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PROSPECTION ON YEASTS FROM STINGLESS BEES HONEY IN BRAZILIAN TROPICAL DRY FOREST (CAATINGA)

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ABSTRACT – The richness of the yeasts associated with honey obtained from the stingless bees *Melipona mandacaia*, *M. asilvai*, *Patarmona* sp. and *Scaptotrigona* sp. living in Brazilian tropical dry forest (Caatinga) was studied. Based on morphological and physiological characters 12 species were identified belonging to the phylum Ascomycota, with eight *Candida* species. This study has shown that the honey of stingless bees is an important source of yeasts, particularly in tropical dry environments, where the diversity of fungi is still largely unknown.

KEY WORDS: CAATINGA ECOSYSTEM; FUNGI; HONEYBEES; MELIPONINI

PROSPEÇÃO EM LEVEDURAS DE ABELHAS SEM FERRÃO EM UMA FLORESTA TROPICAL SECA DO BRASIL (CAATINGA)

RESUMO – A riqueza de leveduras associadas ao mel obtido das abelhas sem ferrão *Melipona mandacaia*, *M. asilvai*, *Patarmona* sp. e *Scaptotrigona* sp. vivendo na floresta tropical seca brasileira (Caatinga) foi estudada. Com base nas características morfológicas e fisiológicas, um total de 12 espécies foram identificadas, todas pertencem ao filo Ascomycota, sendo oito espécies de *Candida*. Este estudo mostrou que o mel de abelhas sem ferrão é uma importante fonte de leveduras, especialmente em ambientes secos tropicais, onde a diversidade de fungos ainda é em grande parte desconhecido.

PALAVRAS CHAVE: ECOSISTEMA CAATINGA; FUNGOS; MEL DE ABELHAS; MELIPONINI

PROSPECCIÓN EN LEVADURAS DE ABEJAS SIN AGUIJÓN EN UNA SELVA TROPICAL SECA DE BRASIL (CAATINGA)

RESUMEN – La riqueza de levaduras presentes en la miel obtenida de abejas sin aguijón *Melipona mandacaia*, *M. asilvai*, *Patarmona* sp. y *Scaptotrigona* sp. que viven en el bosque tropical seco brasileiro (Caatinga) fue estudiada. Con base en las características morfológicas y fisiológicas, se identificaron un total de 12 especies, todos pertenecen a los Ascomycota, siendo la mayoría de los aislados anamorfo de las especies de *Candida*. Este estudio mostró que la miel de abeja sin aguijón es una fuente importante de la levadura, especialmente en ambientes tropicales secos donde la diversidad fúngica es todavía en gran parte desconocida.

PALAVRAS CLAVE: ECOSISTEMA CAATINGA; HONGOS; MIEL DE ABEJAS; MELIPONINI

INTRODUCTION

Stingless bees are present in all tropical regions of the world and subtropical regions of the southern hemisphere (Silveira et al. 2002). All species are eusocial, nesting in pre-existing cavities such as hollow trees, abandoned termite mounds and anthills (Nogueira-Neto 1997; Silveira et al. 2002). These bees belong to the family Apidae, which is subdivided into three subfamilies: Xylocopinae, Nomadinae and Apinae. The subfamily Apinae comprises 19 tribes, including Apini, Meliponini, Bombini and Euglossini, which are bees that have pollen baskets (Michener 2000). Stingless bees are classified in the tribe Meliponini, composed of individuals with variable sizes (Silveira et al. 2002).

Several microorganisms can be found in the hives of Meliponini bees, including bacteria (mostly *Bacillus* species), filamentous fungi (*Penicillium*, *Mucor* and *Aspergillus*) and yeasts (*Saccharomyces* and *Candida*) (Alves et al. 2009).

Yeasts can be associated with various substrates, including plants, insects, and humus (Phaff 1990). Yeast populations played an important role in the trophic chain, and their spread among

environments or microhabitats may occur through the action of insects like *Drosophila* spp. which maintain a mutualistic association with yeasts serving as source of nutrients for these insects and, in addition, to detoxifying the substrates used as food. The flies, in turn, disperse these microorganisms in the ecosystem (Morais and Rosa 2000). Some *Candida* species are mutualistic in the nests of *Ptilotrigona lurida* bees, contributing to the dehydration and preservation of the stored pollen, making the nest less attractive to natural enemies (Camargo et al. 1992). Few studies have assessed the presence of yeasts and other microorganisms with Meliponini bees, including their habitat and the by-products produced by them (Rosa et al. 1999; Hong et al. 2003; Teixeira et al. 2003; Brysch-Herzberg 2004; Brysch-Herzberg and Lachance 2004; Ergin et al. 2004; Pimentel et al. 2005). Such have been carried out mostly in humid tropical environments. Caatinga (Tupi language: *caa*= forest and *tinga* = white) is a tropical dry forest in Brazil. In spite of its socioeconomic importance, it is submitted to intense exploration for its natural resources, and has been poorly protected or studied (Andrade et al. 2006).

Despite the efforts in understanding the biology of bees, there have been few studies that deal with the examination of the association of these insects with yeasts, especially with the bees under tribe Meliponini in semi-arid environments. This study aims to investigate the yeast communities associated with the honey of Meliponini bees. Specifically, this research seeks to understand how these bees contribute to the distribution of yeasts in semi-arid environments and the extent of similarity between yeast communities composition among the species of eusocial bees of the Caatinga.

MATERIAL AND METHODS

Honey sampling

Honey sampling was conducted at Caatinga at the Federal Rural University of Pernambuco Serra Talhada campus (UAST/UFRPE), Pernambuco, Brazil (7°57'22.3452"S, 38°17'45.5994"W) between December 2010 and January 2011 (Figure 1). Nest of each of the following Meliponini bees, *Melipona mandacaia* Smith, *M. asilvai* Moure, *Patarmona* sp. Schwarz and *Scaptotrigona* sp. Moure, (Figure 2) found in the area were sampled. Three collections were performed for each nest and only one nest for each species was sampled respecting the necessity to preserve these insects and making minimal disturbance to the beehives. Honey collected presents more than 10% of the stored honey, since each colony stored about 30 ml of honey in its storage pots.

Microbiological analysis

For the microbiological analysis, the honey pots were placed in the isothermic box and transported to the laboratory for processing. The sampling technique used was in accordance to Gilliam et al. (1990). The honey storage pots were disinfected by washing several times with distilled sterilized water and 70% ethanol to remove any visible debris and external microorganisms. After disinfection, 1 ml honey aliquot was collected from the honey storage pot by removing the operculum of wax produced by the bees to close the top of the pot. Three honey storage pots were taken from each hive to form a composite sample, for a total of 12 samples.

Isolation and identification of yeasts

For the isolation of yeasts, the honey aliquot of 1 ml was diluted in 9 ml of distilled sterilized water, thereby producing 10⁻¹ dilutions for each sample. Subsequently, 100 µl of each dilution was seeded in Petri dishes, containing Sabourauds agar culture medium (Himedia®), with 0.2 mg/ml of chloramphenicol (Neo Fenicol – Neo Química®). The experiment was conducted with three replications. The Petri dishes were incubated at 28±2°C for eight days. After the incubation period, the colony-forming units (CFU) were counted, isolated and purified.

Isolates were identified using macro- and microscopic characteristics and physiological and biochemical indicators according to classical taxonomy (Kurtzman et al. 2011).

Data analysis

Distribution of each fungal species were calculated for each sampling from formula $D_i = (N_i/N) \times 100$, where D_i = distribution of the species i ; N_i = number of colony forming units (CFU) of the species i ; N = total number of CFU. Based on this formula, the species frequencies can be classified as: <0.5% = rare, ≥0.5 <1.5% = occasional, ≥1.5 <3.0% = common, ≥ 3.0% = abundant (Schnittler and Stephenson 2000). Simpson's (λ) and Shannon–Wiener's (H') Diversity, Pielou's Equitability and Berger–Parker's Dominance were calculated using the PAST 1.7 software (Hammer et al. 2001).

RESULTS

The number of yeasts (CFU) obtained from the hives of different species of stingless bees ranged from 5.0×10^2 to 1.18×10^3 CFU/mL. The most common genera isolated from the hives of honeybees studied were *Candida*, *Debaryomyces*, *Dekkera*, *Pichia* and *Kloeckera*. Twelve species were identified to be belonging to Ascomycota (Table 1).

Some yeast species were isolated only from a single type of honey such as *Candida diversa* which was isolated only from the honey of *M. mandacaia* bee hive. This honey revealed to have the highest concentration and richness of yeasts. The richness of yeasts from the honey ranged according to the species of bee, for example, in the hive of *M. mandacaia*, six (6) were isolated while in *M. asilvai*, only one species was isolated. The distribution of yeasts in different types of honey is listed in Table 1. Most species identified were classified as abundant, where only *C. buffonii* was considered as rare (Table 1).

The similarity analysis between the composition of yeasts in different types of honey was estimated. The biggest similarity was between the samples of honey from *M. mandacaia* and *Partamona* sp. (36.36%) and between *M. mandacaia* and *M. asilvai* (28.57%). Among the samples of honey from bees of *M. asilvai* and *Partamona* sp., no similarity between the yeast communities was found.

The diversity indices calculated from the honeys studied were the Simpson's (λ) and the Shannon's (H') index. Simpson's index indicated that the honey's *Partamona* sp. has the highest diversity compared to the other. Pielou's evenness index (J') indicated that in the honey's *Scaptotrigona* sp. all yeasts species were equally abundant (Table 2).

Table 1 - Richness of yeasts (CFU/ mL) in honey samples in stingless bees in the tribe Meliponini in Brazilian tropical dry forest.

Yeasts	Stingless bees					Total	D
	<i>M. mandacaia</i>	<i>M. asilvai</i>	<i>Partamona</i> sp.	<i>Scaptotrigona</i> sp.			
<i>Candida apicola</i> (Hajsig) S.A. Mey. & Yarrow	7.0 x10 ¹	0	0	0	7.0x10 ¹	C	
<i>C. bertheti</i> Boidin, Pignal, Mermiér & Arpin	0	0	0	4.2x10 ²	4.2x10 ²	A	
<i>C. buffonii</i> (C. Ramírez) Uden & H.R. Buckley ex S.A. Mey. & Ahearn	1.0x10 ¹	0	0	0	1.0x10 ¹	R	
<i>C. diversa</i> Y. Ohara, Nonom. & Yunome ex Uden & H.R. Buckley	5.7x10 ²	0	0	0	5.7x10 ²	A	
<i>C. glabrata</i> (H.W. Anderson) S.A. Mey. & Yarrow	1.1x10 ²	0	1.7x10 ²	0	2.8x10 ²	A	
<i>C. silvatica</i> (Van der Walt, Klift & D.B. Scott) S.A. Mey. & Yarrow	8.0x10 ¹	0	6.0x10 ¹	0	1.4x10 ²	A	
<i>C. sake</i> (Saito & M. Ota) Uden & H.R. Buckley ex S.A. Mey. & Ahearn	0	0	9.0x10 ¹	0	9.0x10 ¹	C	
<i>C. zeylanoides</i> (Castell.) Langeron & Guerra	0	0	9.0x10 ¹	0	9.0x10 ¹	C	
<i>Debaryomyces hansenii</i> (Zopf) Lodder & Kreger-van Ri	0	0	9.0x10 ¹	0	9.0x10 ¹	C	
<i>Dekkera bruxellensis</i> Van der Walt	5.1x10 ²	5.0x10 ¹	0	0	5.6x10 ²	A	
<i>Pichia anomala</i> (E.C. Hansen) Kurtzman	0	0	0	3.4x10 ²	3.4x10 ²	A	
<i>Kloeckera africana</i> (Klöcker) Janke	0	0	0	4.2x10 ²	4.2x10 ²	A	
Total	1.35x10 ³	5.0x10 ¹	5.0x10 ²	1.18x10 ³			

Table 2 – Simpson's (λ) and Shannon–Wiener's (H') Diversity, Pielou's Equitability and Berger–Parker's Dominance of yeasts isolated from honey samples of meliponini bees collected sampled between December 2010 and January 2011.

Stingless bees	λ	H'	J'	Dominance
<i>M. mandacai</i>	0,67	1,29	0,72	0,42
<i>M. asilvai</i>	0	0	-	1
<i>Patarmona</i> sp.	0,77	1,54	0,96	0,34
<i>Scaptotrigona</i> sp.	0,66	1,09	0,99	0,35

DISCUSSION

Prospecting of yeasts remains poorly studied in the honey of stingless bees. Osmophilic yeasts are able to grow in honey as they tolerate the low water activity and high concentrations of sugar found in this medium, and are even able to grow in mature honey, where the humidity is lower, fermenting it easily (Calaça 2011). Scores ranging from 1.0×10^1 to 4.4×10^3 CFU's of molds and yeasts per gram of honey were observed for Meliponini bees (Souza et al. 2009).

The presence of yeasts in honey can be related to the grazing activity of bees, which can carry yeasts to the hive from external sources such as plants, animals, soil, and water, in addition to sources related to human activity (Nogueira-Neto 1997). Physiologically, yeasts tend to adapt to more severe conditions than most microorganisms, for example, substrates with high concentration of fatty supporting variations pH between 2 and 9, while the optimum for most species is situated around 5,6 (Lacaz-Ruiz 2000). Thus, correlation between yeasts richness and moisture, pH, acidity and others factors in the stingless bees honey samples may be evaluated in future studies.

Pollen stored in the nests of honeybees, known as 'saborá' (Nogueira-Neto 1997) passes through fermentation processes, for example fermentation of lactic acid performed by bacteria and yeasts (Snowdon 1999). Certain yeasts tolerate conditions of high sucrose levels and are able to grow in honey, while others are considered osmophilic by thriving when the osmotic pressure is high, including in mature honey, fermenting it easily (Souza et al. 2009).

Some species of yeasts isolated have biotechnological or agricultural application, such as *C. sake* that has been used in the control of fungi that decay fruit in the post-harvest stage (Cañamás et al. 2008) and *C. silvatica* which is used in the production of proteases (Kreger-Van Rij 1984), enzymes used in the manufacture of detergents and in the food industry (Coelho et al. 2008). On the other hand, there are strains of *C. glabrata* that are of clinical important, common in hospital-acquired infections and resistant to fluconazole (Zardo and Mezzari 2004) and considered a secondary and opportunistic agent that mainly affects immunocompromised patients (Kreger-Van Rij 1984).

Among the yeasts isolated, *Debaryomyces hansenii* is considered a generalist species, halotolerant and osmotolerant, commonly observed as a food spoilage agent (Brysch-Herzberg 2004; Souza et al. 2009). This fungus has already been reported, along with

Zygosaccharomyces mellis, causing deterioration in the honey of *Apis mellifera* (Snowdon and Cliver 1996), and has also been isolated from the honey and from adult specimens of *M. quinquefasciata* (Peruquetti 2000).

In the microbiota of meliponaceous honey, yeasts such as *Saccharomyces*, *Zygosporomyces* and *Torula* were found (Peruquetti 2000). However, the results obtained from this study did not corroborated with the findings in any of the cited genera of yeasts. The mandibular glands of bees are known to produce bactericidal and fungicidal substances, such as linalool, citral, geraniol, citronellol and geraniol and nerol acetates, which can prevent the growth *Candida* and others fungi (Nogueira-Neto 1997). Nevertheless, eight species of *Candida* were found in honey of the bees studied, except in the honey of *M. asilvai* where only *Dekkera bruxellensis* was found. The yeast *C. apicola* had already been isolated from samples of honey of *Melipona quadrifasciata*, being considered frequent in that honey (Rosa et al. 2003). However, in the present study, its distribution was only 2.27% and was found only in the honey of *M. mandacaia*.

The occurrence of yeasts, including new species, associated with the provisioning of larvae, pupae and adults of stingless bees, have been reported in several papers (Rosa et al. 1999; Hong et al. 2003; Teixeira et al. 2003; Brysch-Herzberg 2004; Brysch-Herzberg and Lachance 2004; Ergin et al. 2004; Pimentel et al. 2005). *Candida riiodocensis* and *C. cellae* were isolated from solitary bees, which do not produce honey, in the Atlantic forest. The nectar and pollen carried by females are used by the bees and yeasts alike as sources of energy (Pimentel et al. 2005). *Starmerella meliponinorum* was described in association with nests of the eusocial stingless bee, *T. angustula*, and could also be associated with food, both honey and pollen, propolis, detritus, and adult individuals of *M. quadrifasciata*, *M. rufiventris*, *T. angustula*, and *T. fulviventris* (Rosa et al. 2003; Teixeira et al. 2003). However, to our knowledge and experimental conditions among our isolates, only the yeast *C. apicola* had already been reported by Teixeira et al. (2003) who studied honey samples of *M. quadrifasciata*.

Stingless bees from Caatinga usually visit several species of native plants, including *Myracrodruon urundeuva* Allemão, *Pityrocarpa moniliformis* Benth, *Spondias tuberosa* Arruda, *Handroanthus impetiginosus* (Mart. ex DC.) Mattos and *Ziziphus juazeiro* Mart., which is fundamental in maintaining the nests during periods of drought (Maia-Silva et al. 2012). It is possible that the yeasts richness in the honey of bees is related to the plant species visited, but in a later analysis (unpublished data), yeasts were not found in the nectar of *S. tuberosa* or *Z. joazeiro*. Stingless bees visit several food sources, including animal carcasses, and can collect feces or clay to close cracks in the hives, which are considered unhygienic habit that can contaminate the honey (Nogueira-Neto 1997). On the other hand, honey can adapt to environment with high osmotic pressure that impedes the growth of various types of microorganisms. Nevertheless, the yeasts isolated were able to persist in the honey and resisted the great osmotic pressure found in this environment, despite not all are considered osmophilic species. The results obtained can serve as a reference for studies that seek to identify the physiological and biochemical characteristics of these yeasts with biotechnological potential for industrial or agricultural application.

Further studies of yeast from body bees, pollen storage and native plants are needed to understand the relationships between bees, plants and fungi from arid and semi-arid regions, and the benefits that such fungi can possibly confer on their hosts, as well as the mechanisms evolved to adapt these fungi to these extreme living conditions.

According to the results, *M. mandacaia*, *M. asilvai*, *Scaptotrigona* sp. and *Partamona* sp. are disseminators of yeasts in the semi-arid region of northeastern Brazil. Species of *Candida* are the most benefited, in view of the abundance of these yeasts in honey samples of bees naturally found in the Caatinga. The Meliponini bees visits different plants in order to feed on nectar and pollen, and many other substrates to maintain the hives, including dung of vertebrates, which probably contributed to the abundance of yeasts species in the honey. However, this preliminary study found that the similarity in the composition of yeast communities in hives of different species of bees was low, and it was not possible to assume that there is a co-evolutionary relationship between these taxa. Further studies will contribute to the understanding of the relationship between bees-yeasts-honey and possibly will be able to verify this co-evolutionary relationship, among other factors such as the influence of the composition of honey in the richness of yeasts. Altogether, this study has shown that yeasts richness in the honey of different bee species of the Tribe Meliponini is variable and that yeast species that can be isolated from honey may present interesting features and biotechnological potential.

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