Fungal diversity associated with the hive stored pollen of stingless bees *Tetragonula travancorica* Shanas and Faseeh

ABSTRACT

The process of collecting and storing pollen by stingless bees involves a complex fermentation process, enriching it with nutrients and probiotics, making it valuable as a dietary supplement. However, the presence of mycotoxins-producing fungi in bee pollen poses health risks to humans. Thus, the study aims to characterize the fungi associated with hive-stored pollen of stingless bees. The results revealed the presence of various fungal species, including *Penicillium*spp. *P. chrysogenum,Aspergillus flavus, and A. aculeatus.* This species has a symbiotic association with hive-stored pollen and is capable of producing mycotoxins.This necessitates post-harvest processing to reduce microorganisms ensuring the safety and quality of bee-derived food products for consumer health

Keywords: Stingless bees, *Tetragonula travancorica,* Microbiota,hive-stored pollen,Fungi.

1. INTRODUCTION

Pollen is often regarded as “the world's best food product” [1]. Stingless bees gather pollen during foraging, transport it to the hive in their specialized pollen baskets, and store it in designated pollen pots. While packing the pollen in pots, bee pollen is enriched with honey, as well as digestive enzymes and organic acids from the salivary glands secretions of bees [2]. This process initiates spontaneous lactic fermentation of the pollen by Lactobacillus bacteria within the pollen pots. Pollen sheaths are dissolved in the process of pollen transformation. Fermentation not only protects the pollen against the loss of properties but also gives rise to new components as a result of enzymatic transformations. Proteins in the pollen degrade into peptides and amino acids during this fermentation process. Studiesrevealed that pollen contains higher protein concentrations than bee bread, although amino acid levels are generally lower. Elevated levels of free amino acids may result from specific proteolytic enzyme activity breaking down polypeptide chains. The compound content in bee bread can be influenced not only by the pollen source but also by the genotype of the bees converting it. Furthermore, lactic acid concentration in bee bread is approximately six times higher than in pollen [3]. The presence of lactic acid helps preserve bee bread, thereby extending its shelf life.

The combination of protein content and added probiotics renders bee bread a valuable dietary supplement. While the recommended daily intake for adults stands at approximately 20 grams. Due to its health-promoting attributes, it's crucial to monitor bee pollen for contamination with harmful substances from a food safety perspective [4,5]. Thepresence of mycotoxin-producing fungi in bee pollen was documented by [6]. Many fungi produce mycotoxins, which can cause acute or chronic intoxication and pose risks to human health upon consumption of contaminated food [7]. Thus, the present study was carried out to characterize the fungi associated with the hive-stored pollen of stingless bees.

2. MATERIAL AND METHODS

2.1. Collection of hive-stored pollen

The hive-stored pollen of stingless bees was collected from commercial stingless beekeepers of Kerala.The sample was collected from the 14 districts of Kerala during the dry season (February, March, and April).Pollen was collected by taking out the pollen pots and removing the caps of the pollen pots and the extracted pollen was placed inside a plastic container. The procedure was repeated at least 4 times to gather a minimum of 5 grams of pollen. Later the pollen samples were labelled with a sample code for easy identification and stored in a refrigerator (-20◦ C).

2.2. Isolation and purification of fungi associated with hive-stored pollen

The fungi associated with hive-stored pollen of stingless bees were isolated using serial dilution and plate count technique using Potato dextrose agar (PDA) [8]. The isolated plates were incubated at room temperature for 5 days. After this incubation period, the plates were examined for colony growth, and the cultural and morphological characteristics of the isolates obtained in the isolated plates were recorded. The distinct fungal colonies from each plate were purified separately using the disc method and stored in a refrigerator for identification.

2.3. Molecular characterization of fungal isolates obtained from the hive-stored pollen

The fungal isolates were identified through molecular techniques by sequencing the ITS region. The pure fungal cultures were sent to Biokart, Bangalore for genomic-level sequencing and identification. BLASTn was utilized to search for homologous sequences using nucleotide data obtained from Biokart. Subsequently, all isolate sequences were deposited into the NCBI GENBANK database.

3. RESULTS

The fungal composition present in the hive-stored pollen of stingless bees was analyzed through serial dilution and plate count techniques. The morphological and cultural characteristics of fungal isolates were used for the identification of fungi. Further confirmation is through molecular characterization.

A total of 21 fungal isolates were identified in the hive-stored samples collected from Kerala. Among them, four distinct fungal isolates were identified from all the samples. The morphological and cultural characteristics of fungal isolates aid in the identification of fungi such as *Penicillium* spp., *Penicillium chrysogenum*, *Aspergillus aculeatus,* and *Aspergillus flavus.* The colony characteristics of the *Penicillium* spp. were filamentous, medium-sized, flat with entire margins, rough, and green colonies with white borders. While *Penicillium chrysogenum* colonies were irregular, small-sized, raised elevation, filiform borders, rough surface, and yellow colour with green spores. The fungal genus, well known as laboratory weed Aspergillus is also recorded in the hive-stored pollen samples. The various species of Aspergillus recorded in the hive stored pollen including *Aspergillus flavus* and *Aspergillus aculeatus.* The colony morphology of *Aspergillus flavus* was circular-shaped, small-sized, and raised colonies, with entire margins, wrinkled surfaces, and white margins with brown to black spores. Whereas, the colony morphology of *Aspergillus aculeatus* wasirregular, small-sized, raised elevation, filiform borders, rough surface, and brown to black spores (Plate 1).

Molecular characterization of fungal isolates associated with the hive stored pollen of *T. travancorica* was carried out using ITS gene sequencing from Biokart India Pvt Ltd. The PCR product amplified on 1.2 per cent agarose showed a band between 600-00bp (Fig. 1). The sequence analysis was used to confirm the fungal isolates as *Penicillium sp.* (Accession number: PP296549), *Aspergillus aculeatus* (Accession number: PP291951), *Aspergillus flavus* (Accession number: PP291949), and *Penicillium chrysogenum* (Accession number: PP301322). This confirmed the cultural, and morphological characterization.

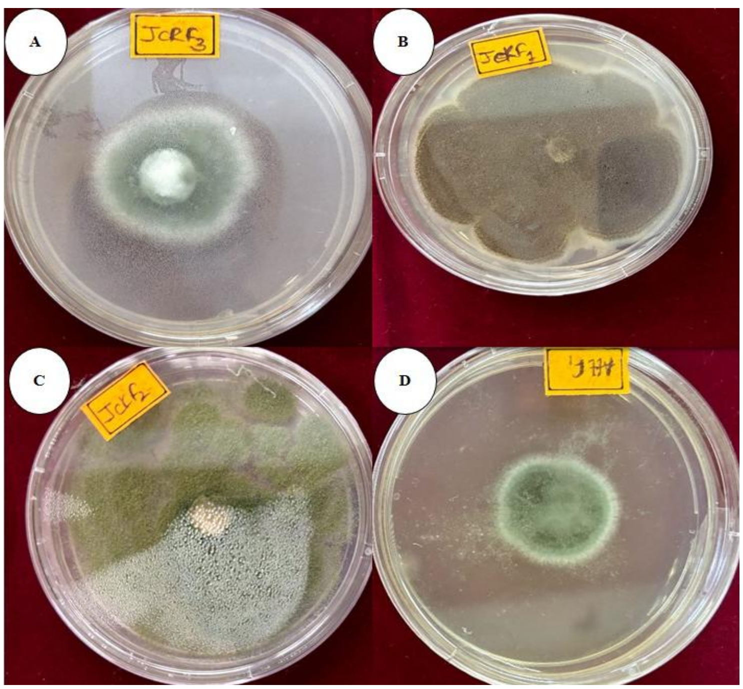

Plate 1. Fungi obtained from the hive stored pollen of stingless bee *T. travancorica,* A-*Penicillium chrysogenum,* B-*Aspergillus aculeatus,* C-*Aspergillus flavus, and* D-*Penicillium* spp.

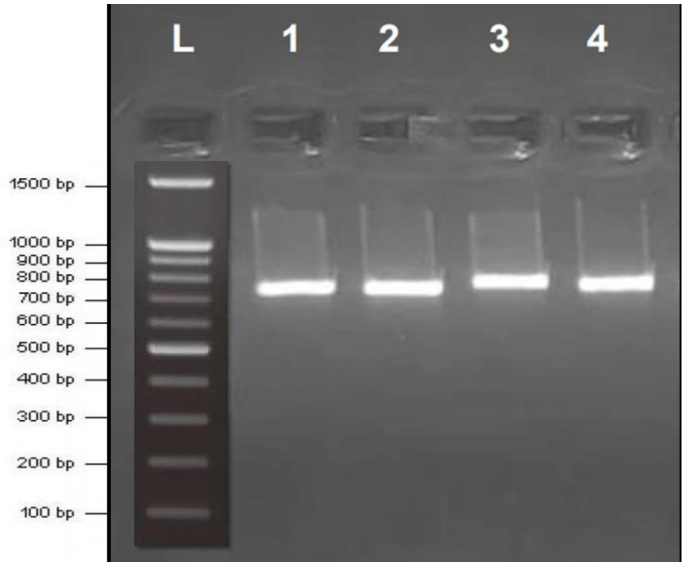

Figure 1. Agarose gel electrophoresis of Genomic DNA isolated from the fungi associated with hive stored pollen. 1-*Penicillium chrysogenum,* 2-*Aspergillus aculeatus,* 3-*Aspergillus flavus, and* 4-*Penicillium* spp.

4. Discussion

The fungi associated with the hive stored pollen of stingless bees revealed the presence of *Penicillium* spp., *Penicillium chrysogenum, Aspergillus flavus,* and *Aspergillus aculeatus*. Similar fungal compositions have been observed in the previous studies of bee pollen and bee bread [9,10,11]. The major factors such as plant source, geographical origin, and bee-keeping practices significantly influence the microbial composition in the bee pollen [12,13]. *Aspergillus* spp. can thrive on decomposing plant matter and are adapted for the degradation of complex plant polymers [14]. These species are often found in association with bees and bee products, particularly pollen. Pollen serves as a point of entry for fungal pathogens into beehives. Studies have shown that spores of Aspergillus spp. can contaminate pollen while it is still on plants [15]. Bees collect pollen, store, and consume pollen, allowing the spores to reach their gut, which is the primary site of infection for bee pathogens [16]. *Aspergillus* spp. are opportunistic pathogens capable of infecting bees at all developmental stages [17]. *Aspergillus flavus* was the causal agent of the stone brood disease of honey bees and is poorly studied in the honey bee pathogens [16,18]. Bee pollen acts as a substrate for the production of mycotoxins [19]. The commercial pollen may still contain *A. flavus* spores [2,6,20]. Thus, posing a risk to human health due to high mould contamination levels and mycotoxins. Therefore, it is important to implement post-harvest processing to reduce microbial contamination.

The association of *Penicillium* spp. with honey and bee bread has been reported earlier, but their specific functions within the hives of stingless bees have been lacking. Studies showed that *Penicillium* spp. is capable of secreting organic acids that aid in the preservation of pollen. The enzymes secreted by the Penicillium contributed to the nutritional value of the pollen [21], and protection against pathogens [22]. Some species of *Penicillium* are producing mycotoxins that impair the health of humans, hence the actual role and specific mechanisms need to be explored.

5. Conclusion

The present work highlights the intricate relationship between fungi and bee products like pollen. The fungi enter the hive from flowers through bees during foraging, proliferate within stored pollen, and produce mycotoxins. These mycotoxins can pose health risks to humans. Hence, it's crucial to implement measures to minimize microbial contamination in food products derived from bee pollen through careful post-handling processes. This emphasizes the importance of ensuring the safety and quality of bee-derived food products for consumer health.

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AUTHORS CONTRIBUTION

Bindu G R experimented and wrote the manuscript, Mani Chellappan and Surendra Gopal Kulkarni formulated the work, Deepu Mathew, Ranjith Matta Thodikayil, and Shanas Sudheer identified a stingless bee, *T. travancorica* and helped in molecular characterization of fungi samples. All authors read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIAL

The corresponding author will provide the datasets created and analyzed during the current work upon reasonable request.

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