

## Amplicon-based gut microbial profiling and their predicted functional potential in *Apis dorsata* across two ecological regions of Pakistan

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### ABSTRACT

**Background and Objectives:** *Apis dorsata* (*A. dorsata*), the giant honeybee, is an important pollinator of Asia. Gut-microbial assemblages contribute to nutrient utilization and metabolism, yet their diversity in the gut of *A. dorsata* remains underexplored. The main objective of the current study was to explore the impact of local geographical conditions on the gut microbial diversity and the functional potential of *A. dorsata* gut-associated bacteria.

**Materials and Methods:** Bacterial community profiling and characterization were performed using high-throughput sequencing on the DNA Nanoball Sequencing (DNBseq) platform.

**Results:** Fifty-four bacterial species were detected from the gut of *A. dorsata*, expanding the known microbial diversity of this host species. The comprehensive analysis of pooled samples from two regions revealed that higher microbial diversity in district Bhimber than in Jhelum. Nevertheless, site-specific putative differences were observed in the pathways related to carbohydrate metabolism, amino acid synthesis, and other cellular activities.

**Conclusion:** This exploratory pilot investigation revealed site-wise preliminary differences in taxonomy and putative metabolic functions of bacterial species. These results provide a baseline for future studies on the diversity and dynamics of functioning of gut-associated microbial communities of *A. dorsata*.

**Keywords:** *Apis dorsata*; 16S rRNA; Next-generation sequencing; Microbiota; Predicted function annotation

### INTRODUCTION

Pakistan supports honeybee fauna across northern mountains, subtropical landscapes, plains, and

Himalayan foothills. *Apis dorsata* (giant honeybee), *Apis cerana* (cavity-nesting), and *Apis florea* (open-nesting) are native species, while *Apis mellifera* is an exotic species (1, 2). However, localized

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high-elevation species, i.e., *Apis laboriosa*, have recently been documented in the Neelum Valley, Azad Kashmir (3). These species contribute significantly to agricultural biodiversity (4). The honeybee gut contains core bacterial taxa, such as *Lactobacillus* spp., *Frischella perrara*, *Commensalibacter* spp., *Gilliamella apicola*, *Bifidobacterium* spp., and *Snodgrassella alvi* (5). These symbionts are involved in host metabolic adaptation and maintenance of health (6). These bacterial taxa play metabolic roles, including vitamin production, the degradation of complex dietary carbohydrates, fermentation of pollen-derived substrates, and suppression of pathogenic microorganisms (7, 8).

Geographical heterogeneity is among the major ecological determinants of microbial communities associated with hosts. Geographical variation, such as climate, vegetation composition, land-use patterns, and floral resource diversity, may produce spatial differences in microbial communities linked with pollinators. It has been demonstrated that the overall similarity of gut bacteria among honeybees tends to decline as geographic distance increases, indicating the joint influence of stochastic processes and environmental selection (9). Abiotic factors such as climate change, exposure to pesticides, the presence of microplastics, phytochemicals, forage type, antibiotics, and air pollution have impacts on the honeybee gut microbial diversity. This environmental stress can alter the microbial community structure and potentially affect host metabolism, immunity, and resistance to pathogens. Other factors capable of disrupting the structure of microbial communities are biotic factors, such as viruses, small hive beetles, fungi, bacteria, wax moth, microsporidia, and Varroa mite (*Varroa destructor*) (10). Furthermore, urbanization disturbs green space, floral richness, and habitat quality, which may have an adverse effect on the abundance of honeybee gut microbes in comparison to rural locations (11).

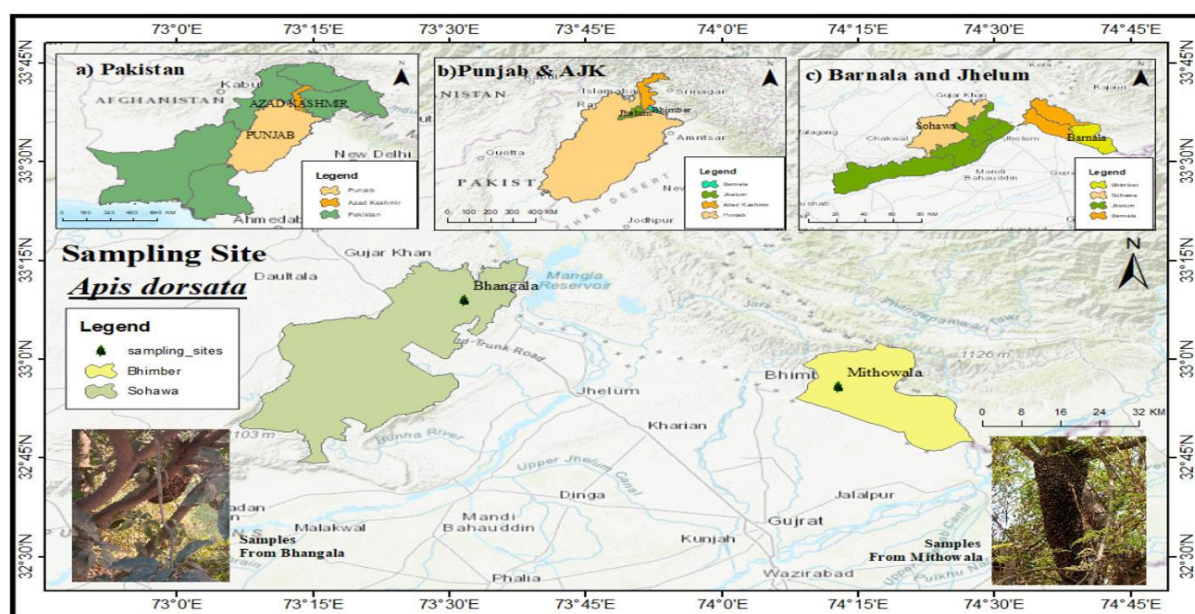
Despite major advances in the study of honeybee microbiomes, most of the available literature has focused on managed honeybee. In contrast, wild honeybee species remain relatively understudied. The comparative analysis of both taxonomic richness and inferred functional capacity of gut microbiota across geographical areas is still scarce for *A. dorsata*. This knowledge gap is especially clear in South Asian ecosystems, where *A. dorsata* plays one of the most significant roles in the pollination process, but

the ecology of its microbes remains poorly characterized. The role of environmental heterogeneity in the development of gut microbial communities in wild honeybee species is very important. The diversity of microbes contributes to host plasticity, ecological stability, and pollinator well-being. In this study, we examined the gut microbial community of *A. dorsata* in two ecologically different areas of Pakistan with the help of the 16S rRNA gene-based characterization of microbial communities. Our objectives were to (i) describe the taxonomic structure of gut-associated bacterial communities, (ii) examine core and potentially novel taxa of *A. dorsata*, and (iii) determine the functional capacity of these bacterial communities in different ecological regions. This study uses a combination of taxonomic and predicted functional analyses to provide a novel understanding of the microbial ecology of *A. dorsata*. This establishes a framework for future research on the role of the gut microbiota in pollinator health, ecological adaptation, and conservation biology.

## MATERIALS AND METHODS

**Study area.** The *A. dorsata* hives were sampled from two districts Jhelum (A1) and Bhimber (A2), Punjab, Pakistan (Fig. 1). Jhelum (32° 56' 25.9728" N, 73° 43' 39.4716" E) is located in the Potohar Plateau in Punjab, Pakistan, and it covers an area of around 3,587 km<sup>2</sup>. The region is characterized by semi-arid weather, hilly topography, and a mixed agricultural environment. Nonetheless, the district of Bhimber (31° 42' 59.9832" N, 73° 59' 6.0864" E), located in Azad Jammu and Kashmir, represents a predominantly mountainous environment with specific ecological and climatic conditions, such as a relatively high percentage of vegetation cover and heterogeneous floral resources (1). These geographically and environmentally contrasting regions were selected to explore the potential effect of habitat variation on the gut microbial diversity of *A. dorsata*. Sampling was conducted during the same season to reduce temporal heterogeneity in microbial assemblage composition associated with seasonal floral availability and environmental conditions.

**Collection of *Apis dorsata*.** Two healthy colonies of *A. dorsata* were selected based on certain indicators of a healthy colony, such as the availability of the



**Fig. 1.** Geographic location of sampling sites for *A. dorsata* in Pakistan. (a) Map of Pakistan highlighting the Punjab province and Azad Jammu and Kashmir (AJK); (b) Regional map showing the sampling areas within Punjab and AJK; and (c) Detailed map indicating the specific sampling locations in Jhelum (Sohawa and Bhangala) and Bhimber (Mithowala).

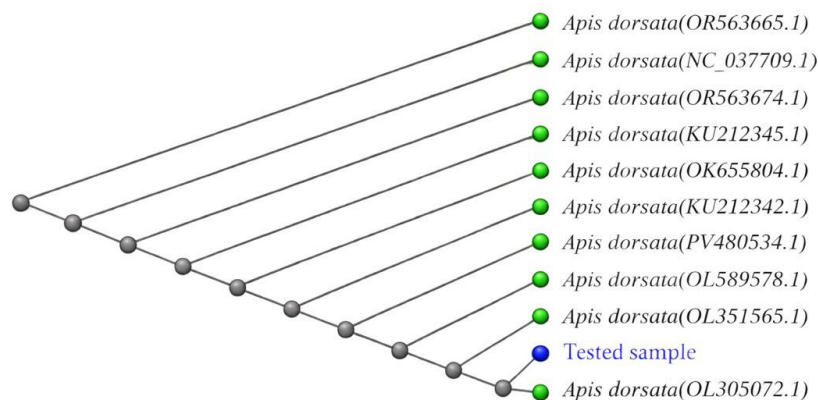
surrounding floral resources, uniformity of the brood, and the absence of visible pests and pathogens. A total of, 30 adult worker bees were selected randomly from the outer comb area with sterile forceps from each location. The samples were placed in sterile and DNA-free collection tubes for gut dissection and subsequent analysis of bacterial diversity and functional potential. To mitigate the risks of contamination, sampling instruments were sterilized using 70% ethanol between collections, and samples were carried to the laboratory on ice and processed within 24 hours of collection. Due to logistical and field constraints associated with sampling wild *A. dorsata* colonies, composite gut samples were made by pooling the digestive tracts of multiple worker bees per hive. Although this method enables the characterization of the microbial communities at the colony level, it can limit the variability of microbiomes in the bees at the individual level. Therefore, the study should be considered descriptive in nature.

**Identification.** *A. dorsata* individuals collected were anesthetized by the deep-freezing method at  $-20^{\circ}\text{C}$  before processing. Identification of species was done by using both morphological and molecular methods. Morphological identification was done through microscopy according to existing taxonomic keys, and molecular validation was done by compar-

ing the sequence with the reference *A. dorsata* sequences obtained from publicly available databases, and phylogenetic relationships were inferred (Fig. 2).

**Dissection.** Samples were surface sterilized using ethanol (70%), then washed with 1x sterile phosphate-buffered saline (PBS) to eliminate any exterior microbial pathogens. Each honeybee was dissected under aseptic conditions in a laminar airflow cabinet, and the entire digestive tract of every honeybee was removed carefully in sterile saline solution. The gut tissues were dissected and placed in 1mL sterile PBS and kept at  $-80^{\circ}\text{C}$  until further processing. A composite sample was prepared by combining fifteen honeybee digestive tracts from each location to get adequate DNA (positive control) for further analysis.

**Preparation of library and sequencing.** PCR positive control containing high-quality genomic DNA, i.e., 30 ng, was subjected to polymerase chain reaction (PCR) amplification. In the case of bacterial community analysis, 16S rRNA gene-specific fusion primers, targeting hypervariable regions of bacteria, were used (12). PCR amplification was performed using universal bacterial primers 341F (5'-CCTACG-GGNGGCWGCAG-3') and 805R (5'-GACTACHVG-GGTATCTAATCC-3'). The reaction was carried out in a 25  $\mu\text{L}$  volume containing 2  $\mu\text{L}$  template DNA,



**Fig. 2.** Phylogenetic tree showing the relationship of the analyzed sample with reference sequences of *Apis dorsata* obtained from public databases. The tested sample (blue) clusters closely with known *A. dorsata* sequences (green), confirming its taxonomic identity. The tree was constructed using a maximum-likelihood approach based on aligned sequence data, with branch lengths representing evolutionary distances.

12.5  $\mu$ L 2  $\times$  PCR master mix, 1  $\mu$ L of forward primer, 1  $\mu$ L of reverse primer, and 8.5  $\mu$ L of nuclease-free water. Thermal cycling conditions included initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 15s, 48°C for 20s, extension at 72°C for 10s, with a final extension at 72°C for 5 minutes. No-template PCR negative control shows neither primer dimer nor anticipated band on agarose gel.

Purification of all PCR products was done using Agencourt AMPure XP magnetic beads, which were dissolved in elution buffer and then subsequently indexed to complete library construction. The concentration and library size distribution were measured using the Agilent 2100 Bioanalyzer. The sequencing of two qualified libraries on the DNBseq platform was then performed based on the instructions of the manufacturer and the respective size of the insert. Potential false-negative results were reduced by ensuring acceptable library quality and obtaining adequate sequencing depth.

**Data analysis.** During downstream bioinformatics analysis, several quality control procedures were applied to reduce false-positive sequence detection and incorrect taxonomic identifications. Raw sequencing reads were initially assessed for quality using Fqtools fqcheck (v0.25), while adapter trimming and removal of poor-quality reads were conducted with Cutadapt (v2.6). The number of reads retained after quality filtering was an average of 67,902 paired-end read pairs, which was equivalent to 1,35,803 individual

reads per sample. Additionally, Q20 and Q30 values were 95.58% and 90.73%, respectively, for Jhelum (A1). However, the values of Q20 were 95.71% and Q30 was 90.90%, respectively, for Bhimber (A2). Readfq (v1.0) was used to process the FASTQ files (5, 13, 14). FLASH (Fast Length Adjustment of Short Reads, v1.2.11) was used to merge paired-end reads. OTUs (Operational Taxonomic Units) were clustered at a 97% sequence similarity using USEARCH (v7.0.1090). Detection and removal were performed using UCHIME (v4.2.40). In the case of bacterial marker genes (16S rRNA), the reference database used to detect chimeras was the Gold database (v20110519).

Taxonomic annotation of bacterial 16S rRNA gene sequences in bacteria was performed using the SILVA SSU rRNA database (release 138) with a confidence threshold of 0.8 to obtain the correct taxonomic classification (15). After OTUs filtering, good-quality sequences were retained for A1 (64,375 reads) and A2 (62, 621 reads), respectively, with an average final sequencing depth of 63,498 reads per sample. The data were submitted to the NCBI SRA database under the bioproject PRJNA1444343.

A maximum-likelihood phylogenetic tree was constructed from aligned sequences using FastTree, and Interactive Tree of Life (iTOL) was used to visualize it. All samples were normalized by rarefaction to the minimum sequencing depth to ensure comparability across samples. Shannon, Simpson Dominance, Chao1, and ACE diversity matrices were used to calculate alpha diversity. Venn diagrams, and rarefac-

tion curves were made with ggplot2 in R software to visualize relative abundance, shared taxa and species distribution. Functional prediction was performed using PICRUSt2 based on 16S rRNA gene sequences, and pathways were annotated against KEGG, COG, and MetaCyc databases. Since functional profiles were inferred from marker gene data, results were interpreted as predictive rather than experimentally validated metabolic functions.

## RESULTS

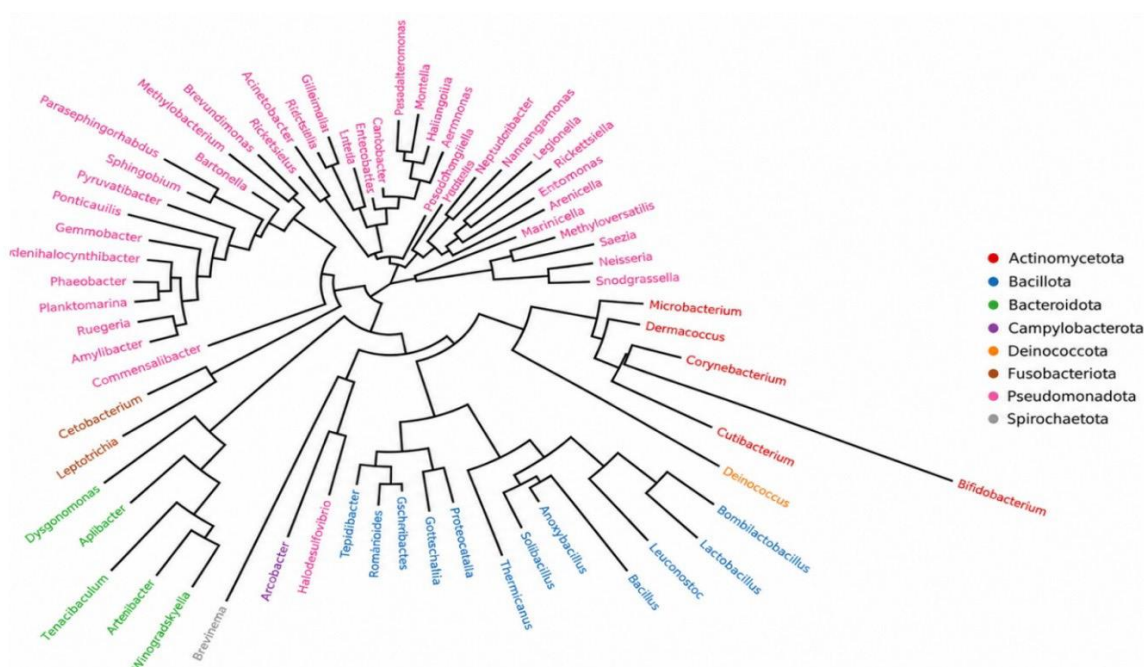
**Phylogenetic analysis of bacterial taxa.** The phylogenetic tree displays clusters of bacterial groups into major phyla, such as *Actinomycetota*, *Bacteroidota*, *Campylobacterota*, *Deinococcota*, *Fusobacteriota*, *Pseudomonadota*, and *Spirochaetota* (Fig. 3).

**Rarefaction analysis and sequencing output.** The sequencing produced 70,907 raw read pairs for Jhelum and 71,104 read pairs for Bhimber, with a mean read length of 301 bp and 300 bp, respectively. The

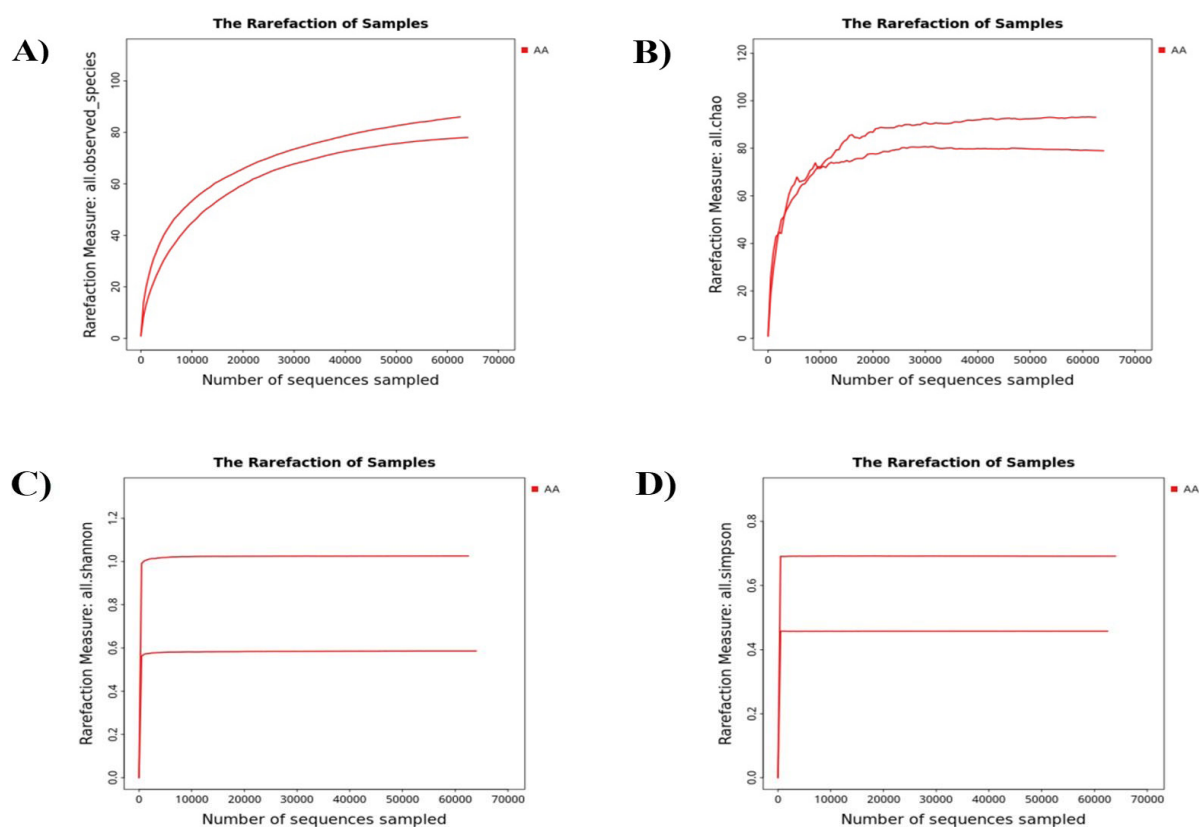
effective reads were then narrowed down after quality filtering and the removal of sequences shorter than 300 bp to 67,786 in Jhelum and 68,017 in Bhimber. The number of reads that were assigned to taxonomic groups was large (95.60% in Jhelum and 95.66% in Bhimber). The taxonomic classification revealed 164 taxonomic units. The rarefaction curves showed that there was enough sequencing depth in all samples (Fig. 4).

**Shared and unique OTUs between sampling sites.** The Venn diagram represents the distribution of the operational taxonomic units (OTUs) in two sampling locations (Fig. 5). There was a total of 66 OTUs that were shared, while 20 OTUs (Bhimber) and 12 OTUs (Jhelum) were unique, indicating site-specific microbial diversity, interpreted cautiously.

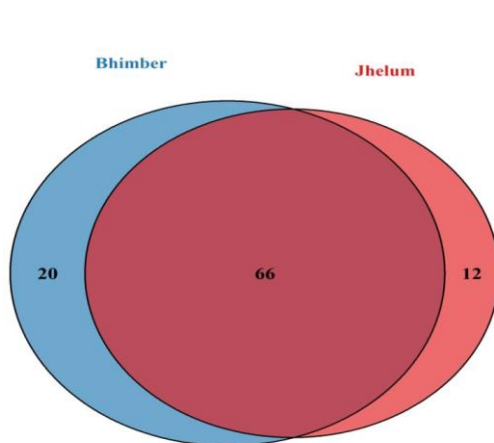
**Rank-abundance distribution of microbial communities.** The rank-abundance curve displays the abundance of the OTUs in the samples. Jhelum has a longer tail than Bhimber, indicating the existence of rarer OTUs and higher species richness (Fig. 6).



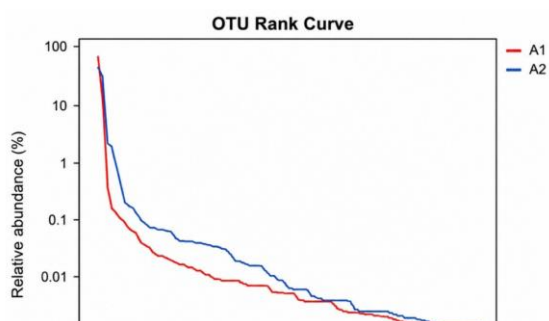
**Fig. 3.** Circular phylogenetic tree showing the taxonomic relationships of bacterial genera identified in the gut microbiota of *Apis dorsata*. Taxa are color-coded according to their respective phyla: Actinomycetota (red), Bacillota (blue), Bacteroidota (green), Campylobacterota (purple), Deinococcota (orange), Fusobacteriota (brown), Pseudomonadota (pink), and Spirochaetota (grey). The tree was constructed using a maximum-likelihood approach based on aligned 16S rRNA gene sequences, with branch lengths representing evolutionary distances. The clustering pattern highlights the dominance of Pseudomonadota and the presence of diverse bacterial lineages contributing to the gut microbial community structure.



**Fig. 4.** The rarefaction curves based on observed species, Chao1 richness, Shannon diversity, and Simpson dominance diversity indices indicate that the sequencing depth was sufficient to capture most of the microbial diversity present in the samples.



**Fig. 5.** Venn diagram showing shared and unique operational taxonomic units (OTUs) between *Apis dorsata* gut microbiota from Bhimber and Jhelum.



**Fig. 6.** The distribution of microbial taxa from *Apis dorsata* gut samples from Jhelum (A1) and Bhimber (A2) is shown in OTU rank-abundance curves. The x-axis denotes the rank of the OTUs, and the y-axis denotes the relative abundance (%). Both samples show a sharp drop in abundance at the start followed by a long tail, meaning that there is a small number of taxa with high abundances and many taxa with low abundances. The slightly longer tail in A2 indicates that there is more richness in A2.

**Variations in gut microbiota across geographical locations.** The present study represents an exploratory pilot investigation based on pooled colony-level samples from Jhelum and Bhimber districts (Table 1). The sequencing depth or effort was adequate because Good's coverage was 0.99 for all samples.

The observed species richness (Sobs) was higher in Bhimber (86) than in Jhelum (78), showing that more bacterial species were observed in district Bhimber. Equally, Chao 1 (estimator of richness) reflects that Bhimber (93.00) had a higher value as compared to Jhelum (78.88), indicating that more rare bacterial taxa exist in Bhimber. Similarly, another estimator of richness, i.e., ACE, increased from Jhelum (80.42) to Bhimber (96.62), indicating greater bacterial species richness in the district Bhimber. However, the Shannon index revealed the highest bacterial diversity in Bhimber (1.025), followed by Jhelum (0.586), indicating higher bacterial diversity and a more balanced taxa distribution in Bhimber. In contrast, the Simpson dominance index (D) was higher in Jhelum (0.69) as compared to Bhimber (0.45), revealing that district Jhelum was dominated by fewer abundant taxa. This indicates greater estimated diversity and species richness in the district of Bhimber than in Jhelum.

Based on beta-diversity analysis of this pilot exploratory study, bray-Curtis value of 0.4416 indicated noticeable dissimilarity between the two microbial communities in context of relative abundance. The Jaccard distance of 0.3265 indicated a high degree of similarity between samples, with a few differences in taxonomic composition. The lowest dissimilarity (0.3014) was observed with Morisita-Horn dissimilarity, indicating comparatively higher similarity in the distribution of dominant taxa. Overall, these results indicate that the Jhelum and Bhimber samples shared a large portion of gut microbiota. However, there was mostly variation in the relative abundance of individual taxa.

**Taxonomic composition of honeybee gut microbiota.** Stacked bar charts illustrate the relative abundance of bacterial taxa in the honeybee gut microbiota across different taxonomic levels (Fig. 7). At the

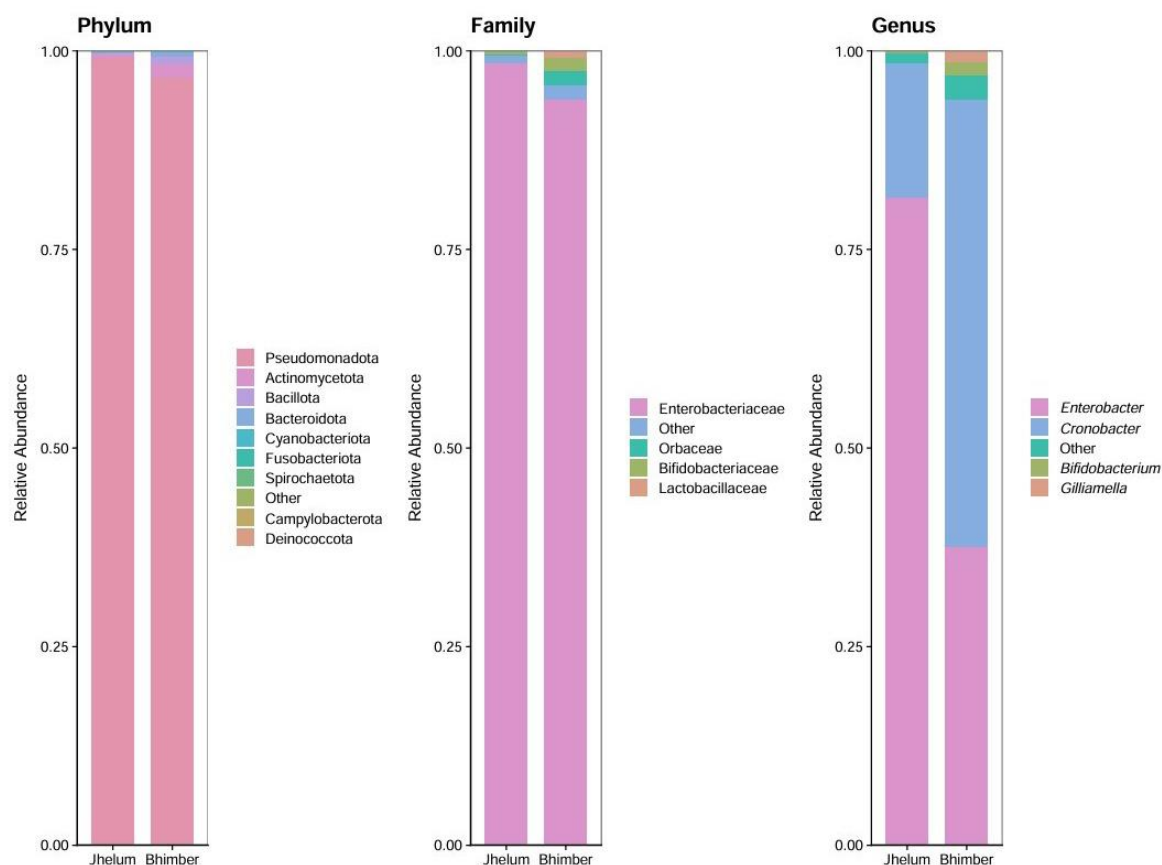
phylum level, the bacterial assemblage was largely constituted by *Pseudomonadota* in district Jhelum (99.07%) as well as district Bhimber (96.57%). In contrast, phyla like *Bacillota*, *Actinomycetota* and *Bacteroidota* made a rare contribution. *Bacteroidota* constituted 0.09% and 0.48% in Jhelum and Bhimber, respectively. *Actinomycetota* and *Bacillota* represented 0.37% and 0.20 % in Jhelum as well as 1.67% and 1.02% in the district Bhimber, respectively. The most abundant classes in Jhelum and Bhimber were *Gammaproteobacteria* (98.74%) and *Enterobacterales* (98.46%), respectively, and the most abundant orders in Jhelum and Bhimber were *Enterobacterales* (95.99%) and *Gammaproteobacteria* (98.46%), respectively.

At the family level, *Enterobacteriaceae* were the most dominant group in Jhelum (98.43%) and Bhimber (93.75%), followed by *Orbaceae*, which represented 0.11% in Jhelum and 1.90% in Bhimber. *Bifidobacteriaceae* constituted 0.33% and 1.65% of Jhelum and Bhimber bacterial taxa, respectively. *Lactobacillaceae* represented 0.169% in Jhelum and 0.90% in Bhimber. Furthermore, at the genus level, *Enterobacter*, *Cronobacter*, *Bifidobacterium*, and *Gilliamella* were dominant genera. *Enterobacter* accounted for 37.53% in Bhimber and 81.39% in Jhelum. In contrast, *Cronobacter* accounted for 17.05% and 56.22% in Bhimber. *Gilliamella* and *Bifidobacterium* were more abundant in district Bhimber, i.e., 1.66% and 1.55%, respectively, as compared to district Jhelum (0.34% and 0.09%, respectively). *Enterobacter hormaei* was predominant in Jhelum (81.39%), while *Cronobacter dublinensis* was predominant in Bhimber (56.22%) at the putative species level.

**Predicted functional profiling.** The functional relative abundance heatmap (log10 scale) of COG pathways reflects predicted gene functional categories in the samples of Jhelum and Bhimber (Fig. 8). Putative pathways indicate that amino acid transport and metabolism, carbohydrate transport and metabolism, and coenzyme transport and metabolism were found to be highly relevant, indicating processing of nutrients by gut microbiota. Other inferred metabolic

**Table 1.** Alpha diversity metrics of *A. dorsata* gut microbiota from Bhimber and Jhelum based on 16S rRNA gene sequencing

| Area    | Sobs | Chao   | Ace     | Shannon | Simpson dominance | Coverage |
|---------|------|--------|---------|---------|-------------------|----------|
| Jhelum  | 78   | 78.882 | 80.4252 | 0.58611 | 0.691481          | 0.999907 |
| Bhimber | 86   | 93     | 96.6268 | 1.02586 | 0.457482          | 0.999776 |



**Fig. 7.** Relative abundance of gut bacterial communities in *Apis dorsata* from two ecological regions (Jhelum and Bhimber) at different taxonomic levels.

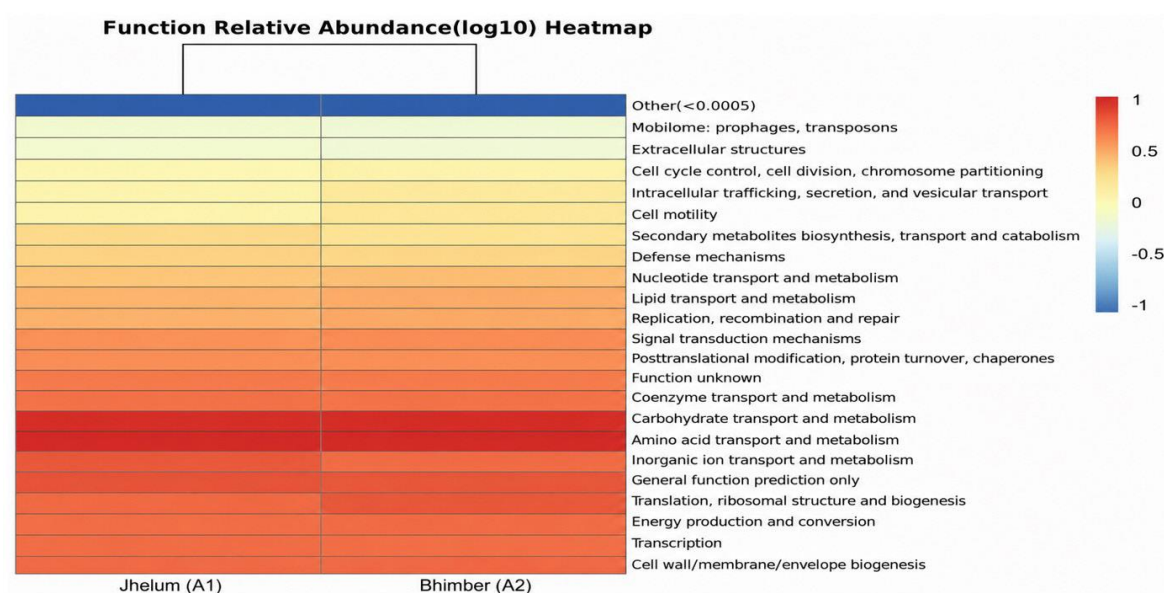
potential included energy production and conversion, translation and ribosomal structure, transcription, and cell wall (membrane) biogenesis, suggesting that microbial communities maintain strong metabolic and cellular activity. Predicted functions related to replication, recombination, and repair, signal transduction mechanisms, and post-translational modification and protein turnover were also well represented. Lower relative abundances were observed for categories such as mobilome elements (prophages and transposons) and extracellular structures. Overall, the predicted functional profiles of samples Jhelum and Bhimber were highly similar, indicating a consistent putative functional potential within the honeybee gut microbial community.

## DISCUSSION

In recent years, the occurrence of wild honeybee species such as *A. dorsata*, *A. florea*, and *A. cerana*

has been reported from different districts of Punjab and AJK, including Jhelum and Bhimber districts (1). These districts have great environmental heterogeneity, human population pressures, and a variety of floral availability for honeybee species to flourish. This has led to the interest of ecologists in exploring the gut microbiota associated with honeybee species, especially due to concerns regarding their conservation (16). In the current study, 16S rRNA amplicon-based sequencing was used to determine the composition of the microbial community associated with the gut of *A. dorsata* from two ecological regions of Pakistan.

The pilot investigation identified sequences affiliated with beneficial bacterial genera, such as *Bifidobacterium*, *Gilliamella*, *Bartonella*, *Frischella*, and *Snodgrassella* (7, 8, 17, 18). It also identified sequences affiliated with environmental or opportunistic pathogenic bacteria, like *Enterobacter* and *Cronobacter* (19, 20). Sequences affiliated with core, beneficial, or probiotic genera accounted for around 20-40%



**Fig. 8.** Heatmap of predicted functional profiles of the gut microbiota in *Apis dorsata* from Jhelum (A1) and Bhimber (A2) based on COG annotation. Values represent log<sub>10</sub>-transformed relative abundances, with red indicating higher and blue lower abundance. Major putative functions include carbohydrate, amino acid, and energy metabolism, showing largely conserved functional profiles between samples.

of the bacterial community. In contrast, sequences affiliated with environmental or potentially opportunistic pathogens accounted for around 50-80% of the bacterial community (21). A preliminary study of the relative abundances of core taxa and other taxa associated with honeybees revealed contrasting trends in the two regions. *Bifidobacterium* occurred in high abundance in Bhimber compared to Jhelum, which accounted for about 1.66% and 0.34% of the bacterial community, respectively (22). Likewise, the proportion of *Gilliamella* was 1.55% in Bhimber, whereas in Jhelum it was 0.09%, which is contrary to the previous studies (23, 24). Similar to Jhelum, *Frischella* was also more abundant in Bhimber (0.36%) than in Jhelum (0.02%), which coincides with (24). *Bartonella* accounted for approximately 0.15% in Bhimber and 0.02% in Jhelum. *Snodgrassella* was found only in Bhimber, with 0.04% relative abundance (25). In contrast, *Commensalibacter* was more abundant in Jhelum, accounting for about 0.12% of the community. It accounted for about 0.04% in Bhimber, which is analogous to the earlier study (22). *Cronobacter* was represented at 56% in Bhimber and at 17% in Jhelum, which is similar to (26). However, *Enterobacter* was detected at 81% in Jhelum and 37% in Bhimber, which is in accordance with (27). This pilot investigation reveals that abundance could be due to

geographical, dietary, seasonal, and methodological variation.

Although the beneficial bacteria are rare, they could still be ecologically important because microbial significance does not necessarily correlate to relative abundance. *Gilliamella* and *Bifidobacterium* species contain carbohydrate-active enzymes that are used for the degradation of plant polysaccharide pectin, hemicellulose, and other complex glycans, which aid in fermentation and utilization of plant polysaccharides (28, 29). In addition, *Bartonella* can break down aromatic plant compounds. It possesses genomic pathways linked with the synthesis of amino acids and other essential cofactors. These abilities could help honeybees adapt their nutrition to a nitrogen-limited pollen- and nectar-based diet (30). *Snodgrassella* plays a role in microbial community organization, metabolic cross-feeding, and gut homeostasis (31, 32). In contrast, the direct effect of *E. hormaechei* and *C. dublinensis* remains unknown in honeybees; their abundance in the gut of honeybees may either be a sign of the harmful influence on their health or may be favorable to the development of other useful microbial species (33-35).

An unexpected observation in this study was the presence of sequences affiliated with *C. dublinensis*, a species that is not commonly identified as part of

the core microbiota of *A. dorsata*. This site-specific abundance may be linked with environmental exposure or floral sources (36). Some sequences were closely affiliated with *Methyloversatilis universalis*, *Saezia sanguinis*, and *Cetobacterium somerae*. To the best of our knowledge, all these species reflect previously unreported bacterial species in association with the *Apis dorsata* gut. However, higher-resolution sequencing approaches such as full-length 16S rRNA sequencing or shotgun metagenomics are required for confirmation. In addition, the current study has a few limitations in scope.

Functional predictions derived from PICRUSt2 represent predicted function and cannot fully reflect direct metabolic activity; future work must integrate metatranscriptomic, proteomic, or metabolomic techniques. Another limitation is the lack of biological replication across geographic locations, which could help explore how changes in local regional conditions affect microbial community stability. Therefore, diversity was interpreted descriptively, without inferential statistical testing. Future research should focus on independent colony-level biological replicates to reveal the statistically significant effect of geographical area on gut microbiota and integrate multi-kingdom metabarcoding approaches to give a more comprehensive understanding of microorganisms associated with the honeybee gut.

## CONCLUSION

The study gives comparative insight into the diversity of gut microbiota of the giant honeybee, *A. dorsata*, in the districts of Jhelum and Bhimber. Both sites harbored both core and non-core bacterial taxa, but Bhimber harbored a relatively high bacterial diversity. At the species level, *E. hormaechei* was the dominant species in Jhelum, representing 81.38% of the bacterial community, and *C. dublinensis* was dominant in Bhimber, comprising 56.2% of species-level assigned sequences. The present pilot study suggests that core bacterial taxa are shared between geographically distinct populations of *A. dorsata*, although further investigations with larger sample sizes are required to confirm these patterns. The ecological and economic implications of such a study are significant because giant honeybees and the microbes that they carry are indispensable to the well-being of the bees, colony stability, and honey

production. Additionally, this research provides a foundation for future investigation into the functional roles of such bacterial communities in *A. dorsata* and their implementation in sustainable beekeeping practices. On the other hand, a limitation of the present study is the fact that the number of sampling sites is relatively small, and the use of composite gut samples represented individual colonies. Although pooling several worker bees allows characterization of the colony-level microbial community, such an approach can decrease the possibility of exploring the colony-level variability. Microbiomes associated with the gut of honeybees vary in individuals due to age, diet, and environmental exposure. Hence, future studies must incorporate larger sample sizes, and individual-level sampling across multiple colonies would give a better understanding of microbiome variability in *A. dorsata*.

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