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Seasonal variations in the gut bacteriome of *Apis mellifera* assessed through 16S rRNA gene sequencing

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Honeybees play a crucial role in global food production and ecosystem maintenance through pollination, yet their health is increasingly threatened by environmental stresses, including changes in diet and climate. The present study investigates the seasonal variations in the gut bacteriome of *Apis mellifera* using 16S rRNA gene sequencing to understand how microbial diversity fluctuates across different seasons. Worker bees were sampled from five colonies during spring and winter at the University of Veterinary and Animal Sciences, Pattoki, Pakistan. DNA was extracted from pooled gut samples and high-throughput sequencing was performed using universal 16S rRNA primers targeting the V3-V4 region. The results revealed that Proteobacteria dominated the gut microbiome in both seasons accounting for 74.4% in winter and 76.2% in spring. However, spring samples exhibited a richer and more balanced bacterial community with a higher Shannon diversity index (1.904 vs. 1.814) and Fisher index (1.296 vs. 1.117) compared to winter. At the genus level, *Commensalibacter* was the most abundant in both seasons but with higher prevalence in spring (37.0%) compared to winter (30.2%). Other key genera included *Gilliamella*, *Pseudomonas* and *Bifidobacterium*, which are essential for nutrient processing and immune defense. Seasonal shifts likely reflect changes in available floral resources, temperature, and beekeeping practices such as sugar supplementation during winter. These findings highlight the importance of a diverse gut microbiome in honeybee health and suggest that targeted interventions, such as probiotic supplementation and dietary management, could help maintain microbial balance throughout the year and improve colony resilience in varying environmental conditions.

Keywords: gut microbiome; *Apis mellifera*; seasonal variation; 16S rRNA sequencing; microbial diversity.

Introduction

Insects are basic components of agro ecosystems and play their role in ecosystem services such as pest control, crop production and pollination of the crops. Honey bees are known to be social insects and considered as a leading pollinator species (Miroslava, 2019). Honey bees are involved in the pollination of 35% of the worldwide food production crops (Guo et al., 2015). Honeybee populations in Pakistan have certain geographic and ecological distributions that influence their role in agriculture and nature. Four species from the genus *Apis* have been reported from Pakistan so far, namely *A. cerana*, *A. dorsata*, *A. florea* and *A. mellifera* (Khan et al., 2020). Their distribution and density are influenced by floral density and resource levels, which in turn influence their pollination efficiency and survival (Ali et al., 2023). Understanding their environmental interactions and ecology is crucial for conservation and management of these valuable insects. Natural honeybee colonies have experienced significant decline globally since 2006. Several factors are involved in reduction of honeybee colonies, such as pollution, environmental pressure, foreign pathogens and pesticides (Romero et al., 2019). However, prevalence of pathogens and parasites are also the main threats in managing the bee colonies (Neal et al., 2018). Climatic factors such as temperature and humidity influence the types of vegetation in a given area. Few plants grow well and few flowers bloom during periods of extreme temperatures while humidity percentage directly affects nectar production. These factors in turn directly influence honey bee behaviour (Becerril-Sánchez et al., 2021; Chatha et al., 2022).

The gut microbiota is key to better health of honeybees (Yanez et al., 2016; Kwong et al., 2017). The gut microbiota play a vital role in synthesis of amino acids and improve metabolism for better immunity in honeybees (Khan et al., 2020). Furthermore, it has been shown that diverse gut microbiota of the hosts can also act as a barrier against pathogens (Anderson et al., 2013). Similiarly, the varied form of gut microbiota also provides the honeybee with an advantage to adapt to seasonal change (Kešnerová et al., 2020; Chen et al., 2024). When floral resources are abundant during summer, the gut microbiota of honeybees is mostly diverse with a wide range of microbial species. During the winter season when honybees are totally dependent upon stored honey and pollen, their gut gut microbiota diversity reduced significantly (Lee et al., 2015; Ludvigsen, 2015; Bleau et al., 2020; Papp et al., 2022).

Recently many studies have been reported on the honeybee's gut microbiota and there is lack of information about the environmental factors that might affect it (Kwong & Moran, 2016). However, a few studies have reported that seasonal changes and environmental factors can easily influence gut microbiota composition of honey bees (Almeida et al., 2022). During the summer and spring seasons, various floral resources provide diverse feed for the honey bees (Forsgren et al., 2018; Papp et al., 2022). The gut microbiota of many invertebrate and vertebrate species changes with the season (Hroncova et al., 2015). However, seasonal changes are mostly linked with various factors such as the feeding habit of the animal or its lifestyle (Lan et al., 2016). The present study explored the impact of seasonal changes on the gut bacteriome of *Apis mellifera* to understand how floral resources and climatic factors affect honeybee gut health and productivity.

Materials and methods

The present research was conducted at the University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Punjab, Pakistan. The climate of Pattoki is semi-arid with hot summers and relatively cool winters. The region experiences relatively little rainfall during the spring (March to May) and winter (November to February) with average temperature also varying between 20 to 35 °C in spring and 5 to 20 °C in winter when agriculture relies on irrigation. Regarding vegetation, the study area is home to several cultivated and wild plants, including a few resources important for pollinators including *Apis mellifera*. In spring, floral diversity is provided by crops such as mustard (*Brassica* spp.), clover (*Trifolium* spp.) Other fruit blossoms provide a good source of nectar and pollen. In winter, the region's plant diversity is lower but winter crops like winter wheat (*Triticum aestivum*) and some shrub types still render resources vital for honey bees (Ali et al., 2018).

Apis mellifera worker bees were collected during spring and winter to assess the seasonal variations in the gut bacteriome. Samples were collected from five distinct colonies of *A. mellifera* from the study area. The strategy to select various colonies was to make sure that variation in foraging behavior and exposure to various floral resources were covered. During each season, a total of two worker honeybees were randomly captured from each colony to minimize sampling bias. The sampling was done at the peak of foraging activity to record seasonal fluctuations in floral resource availability. The honeybees were captured using a sterilized hand net and immediately euthanized to preserve the integrity of their gut bacteriome. The gut of each honeybee was dissected under sterile conditions and a pooled sample made for further DNA extraction.

DNA extraction was done from the pooled gut samples of *A. mellifera* using QIAamp DNA Microbiome Kit (Qiagen, Germany) following the manufacturer's instructions (Chakravorty et al., 2007). The gut samples were transferred into a 1.5 mL Eppendorf tube and vortexed. A cell lysis buffer was then added to break down the microbial cell wall. The buffer contains enzymes that lyse both bacterial and host cells, releasing the DNA. The sample was incubated at 56 °C for 10 minutes. Proteinase K was added to digest proteins that could be co-extracted with the DNA. The solution was incubated at 56 °C for an additional 10 minutes followed by vortexing. The extracted DNA was purified (Fig. 1) and DNA was collected into sterile tubes and the concentration and purity of the DNA were measured using a NanoDrop One spectrophotometer. DNA quality was assessed by measuring the A260/A280 ratio using the NanoDrop One (Table 1). A ratio between 1.8 and 2.0 indicated high-quality DNA, suitable for downstream applications like 16S rRNA gene amplification. The purified DNA was stored at –20 °C until further processing. After the DNA extraction process, the 16S rRNA gene was targeted for amplification using universal primers 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and BS-R1407 (5'-GACGGGCGGTGWGTRC-3') to amplify the V3-V4 variable regions of the gene (Papp et al., 2022). The PCR products were sent to Azenta Life Sciences, China Next-Generation Sequencing (NGS).

The sequencing data was pre-processed by filtering low-quality reads and valid sequences were assigned taxonomic identities using the database. Operational Taxonomic Unit (OTU) picking was performed to group similar sequences, facilitating the analysis of microbial diversity (Hussain et al., 2025). After trimming the DNA reads, a FASTQ file was used for species annotation and abundance analysis was performed using the SILVA database for 16S rRNA gene. Taxonomy was assigned to each DNA read with 99% similarity from the reference database. The Sankey plot was constructed to visualize the taxonomic composition and relative abundance of different bacterial groups from phylum to genus level using Bacterial and Viral Bioinformatics Resource Center (BV-BRC) at www.bv-brc.org.

Alpha-diversity was estimated using metrics such as Shannon's index and Simpson's index to measure both species richness and evenness within the microbial community. The calculation of the diversity indices was performed using Bacterial and Viral Bioinformatics Resource Center (BV-BRC) at www.bv-brc.org.

Results

A total of 10 specimens of *A. mellifera* were captured during field sampling in winter and spring seasons. The captured specimens were tagged with a specific number (Winter: AM-W, Spring: AM-S) and preserved for further analysis. DNA was extracted from gut samples using QIAamp DNA Microbiome Kit as per protocol available with the kit. The extracted DNA was quantified using Thermo Scientific NanoDrop One.

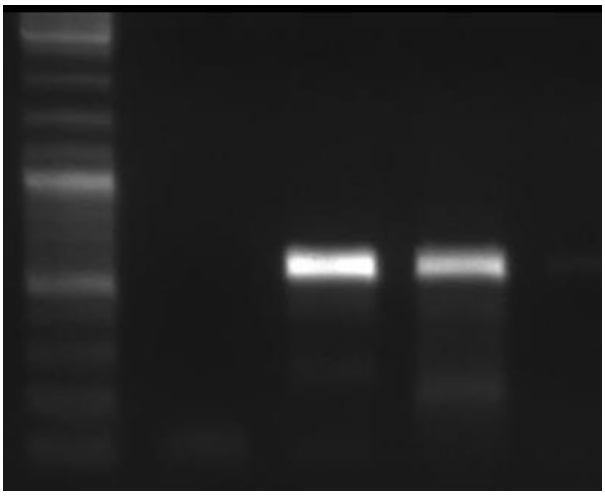

Fig. 1. Gel electrophoresis of extract DNA samples from the gut of *Apis mellifera*

Table 1
The quantity of extracted DNA samples was checked through Nanodrop ONE

Label	Species	Nucleic acid, ng/µL	A260 / A280	A260 / A230	A260	A280
AM-S	*Apis mellifera*	429.04	1.64	0.60	8.58	5.22
AM-W		322.33	1.72	0.83	6.44	3.74

Taxonomic composition of the bacterial community in the gut of A. mellifera during winter. The results revealed that the bacterial community in the gut of *A. mellifera* during winter was dominated by several major phyla. The the relative abundance of phylum Proteobacteria was 74.5% followed by Bacteroidota (15.7%), Firmicutes (8.3%) and Actinobacteriota (1.7%). At the class level, the highest relative abundance of Gammaproteobacteria was 44.0% followed by Alphaproteobacteria (30.3%), Bacteroidia (15.7%) and Bacilli (7.6%) while Actinobacteria and Negativicutes were 1.7% and 0.7% respectively in minor concentration. At the order level, the relative abundance of Acetobacterales was 30.1% followed by Enterobacterales (24.2%), Burkholderiales (16.5%) and Bacteroidales (14.1%). These bacterial orders play a role in metabolic processes including fermentation and nitrogen fixation. A few other orders including Pseudomonadales (3.3%), Lactobacillales (7.6%), Bifidobacteriales (1.7%), Flavobacteriales (1.6%), and Acidaminococcales (0.7%) were also identified. These bacterial orders provide ecological balance within the gut of *A. mellifera*. At the family level, Acetobacteraceae (30.2%) and Orbaceae (24.2%) were the most abundant followed by Comamonadaceae (16.3%) and Dysgonomonadaceae (14.1%). A few other families such as Lactobacillaceae (7.6%), Pseudomonadaceae (3.1%), Weeksellaceae (1.6%), Bifidobacteriaceae (0.4%) and Neisseriaceae (0.2%) were also identified. These families are instrumental in nutrient breakdown and contribute to the maintenance of gut health of *A. mellifera*. At the genus level the relative abundance of *Commensalibacter* was (30.2%), *Gilliamella* (24.2%), *Dysgonomonas* (14.1%), *Lactobacillus* (6.7%), *Pseudomonas* (3.1%), *Bifidobacterium* (1.7%), *Apibacter* (1.6%) and *Bombilactobacillus* was at 0.9%. These genera are believed to play critical roles in nutrient processing and microbial stability. Some of the genera have not been fully characterized or classified. The presence of 17% of uncultured genera highlights the limitations in our understanding of the full diversity of the honeybee gut microbiome (Fig. 2). One significant limitation of the present study was the incapability of classifying bacterial taxa up to the species level. The uncultured genera reflects a challenge in characterizing the full spectrum of microbial diversity as many microbes have yet be isolated or classified in reference databases. Future studies using more refined genomic techniques or additional marker genes may allow more accurate species level classification and a deeper understanding of microbial diversity and function.

The data revealed that the gut microbiota of *A. mellifera* possess a bacterial community that significantly influences the bees' health, particularly during the winter season when survival is most challenging. It is dominated by Proteobacteria, Gammaproteobacteria and Alphaproteobacteria showing a complex ecosystem which supports vital functions such as nutrient processing, energy production and immune support by providing defense against pathogens. Similarly, bacteria belonging to the phylum Bacteroidota are specialized in breaking down complex carbohydrates and Firmicutes support the fermentation process, which provides fuel essential metabolic functions. The bacterial families including Acetobacteraceae and Orbaceae contribute to fermentation while the dominant genera like *Commensalibacter* and *Gilliamella* play a role in nutrient synthesis and gut stability. The presence of 17% of uncultured genera highlighted the fact that many bacterial groups remained unidentified but may play a vital role in maintaining gut health. The findings of the current study provide valuable information on how the honeybees' gut microbiota helped them in survival during under the stressful conditions of changing seasons. Furthermore, it is revealed that the gut microbiota of *A. mellifera* provided them with a unique balance of bacterial diversity to perform vital functions such as nutrient breakdown and immune support. The findings provide baseline information for future studies to enhance our understanding by providing information on how beneficial bacterial groups can be used in hive management.

The alpha diversity indices provide valuable insights into the structure and diversity of the microbial community in the gut of *A. mellifera*. The Berger-Parker Diversity Index was calculated as 0.302 indicating a relatively balanced composition, which suggests that the bacterial community in the gut of *A. mellifera* during winter has a moderate level of dominance by certain bacterial groups. Fisher's Index was calculated as 1.117, which indicated that moderate species richness was present in gut microbiome. The Shannon Diversity Index (1.814) further reinforces this finding. This index revealed that there are a variety of species present but some are likely to be more dominant. Similarly, the Simpson's Reciprocal Index (4.948) indicated a relatively low probability of dominance by a single species. Finally, Simpson's Index of Diversity was calculated as 0.798, which aligns with the previous indices, suggesting moderate bacterial diversity in the gut of *A. mellifera* during winter. Overall, the microbial community in the *A. mellifera* gut exhibits a healthy level of diversity. However, the relatively low values of Fisher's index and Berger-Parker's diversity suggested that there may still be potential for greater richness and balance in the gut microbiome (Table 2).

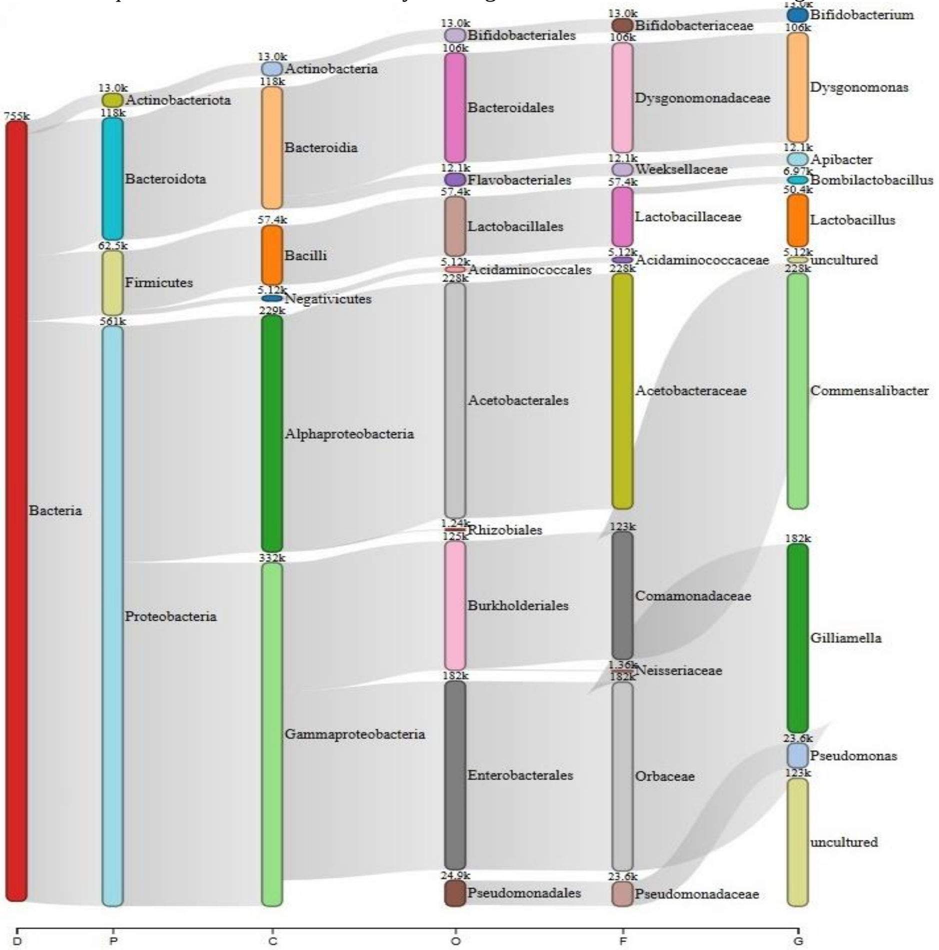

Fig. 2. Sankey plot showing diversity of bacteria in the gut of *Apis mellifera* specimens collected during winter

Table 2
Alpha diversity indices of bacterial community
in the gut of *Apis mellifera* specimens captured during winter

Alpha Diversity Tests	Values
Berger-Parker's diversity	0.302
Fisher's index	1.117
Shannon's diversity	1.814
Simpson's reciprocal index	4.948
Simpson's index of diversity	0.798

Taxonomic composition of bacterial community in the gut of A. mellifera during spring. The gut microbiome of *A. mellifera* during spring was dominated by Proteobacteria (76.1%) followed by Firmicutes (8.2%), Bacteroidota (7.5%), and Actinobacteriota (5.9%). At the class level, Gammaproteobacteria and Alphaproteobacteria were the dominants represented by 39.2% and 37.0% respectively. Other classes includes Bacteroidia (7.5%), Bacilli (7.1%), Actinobacteria and Deinococci were also present in smaller proportions, suggesting their specialized role in the microbial community. At the order level, Acetobacterales (37.0%) was the most abundant followed by

Burkholderiales (14.6%), Enterobacterales (13.3%), and Pseudomonadales (11.3%). These orders play a key role in the fermentation of complex organic compounds and contribute to the overall metabolic stability of the gut microbiome. The relative abundance of Corynebacteriales was 10.6% followed by Lactobacillales 7.1%. At the family level, Acetobacteraceae was was the most abundant at 37.0% followed by Comamonadaceae (14.6%) and Orbaceae (13.1%). A few other families were also identified including Lactobacillaceae (7.1%), Bifidobacteriaceae (5.9%), Dysgonomadaceae (5.6%), Weeksellaceae (1.9%) and Acidaminococcaceae (1.0%). At the genus level, the relative abundance of *Commensalibacter* was 37.0% followed by *Gilliamella* (13.1%), *Pseudomonas* (11.1%), *Bifidobacterium* (5.9%), *Dysgonomonas* (5.6%), *Lactobacillus* (4.4%), *Bombilactobacillus* (2.7%), and *Apibacter* (1.9%). These genera are vital in the digestion of nectar and pollens collected by honeybee. The uncultured and unidentified taxa was 15.6%, which highlighted the limitations of current taxonomy databses. In summary, the microbial community in the gut of *A. mellifera* during spring showed a highly diverse structure with Proteobacteria, Gammaproteobacteria, and Alphaproteobacteria being the most abundant (Fig. 3).

Several key differences were oberseved in gut microbiota of *Apis mellifera* during spring. Firmicutes and Actinobacteriota remained the dominant phyla in both seasons but their relative abundance increased slightly in spring as compared to winter. However, the relative abundance of Bacteroidota decreased from 15.7% in winter to 7.5% in spring. It can be attributed to many ecological factors as honeybees have access to a various floral resources and fresh pollen as compared to winter. This seasonal shift in diet could favor the growth of certain bacterial phyla that are more suited to processing springtime nutrients. At the class level, Gammaproteobacteria and Alphaproteobacteria dominated in spring as compared to winter while Bacteroidia and Bacilli showed a slight increase in relative abundance as compared to winter. The order Acetobacterales was dominant, followed by Burkholderiales and Pseudomonadales. At the genus level, *Commensalibacter* remained dominant in both seasons but the relative abundance of *Gilliamella* and *Pseudomonas* was higher in spring as compared to winter.

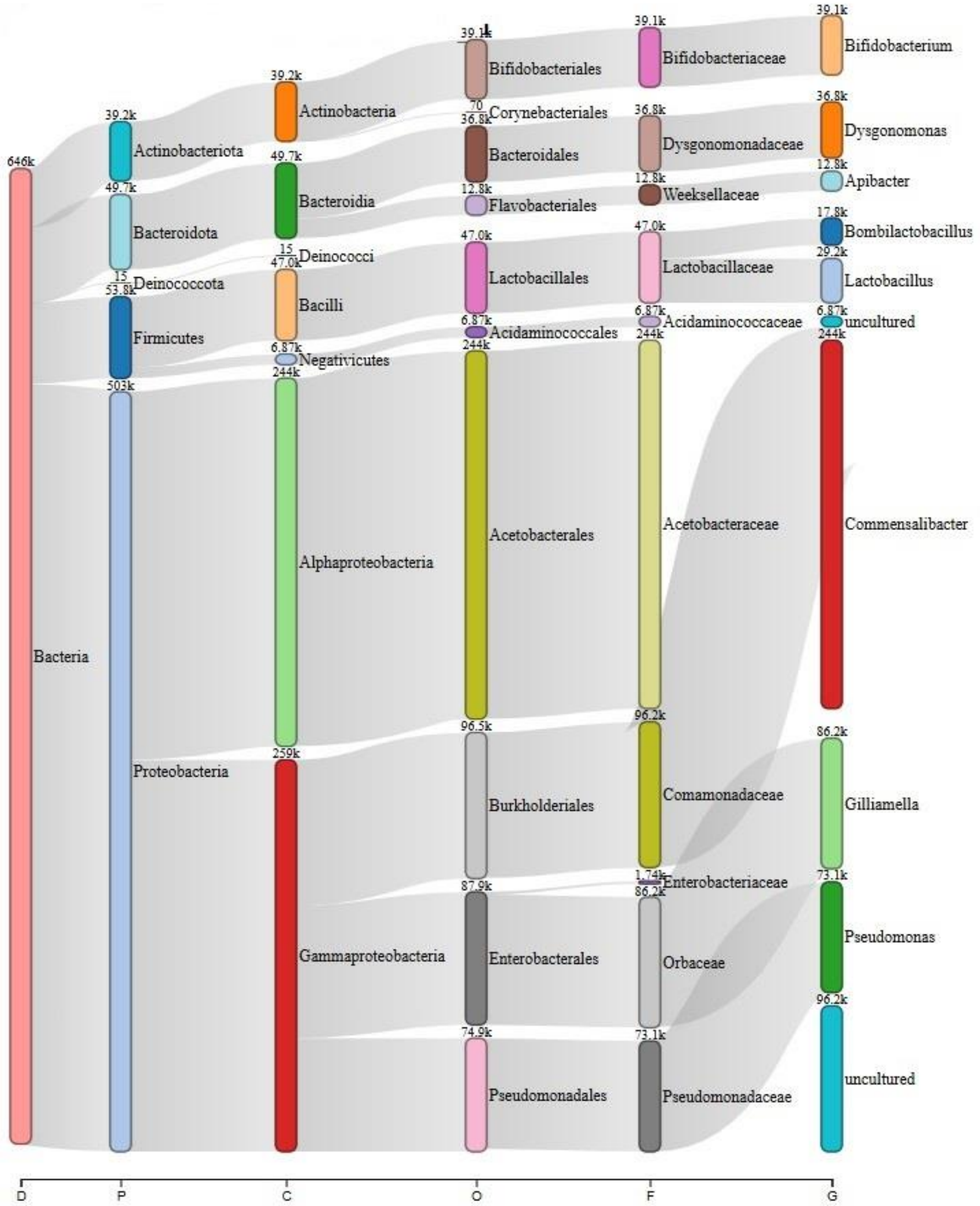

Fig. 3. Sankey plot showing diversity of bacteria in the gut of *Apis mellifera* specimens collected during spring

The Berger-Parker Diversity Index was calculated as 0.377, suggesting a moderate dominance. The finding indicated that a reasonable level of diversity is present in the gut of *A. mellifera* specimens collected during spring. The Fisher Index (1.296) indicated a reasonable variety of diversity in the gut microbiome. This finding revealed that the gut microbiome has a balanced microbial composition. This is further supported by Shannon's Diversity Index, which was calculated as 1.904. This indicated that the overall diversity remains significant, contributing to a healthy and functional gut microbiome. Similarly, Simpson's Reciprocal Index (4.866) suggested that the bacterial community is fairly balanced with a relatively low probability of dominance by a single group. Lastly, the Simpson's Index (0.795) suggested that the gut microbiome is moderately diverse. Overall, the diversity indices indicated that the gut microbiome of *A. mellifera* is diverse and balanced with a healthy mix of bacterial groups (Table 3).

Table 3
Alpha diversity indices of bacterial community in the gut of *A. mellifera* specimens captured during spring

Alpha diversity tests	Values
Berger-parker's diversity	0.377
Fisher's index	1.296
Shannon's diversity	1.904
Simpson's reciprocal index	4.866
Simpson's index of diversity	0.795

The comparison of the bacterial richness between the two samples of *A. mellifera* gut microbiota taken during spring and winter seasons revealed significant insights into how seasonal changes influence microbial diversity. The gut of specimens taken during the spring season showed slightly greater bacterial richness than the winter samples. At the phylum level, Proteobacteria remained the dominant phylum in both samples but its relative abundance was slightly higher in the spring (76.1%) as compared to winter (74.5%). Moreover, during spring the relative abundance of Bacteroidota was 7.5% as compared to 15.7% in the winter, which reflects seasonal variations influenced by available floral resources. In terms of alpha diversity, the spring sample showed higher diversity as indicated by the Shannon's Diversity Index (1.904) as compared to 1.814 during winter. This suggested that the spring microbiome is more diverse and less dominated by a few species. The Berger-Parker diversity index during spring was 0.377, indicated a moderate dominance by certain species, but still greater balance compared to the winter's sample 0.302. The Fisher index for the spring sample was higher (1.296) as compared to the winter samples (1.117), which further revealed that the spring microbiome has more richness. Overall, during the spring season the *A. mellifera* gut demonstrated a more diverse and richer bacterial community, which was likely due to the increased availability of floral resources and more favorable environmental conditions.

Discussion

Bees as pollinators are essential for both ecology and agriculture. The gut microbiome of *A. mellifera* contributes to nectar and pollen digestion, nutrient processing, immune defense, and bee health. Several results have been reported on the composition of their gut bacteria. However, the effect of diet, environment, and seasonality on honeybees gut microbiome composition has been an area of active research. In this study, we evaluated the seasonal dynamics of honeybee gut microbiota during winter and spring in Pattoki, Punjab, Pakistan. The results revealed significant shifts in microbial diversity between the spring and winter months, which can be attributed to variations in floral resources, diet, and environmental factors, including temperature fluctuations and limited foraging opportunities during the colder months.

Kesnerova et al. (2017) reported that winter honeybees had the higher number of bacteria and low bacterial community. The results of our study are in line with Kesnerova et al. (2017) as variation in bacterial number was not significant. Similarly, Anderson et al. (2022) documented that the gut microbiome of honeybees can significantly change across different seasons. The results of our study reveled that there was a decrease in bacterial diversity during winter as honeybees depend on stored honey. he findings of Papp et al. (2022) and Wang et al. (2020) documented that during winter the honeybee's gut microbiomes showed lower bacterial diversity and significant increases in the relative abundance of specific bacteria such as *Bartonella* and *Commensalibacter*.

Moreover, Kešnerová et al., (2020) reported that diet played a vital role in the composition of bacterial diversity in the gut of honeybees. During winter, when less floral resources are available the gut microbiota has less bacterial diversity. This loss of bacterial diversity may hinder the bees' immune system making them more vulnerable to infections and less able to resist environmental threats. The findings of our study also emphasizes the direct effect of season on microbiome composition. Diet plays a crucial role in shaping the composition of the honeybee gut microbiome. Our research found that bees nourished by varied floral resources exhibited higher bacterial diversity compared to those fed with sugar especially during winter. This aligns with findings from Brar et al., (2025), who showed that honey fed bees shown more diverse microbiome than those given sugar water only. Naturally, honeybees consume a variety of floral nectar supporting a wide range of intestinal bacteria including *Lactobacillus* and *Bifidobacterium* essential for breaking down complex carbs and sustaining immune system.

In contrast, beekeepers use sugar solution during the winter months lacking any nutritional composition like natural pollen and nectar, leading to a microbiome that is less diverse. Hroncova et al. (2015) reported similar findings and reported that honeybees fed with pollen and honey had a significantly different microbiome profile as compared to those fed with sugar syrup. This difference in gut microbiota composition may be due to the lack of varied nutritional contents in the diet such as amino acids, lipids and bioactive compounds which support the growth of different bacterial species in the gut. The reduction in microbial diversity due to poor-quality diets can make honeybees more vulnerable to pathogens and diseases during the winter months potentially contributing to colony losses. Environmental factors such as temperature and humidity play an essential role in shaping the gut microbiome of honeybees. We observed in our research a substantial decrease in microbiome diversity during winter, which we hypothesize can be due to the cold weather and the honeybee's limited access to floral resources. Bleau et al. (2020) validates this notion, making clear that the intestinal microbiota of honeybees undergo profound alternations over the winter period with reduced bacterial diversity. These changes are likely a response to the environmental stresses associated with low temperatures and limited food availability.

In addition, our findings are supported by Papp et al. (2022) and Chen et al. (2024), who have shown that climatic factors such as temperature will directly influence the composition and diversity of honey bee gut bacteria. In warmer regions or during warmer months honeybees have access to a wider variety of floral resources which can support a more diverse and resilient gut microbiome. In contrast, during colder months, bees are confined to their hives and rely on stored food which may not provide the same level of microbial diversity leading to reduced gut microbiome diversity and increased vulnerability to pathogens.

The seasonal variations in honeybee gut microbiota observed during the present study have important implications for colony health and management. A healthy, diverse gut microbiome is crucial for maintaining the honeybees' immune system and overall health. Our results suggested that during the winter months when honeybees are more likely to experience microbial imbalance there is need to add probiotic supplementation or that improved diet could help restore microbial balance and enhance honeybee health. Recent researches by Ludvigsen et al. (2015) and Brar et al. (2025) have shown that maintaining a varied diet for honeybees, particularly one that includes fresh pollen and nectar, can help to sustain a healthy gut microbiome and improve the honeybees' ability to survive during harsh conditions.

Moreover, understanding the role of gut microbiome in honeybee health could lead to more effective management strategies for combating diseases and improving colony survival. For example, providing bees with a more varied diet during the winter months, or supplementing their diet with probiotics, could help mitigate the negative effects of dysbiosis and support immune function. This would be particularly important in regions where honeybee colonies face extreme winter conditions or where floral resources are scarce.

Conclusion

The results revealed that seasonal changes directly influence the gut bacteriome of *A. mellifera*. It was recorded that the gut of

A. mellifera had higher bacterial diversity during spring as compared to winter. During winter the honeybees had limited foraging resources and used stored honey. This resulted in decreased in bacterial diversity in the gut and this can be linked with increased vulnerability to pathogens. It is recommended that future studies should focus on adding probiotic supplementation during the winter for better honeybee health throughout the year.

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