

Microbial analysis of hive stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius) (Hymenoptera, Apidae)

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ABSTRACT: The study was conducted to identify the microbial communities associated with the hive stored pollen of *Apis cerana indica* colonies in Palakkad and Wayanad region of Kerala. Investigation revealed that samples collected from Palakkad contained two bacteria viz., *Pseudomonas aeruginosa* and *Bacillus megaterium* and one fungus viz., *Trichoderma reesei*, while pollen from Wayanad had two bacteria viz., *B. aryabhatai* and *B. megaterium* and two fungi viz., *Westerdykella multispora* and *Neurospora crassa*. Microbial load was more in samples from Wayanad (6.6×10^4 cfu g⁻¹) compared to Palakkad sample (3.3×10^4 cfu g⁻¹). © 2025 Association for Advancement of Entomology

KEY WORDS: Microbial communities, bacteria, fungi, microbial load

INTRODUCTION

Honey bee colonies rely heavily on flower nectar and pollen for nutrition, which requires protection from microbial degradation. Bee substances like plant resins, nectar, pollen, wax, honey, and propolis exhibit antimicrobial properties, playing a crucial role in preserving stored pollen (Kujumgiev *et al.*, 1999; Ohashi *et al.*, 1999). Microbial activity (microbial succession, anaerobic breakdown and pollen predigestion) improves the nutrition of stored bee bread, which has been reported to possess antimicrobial, antioxidant, and anti-radiation properties (Loper *et al.*, 1980; Anderson *et al.*, 2011; Matilla *et al.*, 2012; Homan *et al.*, 2013; Disayathanoowat *et al.*, 2020). There are diverse and abundant microbial communities in the bee pollen that not only play roles in pollen processing

and preservation (Martinson *et al.*, 2011; Corby-Harris *et al.*, 2014), but also may serve as significant protein sources for developing bees. The microbial composition in pollen and hive-stored bee bread is influenced by various factors, including land use, floral sources and seasonal variation (Anderson *et al.*, 2014; De Palma *et al.*, 2016; Anderson *et al.*, 2013). While the chemical composition of bee pollen from different plants and geographical origins has been well-studied, information on microbial diversity in hive-stored pollen is limited. This knowledge gap is significant, particularly considering the importance of bee pollen in human nutrition and the potential risks associated with microbial contamination. Studies have shown that diverse microbial communities are associated with honey bee gastrointestinal tracts and hive-stored pollen, highlighting the need for further research. This study

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aims to investigate the microbes associated with hive-stored pollen of *Apis cerana indica* colonies, contributing to our understanding of pollen preservation and informing microbiological standards for safe consumption.

MATERIAL AND METHODS

The study conducted at Department of Agricultural Entomology, College of Agriculture, Vellanikkara, Kerala Agricultural University during 2020-2021. Survey was conducted for the collection of hive stored pollen from the bee hives of Indian honeybees. Samples were collected from two different locations *viz.*, Palakkad and Wayanad districts, which represented the central midlands and highland areas of Kerala. The samples collected from Palakkad were given the code PKD, which includes PKD-1: Mannarkkad, PKD-2: Nellipuzha, PKD-3: Nottanamala and similarly, from Wayanad as WYD, which includes WYD-1: Kenichira, WYD-2: Kayakunnu and WYD-3: Cheengode. Serial dilution and plate counting technique was followed for isolation of microbes and readymade media was used such as nutrient agar (MOO1), Potato Dextrose Agar (MHO96), Kenknight and Munaier's medium (M695) and Sabouraud Dextrose Agar, granulated medium (GM063) were used to isolate bacteria, fungi, actinomycetes and yeast, respectively. The media were prepared and autoclaved for 20-30 minutes at 121°C and 15 lbs of pressure. The medium was stored at room temperature for further use. One gram of Indian bee pollen samples was serially diluted up to 10^{-4} and a series of dilutions were performed. The samples collected from each location were plated carefully in respective media for the isolation of bacteria, fungi, actinomycetes and yeast using nutrient Agar (NA), Potato Dextrose Agar (PDA), Kenknight and Munaier's medium and Sabouraud Dextrose Agar (SDA), respectively. Three replications were maintained for each sample. After incubation, colony-forming units were calculated (Anderson *et al.*, 2014). Bacterial colonies were sub-cultured by performing quadrant streaking method on NA media and purified by performing repeated streaking on NA slants. Fungal colonies were sub-cultured by placing the hyphal

disc at the centre of PDA media and purified by placing the hyphal discs in PDA slants. Morphological characters of bacteria were identified by performing gram staining and fungi isolates were identified by slide preparation and under phase-contrast microscope. Further, molecular characterization of bacterial isolates was carried out based on 16S rRNA gene sequencing.

Sequencing of ITS region of fungi and bacteria:

The 16S rRNA and ITS (The Internal Transcribed Spacer) regions of bacteria and fungi were amplified with universal primers specific to bacteria and fungi. Purified cultures of bacteria and fungi were given for sequencing at the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala. Genomic DNA of bacteria was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. Part of the culture was taken in a microcentrifuge tube. At 56°C, 180 μ l of T1 buffer and 25 μ l of proteinase K were added and incubated in a water bath until they were completely lysed. After lysis, 5 μ l of RNase A (100 mg ml^{-1}) was added and incubated at room temperature for 5 minutes. After incubating at 70°C for 10 minutes, 200 μ l of B3 buffer was added and incubated for another 10 minutes. Then 210 μ l of ethanol (100%) was added and mixed thoroughly by vortexing. The mixture was pipetted into a NucleoSpin® Tissue column placed in a 2ml collection tube and centrifuged at 11000 $\times$ g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2ml tube and washed with 500 μ l of BW buffer. The wash step was repeated using 600 μ l of B5 buffer. After washing, the NucleoSpin® Tissue column was placed in a clean 1.5ml tube and DNA was eluted out using 50 μ l of BE buffer. The quality of the DNA isolated was checked using agarose gel electrophoresis. One μ l of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5 μ l of DNA. The samples were loaded onto a agarose gel (0.8%) prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 μ g ml^{-1} ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until the bromophenol dye front had migrated to the bottom of the gel.

The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using the Gel Documentation System (Bio-Rad). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) by using universal primers. Five μ l of PCR product was mixed with 0.5 μ l of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 85°C for 5 minutes.

For fungus, about 100mg of the fungal tissue or mycelium was homogenized using liquid nitrogen and the powdered tissue was transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 was added and vortexed for one minute. 10 μ l of RNase A solution was added and inverted to mix. For 10 min, the homogenate was incubated at 65°C. The lysate was transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 min. The flow through liquid was collected and the filter was discarded. 450 μ l of buffer PC were added and mixed well. The solution was transferred to a Nucleospin Plant II column, centrifuged for 1 min and the flow through liquid was discarded. The column was filled with 400 μ l of buffer PW1, centrifuged at 11000 x g for 1 min and the flow through liquid was discarded. Then 700 μ l of PW2 was added, centrifuged at 11000 x g and the flow through liquid was discarded. Finally, 200 μ l of PW2 was added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column was transferred to a new 1.7 ml tube and 50 μ l of buffer PE was added and incubated at 65°C for 5 minutes. The column was then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4°C. Gel electrophoresis was carried out in the same way for bacterial DNA. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) by using universal primers.

Agarose Gel electrophoresis of PCR products:

The PCR products were checked on 1.2 per cent agarose gels prepared in 0.5X TBE buffer containing 0.5 μ g ml⁻¹ ethidium bromide. For loading, 1 μ l of 6X loading dye was mixed with 4 μ l of PCR products and electrophoresis was performed at 75V

power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using the Gel Documentation System (Bio-Rad).

Exo SAP-IT Treatment: Exo SAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

NCBI Blast: The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1. The rRNA homology searches were performed using the BLAST program.

The pollen sample data analysis with three replications was carried out. Using R software, the obtained data were analysed using Welch's two sample t test.

RESULTS AND DISCUSSION

In the investigation, two bacteria and one fungus were identified from Palakkad sample, whereas from Wayanad sample two bacteria and two fungi were identified. Total four colonies of bacteria were obtained from hive stored pollen of *Apis cerana indica* from the sampling locations. Isolate B1 (from sample PKD-1) and isolate B2 (from sample PKD-3) were obtained from pollen samples collected from Palakkad, whereas isolate B3 (from sample WYD-3) and isolate B4 (from sample WYD-2) obtained from Wayanad. Out of four bacterial isolates, B1 which appeared in red when observed under a compound microscope, inferred that the bacteria were gram negative, while the isolates B2, B3 and B4 which appeared in crystal violet inferred that all three bacteria were gram positive (Fig. 1). Morphological and cultural characters of different

Table 1. Morphological and cultural characters of different bacterial isolates obtained from hive stored pollen of *Apis cerana indica*

Location	Bacterial isolate	Gram reaction	Shape	Color	Margin	Elevation of colony	Form colony
Palakkad	B1	Negative	Rod	Slight yellowish	Irregular	Flat	Round large
	B2	Positive	Rod	Creamy white	Entire	Flat	Round large
Wayanad	B3	Positive	Rod	Creamy white	Entire	Flat	Round large
	B4	Positive	Rod	Creamy white	Entire	Flat	Round large

Table 2. Molecular identification of bacterial isolates obtained from Palakkad and Wayanad districts of Kerala

No.	Sample code	Isolate	Bacteria identified	Accession number	Query coverage (%)	Identity (%)
1	PKD-1	B1	<i>Pseudomonas aeruginosa</i>	MH010896.1	100	99.84
2	PKD-2	B2	<i>Bacillus megaterium</i>	MN826585.1	100	100
3	WYD-3	B3	<i>B. megaterium</i>	KJ500011.1	100	99.69
4	WYD-2	B4	<i>B. aryabhattai</i>	MT184818.1	99	100

bacterial isolates obtained from hive stored pollen indicated that, isolate B1 produced large round colonies in the media with irregular margin and flat elevation. Isolates B2, B3 and B4 produced numerous small round colonies with entire smooth margin and flat elevation (Table 1).

When molecular identification of bacterial isolates obtained from Palakkad and Wayanad districts of Kerala were analysed through, 16S rRNA sequencing from the hive stored pollen of *A. cerana indica* revealed the presence of B1 as *Pseudomonas aeruginosa*, B2 and B4 as *Bacillus megaterium* and B3 as *B. aryabhattai* in the hive stored pollens (Table 2).

Total three colonies of fungi were obtained. Isolate T1 (from sample PKD-2) was obtained from Palakkad, whereas isolate W1 (from sample WYD-2) and isolate A1 (from sample WYD-1) were obtained from Wayanad. Morphological and cultural characters of different fungal isolates obtained from hive stored pollen, indicated that, isolate T1 had green large round colonies with entire smooth margin having flat elevation. Isolate W1 had white

small round colonies with entire smooth margin having flat elevation. Isolate A1 had orange small round colonies with smooth margin having flat elevation (Table 3).

When molecular identification of fungal isolates obtained from Palakkad and Wayanad were analysed through, ITS sequencing of three fungi resulted T1 as *Trichoderma reesei*, W1 as *Westerdykella multispora* and A1 as *Neurospora crassa* (Table 4).

Kacaniova *et al.* (2012) performed dilution plating analysis for the collected samples and isolated various fungal species like *Penicillium*, *Aspergillus* and *Cladosporium* respectively. Other fungal species like *Botrytis*, *Eurotium*, *Trichoderma*, *Phoma*, *Fusarium*, *Mucor*, *Acremonium*, *Alternaria* and *Epicoccum* were also identified from the samples.

When microbial load of hive stored pollen samples collected from Palakkad and Wayanad districts of Kerala were analysed, the pollen collected from Wayanad recorded the highest microbial load

Table 3. Morphological and cultural characters of different fungal isolates obtained from hive stored pollen of *Apis cerana indica*

Location	Isolate	Color	Margin	Elevation of colony	Form of colony
Palakkad	T1	Green	Entire	Flat	Round
Wayanad	W1	White	Entire	Flat	Small round
	A1	Orange	Entire	Flat	Small round

(6.6×10^4 cfu g⁻¹) compared to Palakkad sample (3.3×10^4 cfu g⁻¹). From Palakkad, the fungal population varied between 0.6×10^4 to 1.3×10^4 with a mean of 1×10^4 (cfu g⁻¹), whereas the bacterial population varied between 1.3×10^4 and 3.3×10^4 with a mean of 2.3×10^4 (cfu g⁻¹). From Wayanad, the fungal population varied between 3×10^4 and 3.6×10^4 with a mean of 3.3×10^4 (cfu g⁻¹), whereas bacterial population varied between 2.66×10^4 and 4×10^4 with a mean bacterial population of 3.3×10^4 (cfu g⁻¹) (Table 5).

Bonvehhi and Jorda (1997) reported that total

coliform bacteria population present in the pollen sample ranged from 3-43 cfu g⁻¹. Whereas, *Clostridium*, *Bacillus* and *Streptococcus* was in the range of 5-10, 5-12 and 5-550 cfu g⁻¹ respectively. Kacaniova *et al.* (2012) has reported that total fungal population varied from 2.00×10^2 cfu g⁻¹ with an average value of 1.20×10^2 cfu g⁻¹ when inoculated on czapek-Dox agar, whereas the fungal population varied from 1.58×10^2 - 9.66×10^2 cfu g⁻¹ when inoculated on Malt Extract Agar. Similar study was reported as total fungal population in hive stored pollen samples (Nogueira *et al.*, 2012; Sinacori *et al.*, 2013; Thakur *et al.*, 2024).

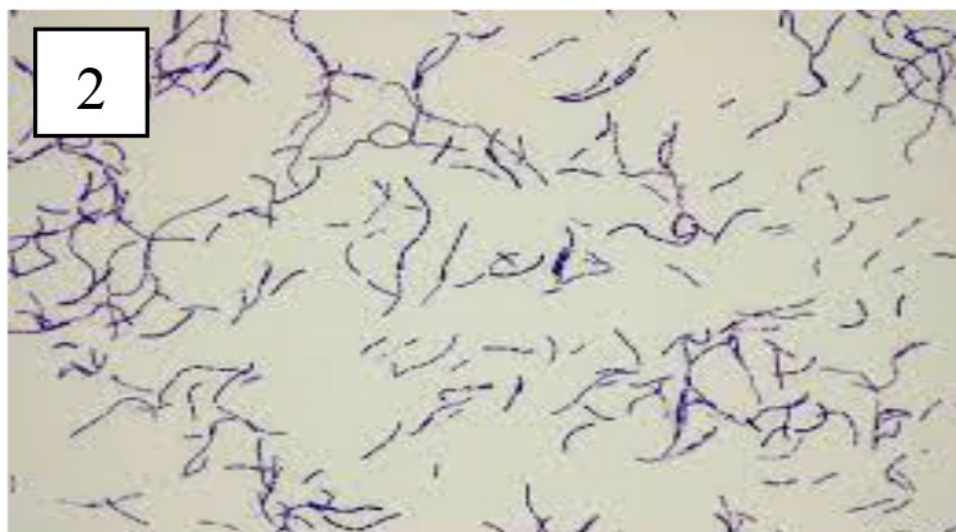
Total bacterial and fungal population of hive stored pollen of *A. cerana indica* obtained from Palakkad and Wayanad districts of Kerala: The fungal population of the hive stored pollen samples collected from Palakkad ranged between 0.6×10^4 to 1.3×10^4 with a mean of 1×10^4 (cfu g⁻¹), whereas the bacterial population ranged from 1.3×10^4 to 3.3×10^4 with a mean of 2.3×10^4 (cfu g⁻¹). The fungal population of the hive stored pollen samples from Wayanad ranged from 3×10^4 to 3.66×10^4 with a mean of 3.33×10^4 (cfu g⁻¹). Whereas the bacterial population was in the range of 2.66×10^4 to 4×10^4



Fig. 1 Microscopic image: Gram-negative bacteria

Table 4. Molecular identification of fungal isolates obtained from Palakkad and Wayanad districts of Kerala

No.	Code	Isolate	Fungus identified	Accession no.	Query coverage	Identity
1	PKD-2	T1	<i>Trichoderma reesei</i>	MW048853.1	87%	96.23%
2	WYD-2	W1	<i>Westerdykella multispora</i>	MH857224.1	100%	99.79%
3	WYD-1	A1	<i>Neurospora crassa</i>	MT557113.1	100%	100%

**Fig. 2** Microscopic image: Gram-positive bacteria

with a mean of 3.3×10^4 (cfu g⁻¹). Among the sample from Palakkad, PKD-1 recorded the highest fungal population (1.3×10^4 cfu g⁻¹), followed by PKD-2 (1×10^4 cfu g⁻¹) and PKD-3 (0.6×10^4 cfu g⁻¹). Similarly, PKD-1 recorded the highest bacterial population (3.3×10^4 cfu g⁻¹), followed by PKD-2 (2.3×10^4 cfu g⁻¹) and PKD-3 (1.3×10^4 cfu g⁻¹). In Wayanad samples WYD-1 recorded the maximum fungal population (3.66×10^4 cfu g⁻¹), followed by WYD-2 (3.33×10^4 cfu g⁻¹) and WYD-3 (3×10^4 cfu g⁻¹). Similarly, WYD-1 recorded maximum bacterial population (4×10^4 cfu g⁻¹) which was followed by WYD-2 (3.33×10^4 cfu g⁻¹) and WYD-3 (2.66×10^4 cfu g⁻¹).

The pollen sample data analysis using R software, Welch's two sample t test, revealed high significant differences among fungal and bacterial population in the samples collected from the hive stored pollen of *A. cerana indica* obtained from Palakkad and

Table 5. Microbial load of hive stored pollen samples collected from Palakkad and Wayanad districts of Kerala

Location	Load (cfu g ⁻¹)	t value	p value
Palakkad	3.3×10^4	-4.7295	4.413e-05
Wayanad	6.6×10^4		

Wayanad districts of Kerala (Table 6). In the present study even after 72-96 hours of incubation, actinomycetes and yeast colonies were not recorded on the plated Kenknights and Munaiers medium and Sabouraud Dextrose Agar (SDA) medium.

Pseudomonas is an inhabitant of bee pollen, contributing to the decomposition of pollen walls, which is commonly detected in honey (Stavropoulou *et al.*, 2023). *Bacillus* sp. possesses the antimicrobial property, which may contribute to

Table 6. Welch two sample t-test statistical analysis of fungal and bacterial population

Location	Colony (cfu g ⁻¹)	
	Fungi	Bacteria
Palakkad	1 × 10 ⁴	2.3 × 10 ⁴
Wayanad	3.3 × 10 ⁴	3.3 × 10 ⁴
t value	-5.7155	-2
p value	4.788e-05	0.06473

regulation of host's microbiota as a beneficial microbe or probiotic (Jia *et al.*, 2020). Comparative analysis of microbial communities in the examined honey samples revealed that their major taxa were either indigenous microbiota of honeybees, mostly beneficial and to a lesser extent potential foodborne and human pathogens and/or inhabitants of bee pollen and flowering plants, including both plant protective and phytopathogenic microorganisms.

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