalis*) trees taken from the natural environment for the construction of the Pajeú pipeline. Beehives were sheltered in beekeeping boxes and transferred to the apiary of the Universidade Federal Rural de Pernambuco/Academic Unit of Serra Talhada. Three pots of honey were collected from each beehive, making a total of 12 samples.

Initially, the pots were washed with 70% sodium hypochlorite and then with sterile distilled water. Then the contents of the pots were aseptically removed using disposable syringes, and then subjected to decimal serial dilutions in saline solution (0,85%). The presence of fecal and thermotolerant coliforms was determined by the most probable number (MPN) method at 95% of confidence (SILVA; JUNQUEIRA; SILVEIRA, 2001). The dilutions were seeded onto Sabouraud agar (Himedia®) and malt agar (Himedia®), and incubated at 30 ºC for 14 days to evaluate growth and to count the colony forming units (CFU) of filamentous fungi and yeast. The dilutions were subjected to hyperthermia (80 ºC) for 20 minutes followed by water cooling at 5 ºC to activate spores and eliminate vegetative cells, adapting the methods described by Vittori et al. (2008). Aliquots were then seeded in 9 cm Petri plates, with nutrient agar (Himedia®), and incubated at 30 ºC for up to 72

hours for the CFU counting.

The CFU were morphologically characterized according to color, texture, the elevation and edges of colonies, and also the shape, arrangement, and size of the cells. The isolates (I) were grown on Nutrient Broth (Himedia®) and incubated at 30 ºC for up to 72 hours to verify the ring or sediment formation. Gram and spore staining procedures were performed, and the ability of the isolates to ferment glucose via the butylene glycol pathway, and to produce acetylmethylcarbinol, were assessed. Starch hydrolase and Voges-Proskauer biochemical assays were tested (GARRITY et al, 2004). The catalase assay was performed to evaluate the capability of the isolates to hydrolyze hydrogen peroxide. The ability to use citrate as sole carbon source and ferment glucose were tested using phenol red and methyl red, respectively, as pH reduction indicators. The reduction of nitrate to nitrite, the ability to grow in nutrient broth containing 6.5% NaCl, and the ability to ferment arabinose, were also assessed.

Each isolate was subjected to the agar block antagonism assay, as previously described by Stern et al. (2006) and Bonfim (2010), using de Man-Rogosa-Sharpe (MRS) agar (Himedia®). First, isolates were diluted in 0.85% saline solution to a final concentration of 1 x 10^8 cells/mL, and then seeded in triplicate onto dishes containing MRS agar. Dishes were incubated at 37 ºC for 24 to 36 hours until a layer of cells formed. After the cells had grown, 6 mm disks of MRS agar were cut out. *Escherichia coli*, *Klebsiella* sp., *Sarcina* sp., *Pseudomonas aeruginosa, Salmonella* sp., and *Staphylococcus aureus* test microorganisms were diluted in 0.85% saline solution to a final concentration of 1 x 10^8 cells/mL, and then seeded in triplicate onto Mueller-Hilton (MH) agar (Himedia®). The isolate culture agar disks were inverted onto the MH agar surface, previously seeded with the test microorganisms. MRS agar disks without cultures were used as controls. Dishes containing disks and the test microorganism cultures were incubated at 37 ºC for 24 to 36 hours. After that, the dishes were examined for inhibition ring formation, whose diameters were measured and classified according to Matsuura (2004): low (7–10 mm), moderate (11–14 mm), and high (above 14 mm) inhibition capability.

RESULTS AND DISCUSSION

The density of sporogenic bacteria varied from 200 to 260 CFU/g of honey. Seven isolates (I1–I7) were obtained, all aerobic, mesophilic, Gram -positive, resistance spore producers, and belonging to the *Bacillus* genus. Isolates were present in nine of

Table 1. Distribution of isolates from honey pot samples (1 to 12) from four beehives (A, B, C, and D) of *Melipona* sp. in a Caatinga area in the Serra Talhada - PE municipality.

BEEHIVE	HONEY POT	ISOLATE
A	1	None
A	2	None
A	3	None
B	4	I-1
B	5	I-2
B	6	I-3
C	7	I-4
C	8	I-5
C	9	I-4
D	10	I-5
D	11	I-6
D	12	I-7
Total	12	7

I = Isolate

Bacillus bacteria are allochthones to the honey of indigenous stingless bees; their presence in honey is due to the feeding habit of the bees and the collection of material such as clay and animal excreta for the maintenance of the nest structure, as there are no scientific reports of a co-evolutionary association between *Melipona* sp. bees and bacteria. The absence of sporogenic bacteria in the honey of colony A might be related to the type of food source that workers had been visiting at the time of the sample collection. Besides, colony A had a large number of subjects in a well-structured nest, where no clay or excreta had recently been collected.

Biochemical assays are essential for phenotypic characterization and are also important tools for species identification. Isolate I-5 produced organic acid from arabinose fermentation, a usual characteristic of *B. alcalophilus* and *B. circulans*, however none of the isolates performed acid production by glucose fermentation (Table 2), excluding the possibility of I-3, I-4, and I-6, which did not produce tumid spores, being classified as *B. insolitus*. This microorganism also does not produce tumid spores, but is able to ferment glucose and produce acid, which is an important characteristic that differentiates it from *B. marinus*.

Table 2. Biochemical characterization assays of *Bacillus* sp. isolated from *Melipona* sp. honey from a Caatinga in the Serra Talhada - PE municipality.

Assay	Isolate						
	I-1	I-2	I-3	I-4	I-5	I-6	I-7
Starch hydrolysis	+	-	-	-	+	-	+
Voges Proskaeur	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	-
Citrate	-	-	-	-	-	-	-
Glucose fermentation with acid production	-	-	-	-	-	-	-
Glucose fermentation with gas production	-	-	-	-	-	-	-
Nitrite reduction	-	-	-	-	-	-	-
6.5% NaCl growth	+	-	-	-	+	-	-
Arabinose fermentation	-	-	-	-	+	-	-
Methyl red	-	-	-	-	-	-	-
Tumid spores	+	+	-	-	+	-	+
Probable species	*B. brevis*	*B. sphaericus*	*B. insolitus*	*B. badius*	*B. circulans*	*B. marinus*	*B. pantothenicus*

Positive (+), Negative (–).

Organic acids decrease the pH of the medium, inhibiting the growth of contaminant microorganisms (REZENDE et al., 2008; ROCHA et al., 2010). The absence of glucose fermentation by the isolates indicates that bacteria do not consume the glucose present in honey, because honey is stored in the beehive in pots sealed with wax at the operculum. This sealing promotes an oxygen-free environment which leaves anaerobic metabolism as the only energy source for growth. Glucose fermentation with organic acid production is an important characteristic for the animal industry, since many of these acids have been tested as antibiotic substitutes added to the feed of broiler and laying hens (BASSAN et al., 2008; REZENDE et al., 2008; ROCHA et al., 2010).

Isolates I-1, I-5, and I-7 were able to hydrolyze starch, resulting in the production of amylase. Catalase, an important enzyme that plays a role in the oxidative stress response, was produced by 85.7% of the isolates. Only 28.6% of the isolates were able to grow in the presence of 6.5% NaCl, which indicates that bacteria in these isolates have a response system against osmotic stress (Table 2).

Isolate I-5 inhibited the growth of all tested bacteria except *Sarcina* sp., displaying good antagonistic potential. On the other hand, I-1 and I-2 inhibited *E. coli*, and I-3 inhibited *Pseudomonas aeruginosa* (Table 3).

Table 3. Test of inhibition of growth of bacterial species by *Bacillus* sp. isolated from *Melipona* sp. honey samples from a Caatinga area in the Serra Talhada - PE municipality.

Isolate	Inhibited species
I-1	*E. coli*
I-2	*E. coli*
I-3	*P. aeroginosa*
I-4	None
I-5	*E. coli, P. aeruginosa*e, *Salmonella* sp., *S. aureus, Klebisiella* sp.
I-6	None
I-7	None

E. coli growth was inhibited by three isolates (I-1, I-2, and I-5; 42.86%), while *P. aeruginosa* growth was inhibited by two isolates (I-2 and I-5, 28.57%). On the other hand, *Salmonella* sp., *Klebsiella* sp., and *S. aureus* were inhibited only by I -5, and *Sarcina* sp. was not inhibited by any isolate. The antagonistic reaction to *E. coli* might produce false negative results in the estimation of fecal contamination of the honey, since this bacterium has largely been referenced as the main indicator of food and water contamination by fecal material (SILVA; JUNQUIERA; SILVEIRA, 2001). The presence of I-1 and I-2 can mask the presence of *S. aureus*, *P. aeroginosa*, *Salmonella* sp., *Klebsiella* sp., and *Sarcina* sp. in the honey of native stingless bees, because the isolates inhibited growth of the main contaminant but did not show antagonism to the tested pathogens.

The antagonistic potential of the isolates was determined by measuring the inhibition rings diameters, according to Matsuura (2004) (Table 4).

Table 4. Antagonistic potential of isolates, determined by measurement of the inhibition zone (in mm) against the indicators and pathogens tested by the agar block technique.

ISOLATE	INDICATORS OR PATHOGENS (inhibition zone in mm)					
	E. coli	*Klebsiella* sp.	*Sarcina* sp.	*P. aeruginosa*	*Salmonella* sp.	*S. aureus*
I-1	11.2	0.0	0.0	0.0	0.0	0.0
I-2	10.3	0.0	0.0	0.0	0.0	0.0
I-3	0.0	0.0	0.0	20.3	0.0	0.0
I-4	0.0	0.0	0.0	0.0	0.0	0.0
I-5	24.0	14.5	0.0	10.6	14.2	14.9
I-6	0.0	0.0	0.0	0.0	0.0	0.0
I-7	0.0	0.0	0.0	0.0	0.0	0.0

Results represent the averages of triplicates.

Isolate I-5 showed strong antagonism to most of the tested pathogens, making inhibition rings larger than 14 mm (high inhibition), except to *P. aeruginosa*, where the ring was 10.6 mm (moderate inhibition). The I-3 only inhibited *P. aeruginosa*, however it was the only isolate to strongly inhibit this microorganism. Results showed that isolates had genetic variability related to production of substances antagonistic to the competitor microorganisms. Isolates I-4, I-6, and I-7 did not produce any antagonistic substance that was able to diffuse through the culture medium and inhibit the growth of the tested pathogens.

In vitro assays demonstrated that the *Nannotrigona testaceicornis* honey inhibited the growth of *Escherichia coli*, *Proteus* spp., *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and Coagulase-positive *Staphylococcus* spp. (GONÇALVES; ALVES FILHO; MENEZES, 2005). Bobany et al. (2010) demonstrated that honey from *Tetratrigona angustula* bees presented inhibitory activity against *Staphylococcus* sp., *Bacillus* sp., and yeast. In the present study, honey from *Melipona* sp. can house microorganisms with antagonistic potential to certain pathogens that cause disease in humans and other warm blood animals.

Studies of symbiosis between bacteria and insects have provided new insights into chemically diverse and biologically active compounds, such as bacitracin and amicoumacin obtained from bacteria associated with *Ceratophyllus* sp. and *Coenagrion* sp., respectively (GUIMARÃES; MOMESSO; PUPO, 2010). Although symbiosis between microorganisms and bees has not yet been proved, the honey, pollen, and larval food of the stingless bees could be a source of microorganisms that produce chemically diverse and biologically active compounds that might be used in the control of infectious diseases of humans and animals.

In a pioneering study, Gilliam, Roubik e Lorenz (1990) verified the presence of *B. circulans, B. alvei*, and *B. megaterium*, which produce digestive enzymes such as proteases, galactosidases, and antimicrobial substances, in the pollen, larval food, and honey of *Melipona fasciata* from a rain forest in Panama. This might indicate a co-evolutionary association between bees and bacteria, as also observed between *Apterostigma dentigerum* ants and *Pseudonocardia* and *Streptomyces* bacteria, which secrete antimicrobial substances and control food contamination by *Escovopsis* sp. (CONTI; GUIMARÃES; PUPO et al., 2012). However, there is no strong evidence to support the co-evolutionary association between bees and antimicrobial substance-producing bacteria. The I-5 isolate presented similar morphological and biochemical characteristics to *B. circulans*, a species also found in the honey of *M. fasciata* in a rain forest (GILLIAM; ROUBIK; LORENZ, 1990).

In all of the evaluated samples, the concentrations of total or thermotolerant coliforms were less than 0.3 MPN/g. This result agrees with those presented by Souza et al. (2009), who did not find total or thermotolerant coliforms in 14 samples of Trigonini honey produced in Bahia state, Brazil. The microbiological analysis of *M. compressipes*, *M. subnitida*, and *M. scutellaris* in Piauí state revealed that samples were free from coliforms (MONTE et al., 2013). Souza (2008) analyzed the microbiological quality of 47 samples of honey from many stingless bee species in Bahia and verified that only one was infected by coliforms. However, these authors did not suggest why samples were free from thermotolerant coliforms, and did not speculate on any relationship between the absence of these microorganisms and the presence of antimicrobial substances or antagonistic microorganisms in honey.

In contrast, Matos et al. (2011) found total and thermotolerant coliforms in 5 out of a total of 15 evaluated samples of honey from *Melipona* sp., with concentrations ranging from 23 to 1,100 MPN/mL. Microbiological analysis of *Melipona* sp. honey from the Parintis municipality in Amapá revealed the presence of filamentous fungi in 80% of the samples, and thermotolerant coliforms in 33%, while yeast was found in all of the samples (MATOS et al., 2011). The microbiological quality of meliponine honey depends on the climate characteristics of the region where the nests are housed. Coliform bacteria, filamentous fungi, and yeast depend on substrates with high water content in order to develop. Very dry environments may be unfavorable to the development of these microorganisms. Thus, it is

natural that semiarid environments would be less favorable to contamination of honey by allochthone microorganisms.

It is possible that native bees possess mechanisms to avoid the contamination of stored food by bacteria harmful to the colony, but fungi have usually been found in honey (MATOS et al., 2011; MONTE et al., 2013; SOUZA, 2008). Phenolic compounds and flavonoids from plants were found in the honey of many stingless bee species, which could be responsible for the antimicrobial activity (BAZONI, 2012), however, no correlation has been found between the concentration of such compounds and the minimum inhibitory concentration of the honey against *E. coli* and *S. aureus*. Inhibition of bacteria is due to pH and acidity of honey (PERALTA, 2010).

It is important to consider the role of organic acid-producing bacteria, which can contribute to the acidity of the honey and, consequently, to its antimicrobial properties. Although none of the isolates produced organic acid from the fermentation of glucose, which is normally present in high quantities in honey, some were able to inhibit the growth of pathogenic bacteria, which means that these isolates must secrete antimicrobial substances of a different chemical nature. Filamentous fungi and yeast were not found in the honey samples evaluated in this work possibly due to the fact that samples had been collected during a drought, when there are not many places with humidity high enough to support their development.

CONCLUSION

In brazilian dry forest (Caatinga), in Brazil, the honey of *Melipona* sp. showed good microbiological quality, and is suitable for use as food for humans. Moreover, it is a good source of sporogenic bacteria resistant to high temperatures, producers of antimicrobial substances, and with good antagonistic potential inhibiting the growth of contaminant microorganisms and contributing to the microbiological quality of the stored food in the bee hive. The demonstrated antagonistic feature is not related to the production of organic acids from glucose present at high concentrations in honey.

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