Microbial Biodiversity in Honey and Pollen Pots Produced by *Tetragonisca angustula* (Jataí)

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**HIGHLIGHTS**

- Eight species of bacteria and three yeasts were identified in *T. angustula* products.
- *Bacillus* was the main bacteria present in pots of stingless bee honey and pollen.
- *S. meliponinorum* was the main yeast found in jataí honey, and *Z. bailli* in jataí pollen.
- Symbiotic relationship between microorganisms and honey and pollen pots

**Abstract:** The combination of nutritional and chemical factors in honey and pollen produced by *Tetragonisca angustula* (jataí) reveals a unique ecosystem that is conducive to developing microorganisms. However, there is a lack of information regarding the microbial species grown in the nest pots of this stingless bee species. The objectives of this study were to count, isolate and identify the microbiota associated with the pots of honey and pollen. The samples were collected from three jataí beehives. Microbiological analyses showed that the average total aerobic mesophiles ranged between 3.78 and 3.71 log CFU/g; lactic bacteria were 3.59 and 3.56 log CFU/g; and the yeast was 4.41 and 4.66 log CFU/g for the honey and pollen, respectively. There were four gram-positive cocci bacteria (*Staphylococcus saprophyticus* subsp. *bovis*, *S. vitulinus*, *S. kloosii* and *S. pasteuri*); two spore-forming gram-positive bacteria (*Bacillus pumilus* and *B. thuringiensis*); and one gram-negative bacteria (*Pantoe agglomerans*). One lactic bacteria (*Leuconostoc mesenteroides* subsp. *mesenteroides*) was also identified. Three yeast species were isolated (*Starmerella meliponinorum*, *Candida magnoliae* and *Zygosaccharomyces bailli*). Bacillus was the main bacteria in jataí honey and pollen. *Z. bailli* was the dominant yeast in the pollen, and *S. meliponinorum* was the dominant yeast in the honey. *T. angustula* bees and their products (honey and pollen) are ecosystems that provide sustainable environmental
and technological integration. They also provide a selection of microorganisms that can be applied in the food, cosmetic and pharmaceutical industries.

**Keywords:** stingless bee honey; isolation; characterization.

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**INTRODUCTION**

Stingless bees (SB) are ecologically important because they preserve plant biodiversity; they are considered to be the primary pollinator of Brazilian flora. It is estimated that the honey produced in one day results from at least one million interactions between flowers and bees. SB are eusocial bees that are pantropically distributed; they do not present a functional sting. SB colonies are perennial and can resist long periods of adversity through consuming food stored in the nests (pots containing honey and pollen). Nesting commonly occurs in exposed nests, and in pre-existing holes such as underground and tree cavities; however, SB can also be raised in urban environments using appropriate wooden boxes. Of these, *Tetragonisca angustula* (jataí) is the most adapted and well-known species [1–4].

Inside SB nests, brood combs are surrounded by pots of food used to maintain the nest. SB contain specific substances and enzymes that chemically transform and modify floral nectars from rich vegetation in native environments; the bees deposit, dehydrate, and store the nectar in pots. SB products are stored inside the nests to be used as sources of sugar and protein [1,4].

The honey and pollen from these bees contain bioactive substances that include phenolic compounds, amino acids, vitamin C, carotenoids and other minor compounds related to their antioxidant and antimicrobial potential. They are entirely different from the honey and pollen produced by bees of the genus *Apis mellifera* in terms of consistency, aroma, color and flavor [1,5].

The main microorganisms living in SB colonies are yeasts, molds, and bacteria [6–9]. It has been demonstrated that the microflora of bees can be used as a biocatalyst agent, a potential probiotic, and as a producer of antimicrobial compounds and enzymes [7,8]. Microorganisms from SB can be beneficial because specific products may be produced by them, such as mead, honey-pollen jelly, or a creamy pollen milkshake [10]. Studies indicate that the honey produced by *Tetragonisca angustula* SB has antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli* [11], *Bacillus cereus*, *Pseudomonas aeruginosa*, and against yeasts such as *Candida albicans* [12]. In addition, this honey has shown excellent antioxidant capacity [13].

However, information regarding the association of some microorganisms (*Bacillus* spp., *Clostridium* spp., *Enterococcus* spp., *Fructobacillus* spp., *Lactobacillus* spp. and *Staphylococcus* spp.) with SB honey and pollen is only available for a few bee genera, namely *Heterotrigona itama*; *H. erythrogastra*; *Lepidotrigona terminata*; *Tetrigona apicalis*; *T. melanoleuca*; *T. bingami*; *Geniotrigona thoracica*; *Homotrigona fimбриata*; and *Melipona fasciata* [2,7,8,14–17]. Therefore, this study aimed to explore and characterize the diversity of microorganisms isolated from the sources of *Tetragonisca angustula* bees.
MATERIAL AND METHODS

Samples

The honey and pollen bees were collected from three jataí (Tetragonisca angustula, Latreille, 1811) beehives at the Curitiba Meliponary, Paraná State, Brazil (25°02′40″ S; 49°16′23″ W), during April 2018 (Figure 1). The honey was collected from sealed storage pots in the nest by suction using disposable syringes, and the pollen was collected with sterile spatulas. This material was transferred to sterile flasks and then packed in a refrigerated box for transport to the laboratory.

Microbial counts and isolation

A quantity of 1 g of each stingless bee honey sample (TaM1, TaM2, and TaM3) and pollen (TaP1, TaP2, and TaP3) was weighed aseptically and homogenized with 9 mL of sterile NaCl (145 mM) and then vortexed for 30 s. Aliquots of 100 µL were transferred and spread over standard plate count agar (PCA), Man, Rogosa and Sharpe agar (MRS), Sabouraud dextrose agar (SDA), and M-Green agar (MGA). PCA isolations were incubated at 36 °C for 48 h and 20% pO₂. The MRS isolations were set at 36 °C for 48 h, 10% pO₂ and 10% pCO₂. The isolations in SDA and MGA were incubated at 25 °C for five days and 20% pO₂. The counts obtained in PCA (total count of mesophilic bacteria), MRS (count of lactic bacteria), SDA, and MGA (yeast count) were expressed in log CFU/g.

Bacteria identification

Priming was performed using Gram’s differential stain, with subsequent biochemical, physiological and antimicrobial resistance tests.

The gram-positive cocci were subjected to the following hemolysis assays; production of catalase, coagulase, and DNase; glucose oxidation/fermentation; resistance to bacitracin and novobiocin; hydrolysis of arginine and urea; butylene-glycolic fermentation; acetoin production; nitrate reduction; and fermentation of salicin, sucrose, trehalose, lactose, L-arabinose, D-mannitol, xylose, D-ribose and D-mannose.

The sporulated gram-positive bacilli were subjected to the following hemolysis assays; production of catalase, amylase, gelatinase, lecinthinase and caseinase; tolerance to NaCl (0, 2, 5, 7, and 10%); butylene-glycolic fermentation; nitrate reduction; fermentation of D-glucose, D-mannose, D-mannitol, L-arabinose, D-xylose, salicin and m-inositol; growth at pH 6 and 5 °C; and the presence of parasporal crystal.

The gram-negative bacilli were subjected to the following oxidase assays; production of gas in glucose, H₂S, indole, DNase, urease, phenylalanine deaminase, β-D-galactosidase, lysine decarboxylase and ornithine decarboxylase; motility; esculin hydrolysis; use of citrate; acetoin production; butylene-glycolic...
fermentation and mixed acid fermentation; fermentation of arabinose, sucrose, mannose, melibiose, sorbitol, mannitol, adonitol, galactose, inositol and lactose; and hydrolysis of arginine.

The lactic acid bacteria were subjected to the following hemolysis tests; production of catalase and gas; hydrolysis of arginine; tolerance to 10% ethanol; growth at 37 °C; and fermentation of D-xylose, L-arabinose, maltose, raffinose, galactose, salicin, D-mannose, D-mannitol, ribose and cellobose.

Yeast identification

The yeasts were identified by growth at 37 °C in the presence of 1% acetic acid, 50% glucose; production of urease and lysine decarboxylase; assimilation of glucose, xylose, rhamnose, melibiose, sucrose, starch, maltose, salicin, mannitol, cellobiose, raffinose, ribose, glycerol, trehalose, inositol, galactose, lactose and arabinose; and fermentation (glucose, sucrose, maltose, raffinose, trehalose, cellobiose, starch, galactose, xylose, lactose and melibiose).

RESULTS

The microbial examination of the stingless bee (SB) honey and pollen showed eight bacteria; one was gram-negative and seven were gram-positive. Only two of them were spore-forming, and one was lactic bacteria. Three yeast species were also isolated. The microbial counts for the samples of honey and pollen are shown in Table 1. The microbiological analyses demonstrated that the average total aerobic mesophiles ranged between 3.78 and 3.71 log CFU/g; the lactic bacteria were 3.59 and 3.56 log CFU/g, and the yeast was 4.41 and 4.66 log CFU/g for the honey and pollen, respectively.

A significant diversity in the microorganisms was observed; the bacteria that were found were *Staphylococcus saprophyticus* subsp. *bovis*; *S. vitulinus*; *S. kloosii*; *S. pasteuri*; *Bacillus pumilus*; *B. thuringiensis*; *Pantoea agglomerans*; and *Leuconostoc mesenteroides* subsp. *mesenteroides*. The yeasts that were identified were *Starmerella meliponinorum*; *Candida magnoliae*; and *Zygosaccharomyces bailli* (Table 1).

Table 1. Microflora isolated from pots of stingless bee honey (TaM) and from pollen (TaP)

| Groups                  | Identified species                     | TaM1 | TaM2 | TaM3 | TaP1 | TaP2 | TaP3 |
|-------------------------|----------------------------------------|------|------|------|------|------|------|
| Cocci Gram +            | *Staphylococcus saprophyticus* subsp. *bovis* | +    | -    | +    | -    | -    | -    |
|                         | *S. vitulinus*                         | -    | +    | -    | -    | -    | -    |
|                         | *S. kloosii*                           | -    | -    | +    | -    | -    | -    |
|                         | *S. pasteuri*                          | -    | -    | -    | +    | -    | -    |
| Sporulated Bacillus Gram + | *Bacillus pumilus*                  | +    | +    | -    | +    | -    | -    |
|                         | *B. thuringiensis*                     | -    | -    | -    | +    | -    | +    |
| Bacillus Gram -         | *Pantoea agglomerans*                  | -    | -    | -    | -    | -    | +    |
| Lactic                  | *Leuconostoc mesenteroides* subsp. *mesenteroides* | -    | -    | +    | -    | -    | -    |
| Yeast                   | *Starmerella meliponinorum*            | +    | -    | +    | -    | -    | -    |
|                         | *Candida magnoliae*                    | -    | -    | -    | +    | -    | -    |
|                         | *Zygosaccharomyces bailli*             | -    | -    | -    | -    | +    | +    |

The analyzed honey samples revealed the presence of *S. saprophyticus* subsp. *bovis*; *S. vitulinus*; *S. kloosii*; and *S. pasteuri*; however, *Bacillus* sp. was the most common bacteria detected in the SB honey and pollen. *Bacillus pumilus*; *B. thuringiensis*; and *Bacillus pumilus* were present in all the honey samples. *B. thuringiensis* was present in two pollen samples, and *Pantoea agglomerans* was also isolated in our study. *S. meliponinorum*; *C. magnoliae*; and *Z. bailli* were identified, confirming the presence of yeasts in these micro-communities. *S. meliponinorum* was found in two jataí honey samples and one pollen sample. *Z. bailli* was found in two pollen samples.

DISCUSSION

Biodiverse microorganisms can originate from the digestive tracts of bees; from the nectar and floral pollen, from sources external to the nest, and from secondary contamination from humans, equipment, containers, wind, and dust [8]. Studies have shown that the intestines of bees contain 1% yeast, 27% gram-positive bacteria (*Bacillus; Bacteridium; Streptococcus*; and *Clostridium* spp.), and 70% gram-negative
bacteria (Achromobacter; Citrobacter; Enterobacter; Erwinia; Escherichia coli; Flavobacterium; Klebsiella; Proteus; and Pseudomonas) [18]. The SB honey samples showed a higher incidence of bacteria and yeast than the pollen samples. The predominance of this microbial group could have been correlated to higher acidity, moisture content, and water activity, which are characteristics of SB honey that encourage the growth of bacteria [3,17,18].

Furthermore, these bacteria and yeasts prevent the growth of other microorganisms which are responsible for the deterioration of food storage pots through antimicrobial systems. High osmotic pressure, low water activity, low pH, acid medium, low protein content, low redox potential due to the high content of reducing sugars, and viscosity limiting the solubility of oxygen, may also inhibit the growth of microorganisms [19].

The genus Staphylococcus currently includes approximately 53 species and 28 subspecies, which are described according to their potential to produce coagulase. The staphylococci most frequently associated with human infection are S. aureus; S. epidermidis; S. haemolyticus and S. saprophyticus [20,21]. Four species of Staphylococcus were found in the samples (S. saprophyticus subsp. bovis; S. vitulinus; S. kloosii; and S. pasteuri). Pucciarelli and coauthors [2] also verified the presence of Staphylococcus spp. in T. angustula honey. The aforementioned authors emphasized that this genus is rarely found in honey, whose antibacterial properties have been associated with the non-survival of this microorganism.

Bacillus sp. was the most common bacteria detected in both the SB honey and pollen, suggesting the existence of a symbiotic relationship between this bacterial genus and bees [2]. Bacillus bacteria are not inactivated by the inhibitory properties of honey, and grow well at pH six or higher [22].

Rosli and coauthors [17] studied the bacterial community of honey from eight different SB species (Heterotrigona itama; Heterotrigona erythrogastra; Tetrigona apicalis; Lepidotrigona terminata; Tetrigona melanoleuca; Tetrigona bingami; Geniotrigona thoracica; and Homotrigona fimbriata). They identified eight bacterial phyla, 71 families, 155 genera, and 70 species using 16S rRNA amplicon sequencing technology. Corroborating with the aforementioned study (and other researchers), the genus Bacillus is one of the bacterial genera most associated with SB colonies, followed by Streptomyces and Lactobacillus [2,7,8,15,16,23].

The genus Bacillus comprises rod-shaped gram-positive bacteria with the ability to form spores. There are 60 species of substantial genetic diversity, most of which are non-pathogenic. They can produce bacteriocins [24]. The roles of non-pathogenic microorganisms in honey bee colonies may include the biochemical contributions of intestinal microorganisms to honey bees, the conversion and preservation of pollen stored in comb cells, and resistance to disease [25].

Similarly, researchers isolated Bacillus bacteria in bee honey and pollen derived from different geographical and botanical origins. They found B. pumilus; B. circulans; B. alvei; B. licheniformis; B. amyloliquefaciens; B. subtilis; B. cereus; B. thuringiensis; B. licheniformis; B. megaterium; and B. pumilus. The authors emphasized that this bacterium could be considered a source of amylase, aiding in the degradation of sugars, and consequently contributing to the transformation of nectar into honey. Bacillus may produce digestive enzymes that act in the pre-digestion of stored food and the production of substances antagonistic to competing microorganisms, mainly organic acids and antibiotics. Acetic and lactic fermentation, which occurs in pollen and honey, are also performed by these bacteria [10,16,17,26].

We identified the genus Pantoea in one of the pollen samples. This strain has been previously found in the intestines of honey bees, in the hive environment, or on plant roots, leaves, and flowers [27,28]. According to Loncaric and coauthors [28] and Costa and coauthors [29], P. agglomerans has been studied for its commercial potential as a possible biological control agent in relation to the major post-harvest pathogens on pome and citrus fruits (against E. amylovora).

One of the honey samples evaluated in our study presented Leuconostoc spp., which are considered to be beneficial bacteria. Leuconostoc spp. are mesophilic, gram-positive and obligately heterofermentative cocci bacteria, which can transform glucose molecules into carbon dioxide, ethanol, and lactate. These bacteria play an essential role in food fermentation and preservation. They are used to improve the nutritional and organoleptic quality of food. Some leuconostoc bacteria produce exopolysaccharides, oligosaccharides, folate, riboflavin, vitamin K and bacteriocins [30,31].

Staumerella, Candida and Zygosaccharomyces frequently occur in SB pollen and honey; they provide sensory and conservation characteristics [10,24,32,33]. These species may have potential uses in biotechnology such as in the production of biosurfactants and other microbial polymers [34]. According to Camargo and coauthors [35], Candida yeast seem to dehydrate the pollen stored by Ptilitrigone lurida bees. This dehydration process is efficient in limiting deterioration and preventing phorids (Phoridae, Pseudohypocera) from consuming pollen, which can cause serious damage to the colonies of this bee species.
Floral visits by SB directly influence the frequency and abundance of yeasts in the nectar. The more attractive the characteristics of the composition of the nectar, the greater the probability of visitation and yeast cells being inoculated after opening the corolla of the flowers [36–38].

As was the case in our study, *S. meliponinorum* was previously identified in samples of honey, pollen and propolis of *T. angustula* bees [30,32,33,39], and *Zygosaccharomyces* sp. was found in association with adult bees of *M. quadrifasciata*. *S. meliponinorum* can ferment pollen after storage [10]. Species of *Zygosaccharomyces* are recognized as being osmophilic. Studies have shown that this yeast has high productivity of glycerol [40].

The microorganisms found in SB products (honey and pollen) are fundamental for the life of stingless bees, helping to keep colonies healthier and more productive. They can be explored as new sources of substances for humans (as a biocontrol agent, preservative, antibiotic, probiotic, and as a producer of antimicrobial compounds and enzymes).

**CONCLUSION**

The honey and pollen collected from three beehives of *T. angustula* native stingless bees were dominated by gram-positive bacteria, mainly *Bacillus pumilus* and *B. thuringiensis* species. From the three yeast species that were isolated, *Zygosaccharomyces bailii* was the dominant yeast in jataí pollen, and *S. meliponinorum* was the dominant yeast in jataí honey. It is hoped that this characterization study of bacterial and yeast diversity from the sources of *T. angustula* bees will indicate a pathway towards extending the biotechnological potential of future applications of these products (stingless bee honey and pollen) in the food, cosmetic and pharmaceutical industries.

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