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Cultivable fungal diversity associated with the digestive tube of stingless bees (*Melipona* spp.) in the Brazilian Amazonia

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ABSTRACT. Stingless bees interact with diverse symbiotic organisms, such as bacteria, fungi, mites and insects. We isolated and identified filamentous fungi presents in the digestive tract of stingless bees (*Melipona* spp.) to quantify this association and discussed their importance for the health of these insects. Twenty stingless bees were collected and their digestive tracts were analyzed. The slide micro-cultivation technique was used for morphological identification of the fungi. A total of 1,447 fungal colonies were isolated, identifying nine genera, with *Aspergillus*, *Paecilomyces*, and *Cladosporium* being the most prevalent. The findings highlight fungi's diversity and potential pathogenicity, with implications for bee health and biodiversity conservation. The first record of the genus Lasiodiplodia in stingless bees (*Melipona* spp.) expands the knowledge about the fungal microbiota of these organisms. Although some fungal species can be harmful, understanding these interactions can promote management strategies for stingless bees.

Keywords: microorganisms; microbiota; filamentous fungi.

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Introduction

Stingless bees (Apidae, Meliponinae) occur in tropical and subtropical regions worldwide, including Africa, Australia, and Central and South America (Kwapong, Aidoo, Combey, & Karikari, 2010). However, many species still need to be taxonomically described. Unfortunately, stingless bee populations are declining mainly due to the degradation and loss of native forest habitats, which are the preferred environments for these species (Lopes, Ferreira, & Santos, 2005).

Stingless bees have a wide range of symbiotic organisms, including bacteria (Machado, 1971; Cruz-Landim, 1996), fungi (Gilliam, Roubik, & Lorenz, 1990), mites (Eickwort, 1990) and insects from different orders (Salt, 1929; Wilson, 1971; Kistner, 1982; Wille, 1983; Melo, 1996). However, there is a scarcity of studies dealing with the relationship between fungi and stingless bees (Peruquetti, 2000), and the symbiosis with these bees still needs to be discovered. Little is known about the fungal microbiota in the digestive system of these bees, especially in the case of stingless bees (Liu et al., 2023). Most native species of stingless bees lack information about their behavior, reproduction, and health. A fundamental aspect of the biology of these insects is understanding the fungal community, which can trigger diseases when the bees' immunity is compromised (Ferraz, Lima, Pereira, Freitas, & Feijó, 2010).

Nosemosis is an important disease among bees caused by a microsporidian fungus of the genus *Nosema*. *N. apis* and *N. ceranae*, some of the variants most frequently found in diseased hives, attack the bees' intestinal mucosa. Although it mainly affects species of the *Apis* genus, there are records of *Nosema* occurring in stingless bees (Porrini et al., 2017; Guimarães-Cestaro et al., 2020). Other diseases, such as chalkbrood and stonebrood, are triggered by *Ascophaera apis* and *Aspergillus flavus*, which affect the larvae, causing a mummification process in their intestines, resulting in their death (Liu et al., 2023).

On the other hand, fungi can also be beneficial to bees, performing a protective function by contributing to health by fighting pathogens, providing nutrients, serving as food sources and participating in metabolic and catabolic processes in digestion (Six, 2012; Kellner et al., 2014; Kaltenpoth & Steiger, 2014; Harrison, Urruty, & Forister, 2016; Malacrinó et al., 2017; Gurung, Wertheim, & Salles, 2019). Studies have

characterized the microbial community associated with bees (Morais, Calaça & Rosa, 2012), revealing that these microorganisms contribute to chemical conservation in the intestinal tract, preservation of pollen stored in comb cells, and production of antimycotic substances (Gilliam, 1997).

Unlike bacteria, protozoa, and viruses, fungi can infect insects through multiple routes, including the intestinal tract, spiracles, and the outer surface of the integument. This property allows insects to be infected regardless of their feeding activity (Ferron, 1978; Hajek & Leger, 1994). Investigating and exploiting the industrial potential of fungi associated with bees is important since compounds produced by microorganisms have a lower environmental impact and cost (Ramos et al., 2013).

The intestines of insects are known to harbor a wide variety of microorganisms, and bees have unique intestinal microbiomes. For example, Apis spp. bees have bacterial communities dominated by the genera *Snodgrassella*, *Gilliamella*, *Lactobacillus*, and *Bifidobacterium*, which are acquired through floral visits and the hive environment (Liu et al., 2023). However, there is a gap in the knowledge about fungi in the gut of insects, including stingless bees (Paula, Menezes, Pupo, & Rosa, 2021). For example, there is a lack of information on the gut microbiome of bees, especially on fungi and their interactions with other species (Liu et al., 2023). This limited understanding is mainly linked to the presence of these fungi in food stocks, brood cells and even externally to bees (Rosa et al., 2003; Brysch-Herzberg & Lachance, 2004; Ferraz et al., 2008; Matos, Nunes, Mota, Laureano, & Hoshiba, 2011; Paludo et al., 2018; Dharampal et al., 2020).

In this study, we isolated and microscopically identified the genera of filamentous fungi present in the digestive tract of stingless bees (*Melipona* spp.). These investigations are fundamental to understanding these associations and their relevance to the well-being and survival of these bees, as well as contributing to the maintenance of the ecosystem services of pollination.

Material and methods

Collection of biological material and fungal isolation

Twenty individuals of stingless bees (*Melipona* spp.) were collected in August 2018 from an apiary in the municipality of Oriximiná (1°45'39.5" S 55°52'00.6" W), state of Pará, Brazilian Amazonia. The stingless bees were collected alive using sterile tweezers and transferred to sterilized tubes. The material was placed in thermal boxes under refrigeration at 10ºC to euthanize the stingless bees by hypothermia and preserve the material for transport to the laboratory for later analysis.

The fungi were isolated according to the methodology proposed by King, Hocking, and Pitt (1979), where the stingless bees were superficially disinfected in a 0.5% solution of sodium hypochlorite for 1 minute and rinsed in sterile distilled water. Subsequently, the stingless bees were immersed in a solution of 0.05% ascorbic acid plus 0.05% citric acid for 1 min. At the end of this process, the individuals were dissected, and the digestive tract (alimentary canal) was separated and then immersed in 1 ml of a 0.8% saline solution and placed in tubes.

The spores were removed from the stingless bee structures by shaking in a vortex for 10 minutes. Aliquots of 100 µL of each sample were transferred to Petri dishes containing Sabouraud Dextrose Agar (SDA) with a solution of the antibiotic Amoxicillin 100 mg L^{-1} in triplicate. The pH of the culture medium was set at 6.8. The plates were kept in a BOD incubator at 28 ºC for up to 30 days.

Conventional technique for macro and micromorphological characterization of filamentous fungi

The filamentous fungi were identified based on their macroscopic characteristics, such as color, texture, edge shape, presence of filaments, budding and type of spores. In optical microscopy, the shape, presence of filaments, sprouting and type of spores were observed. The identification and description of the genera of filamentous fungi was based on macro and microscopic characteristics (Pitt, 2000; Watanabe, 2002; David, 1997; Braun, Crous, Dugan, Groenewald, & Sybren De Hoog, 2003; Picos-Muñoz, García-Estrada, León-Félix, Sañudo-Barajas, & Allende-Molar, 2015; Barnett & Hunter, 1972; Mourão, Ságio, Souza, & Santos, 2017).

Slide micro-cultivation technique for morphological identification of filamentous fungi

The micro-cultivation technique on a slide was modified by Riddell (1950). In this technique, a circular piece of filter paper is initially placed on the bottom of a Petri dish, then deposited on the paper with 24 x 24 mm coverslips and a sterilized 76 x 26 mm slide.

Two blocks of Potato Dextrose Agar (BDA) were transferred with a spatula to the central surface of the sterile slide and placed on filter paper in a sterile Petri dish. A portion of the colony was inoculated at the four ends of the agar block with an inoculation loop and sterilized in a Bunsen burner. Two sterile coverslips were placed on the surface of the agar block using sterile tweezers. Using a pipette, a small amount of sterilized distilled water was poured onto the bottom of the plate, enough for five to seven days.

After the fungal growth was visually sufficient, the coverslips were carefully removed so that the mycelium adhered to the underside of the coverslip and did not break off. Colonies that did not develop reproductive structures were re-inoculated and subjected to temperature, pH, light, and nutrient variations to stimulate reproductive development. The coverslips were then placed on a drop of lactophenol blue dye on the surface of a 76 x 26mm slide and analyzed under light microscopy (Smith, 1963) to identify their sexual and asexual structures (Ellis, 1971; Barnett & Hunter, 1972; Von, 1970). Duplicates of the parent colony and two monospores of each isolate were stored according to the Castellani method (Araújo et al., 2002) and kept at room temperature.

Results

A total of 1,447 fungal colonies were isolated and nine genera were identified. Of these, seven colonies were considered *Mycelia sterilia*, and despite the techniques used, these colonies did not develop reproductive structures in the laboratory environment. Of the total number of colonies obtained, 555 (38.36%) were morphologically identified at the genus level. The most abundant genera were *Aspergillus* (42.34% of the total), *Paecilomyces* (20.90%), *Penicillium* (7.57%), *Phoma* (2.34%), *Cladosporium* (18.38%), and *Fusarium* (7.39%) (Figure 1). Together, the predominant genera accounted for 98.92% of the genera identified. These were isolated from all the stingless bees studied. The genera *Lasiodiplodia* (0.18%), *Geotrichum* (0.36%), and *Curvularia* (0.54%) were the least prevalent, representing 1.08% of the genera identified.

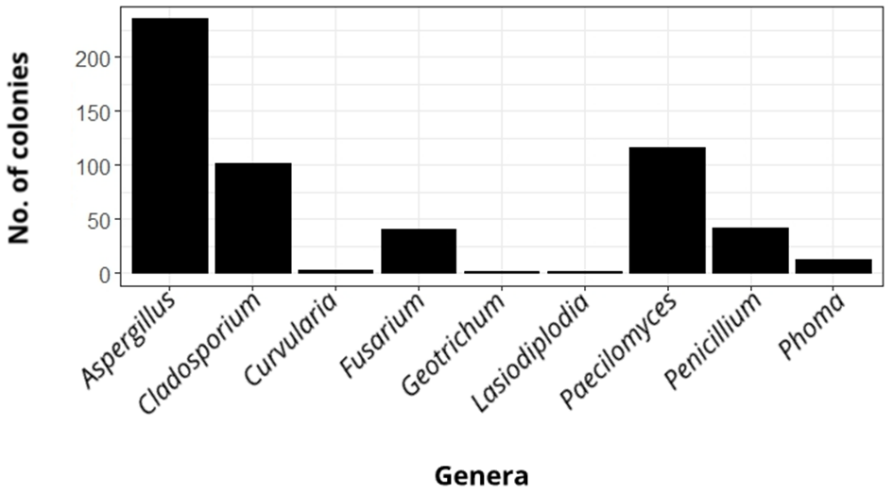

Figure 1. Fungi isolated from the digestive tract of stingless bees (*Melipona* spp.).

Discussion

We identified the presence of nine genera of fungi occurring in the digestive tract of stingless bees (*Melipona* spp.). Three were dominant, with more than 100 spores counted: *Aspergillus*, *Paecilomyces*, and *Cladosporium*. Such findings converge with those of Souza, Costa, Silva, and Silva (2020), who also identified the same fungal genera in their samples of *Melipona seminigra merrilae* C. bees in the same Amazonia region (Figure 2). However, a contrast between Souza et al. (2020) and our results is the low occurrence of the genus *Geotrichum*. This difference can be explained by the methodology used by Souza et al. (2020), who separated the intestine into three parts, while in our study there was no such separation. In addition, other attributes such as diet and exposure to pathogens and parasites may have contributed to the differences observed in the results.

The genera *Colletotrichum*, *Candida*, and *Syncephalastrum* isolated by Souza et al. (2020) were not isolated in our study. Factors that may have influenced this result are environmental conditions and the natural variability of microorganisms. Humidity is a conditioning factor for the appearance of filamentous fungi. Temperature and pH are also determinant factors in the development of fungi (Guerra, Cunha, Silva, & Knop, 2012).

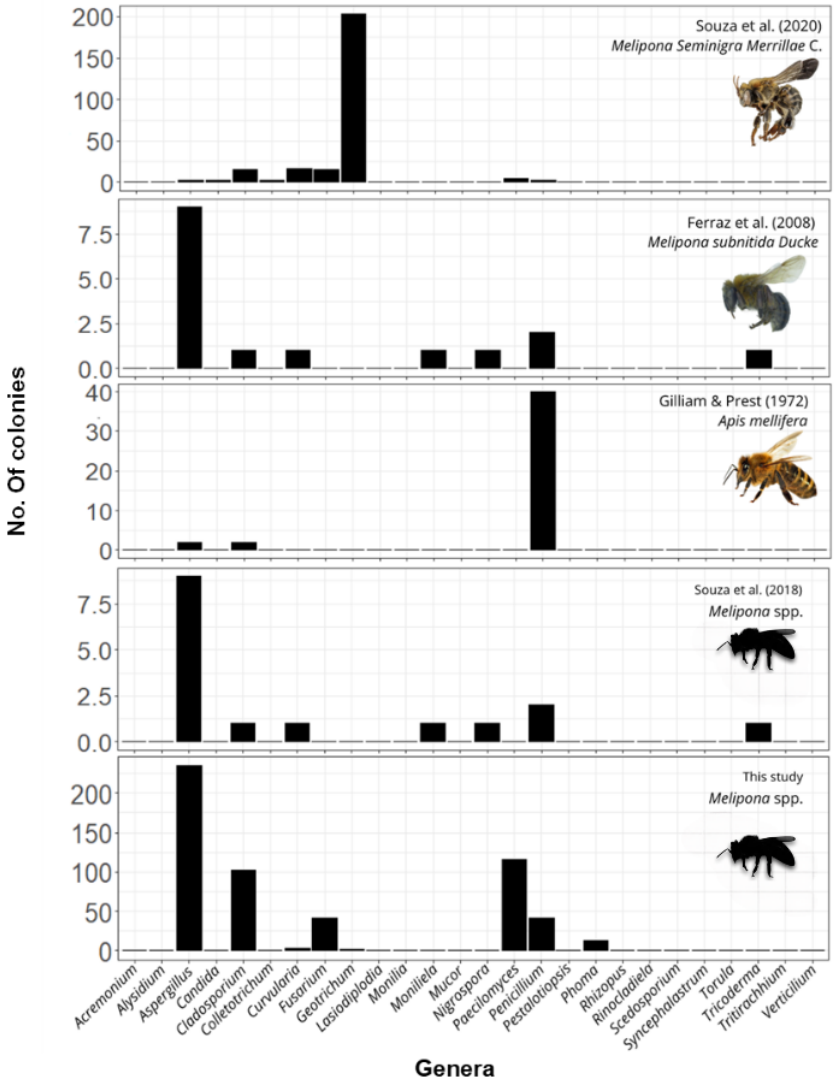

Figure 2. Fungi isolated from the digestive tract of bees. The data was obtained from Souza et al. (2020), Ferraz et al. (2008), Gilliam and Prest (1972), and Souza et al. (2018). Note that the Y-axis scale values are different among plots.

Our first-time record of *Lasiodiplodia* in stingless bees expands the understanding of the microbial diversity associated with these bees. Until now, these genera of fungi had not been described in their digestive tract. The presence of the genus *Lasiodiplodia* in the digestive tract of stingless bees raises questions about its origin and possible ecological role in these bee species. Further studies are needed to investigate whether this fungus is present as a commensal, symbiont, or pathogen in stingless bees and whether its presence is related to any specific aspect of the environment or the diet of these bees.

Gilliam and Prest (1972) isolated the genus *Penicillium* with high frequency from the intestinal contents of *A. Mellifera* L. bees in Arizona/USA, two cultures of *Cladosporium* sp. and *Aspergillus* were also isolated, but less frequently. The authors observed that the genus *Penicillium* was the most prevalent, isolated in 98% of the bees collected (Figure 2). The genera *Paecilomyces*, *Phoma*, and *Fusarium* were not isolated (Gilliam & Prest, 1972). The authors were surprised not to find a greater diversity of fungi in bees since the bees' food (honey) and intestines are rich in sugars. Contrasting with such findings, we verified a predominance of *Aspergillus,* isolated in 97% of the samples. Congruent results have already been observed, where the genera *Penicillium* and *Aspergillus* have been consistently identified in the alimentary tract of *A. mellifera* bees, with the species *Penicillium frequentans*, *Penicillium cyclopium*, *Aspergillus flavus*, and *Aspergillus niger* being the most common (Gilliam, 1997). This organism produces ochratoxins, toxic compounds known to affect kidney function. In addition, the *Aspergillus* genus has been associated with pathogenicity in stingless bees, highlighting its importance in the health of these pollinating insects.

Our findings on the presence of the fungus *Curvularia* sp. corroborate with previous discoveries. This fungus has already been identified as part of the microbiota of *Trigona* sp. bees (Morais et al., 2012) and

Curvularia brachyospora has been isolated from the intestines of *A. melifera* bees (Gilliam, Prest, & Morton, 1974). The genus *Phoma* was predominant in propolis produced by *A. melifera* L. (Ferreira et al., 2021). In Brazil, this pathogen is associated with leaf spots in coffee plantations.

Conclusion

We found that the most common fungal genera in stingless bees were *Aspergillus*, *Paecilomyces*, and *Cladosporium*. Some of these fungi can be pathogenic to bees and even produce toxic compounds that can harm the health of bees and other organisms. The high incidence of fungi corroborates the diverse presence of microorganisms in the digestive tract of stingless bees. The first record of the genus *Lasiodiplodia* in stingless bees expands knowledge about the fungal microbiota of these organisms. Although some fungal species can be harmful, understanding these interactions can promote management strategies for stingless bees.

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