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Research Article

First Metagenomic Insights into the Gut Microbiota and Adaptive Physiology of Migratory Bar-Headed Geese in the Upper Wardha Reservoir, Maharashtra, India

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ABSTRACT

The Bar-Headed Goose (*Anser indicus*) is a migratory bird known for its high-altitude flights across the Himalayas, traveling from North Asian breeding grounds to wintering regions in India. This study aims to characterize the gut microbiota of Bar-Headed Geese inhabiting the Upper Wardha Reservoir, Maharashtra, India, to understand potential microbiota adaptations that support migratory physiology. Faecal samples were collected and examined via whole-genome metagenomic sequencing to detect microbial populations and functional pathways. The findings indicated a microbiome predominantly composed of Firmicutes, Proteobacteria, and Actinobacteria, alongside metabolic pathways related to carbohydrate and cofactor metabolism. The existence of taxa like Campylobacter and Psychrobacillus indicates microbial adaptations to certain environmental and nutritional conditions. These findings highlight the role of gut microbiota in supporting the geese's migratory endurance and offer insights for conservation strategies, particularly in addressing ecological and physiological challenges faced by migratory birds. Further investigation into seasonal and geographic variations in gut microbiota composition is recommended to enhance understanding of microbial adaptations essential for migratory health and resilience.

Key words: Bar-Headed Goose, gut microbiota, metagenomic sequencing, migratory birds, microbial adaptation, high-altitude migration

INTRODUCTION

The Bar-Headed Goose (*Anser indicus*) is native to Asia, breeding in specific wetlands of the central Asian high plateaus (Takekawa *et al.* 2009) and spending the winter in south-central Tibet (Bishop *et al.* 1997) and India (Javed *et al.* 2000). It is a migratory waterfowl species renowned for its extraordinary high-altitude flight across the Himalayas, a journey that requires exceptional physiological and environmental adaptations (Takekawa *et al.* 2017; Scott and Milsom 2007; Wang *et al.* 2016a). A large portion of the global population of Bar-Headed Geese undertakes migrations twice a year between their breeding grounds in Mongolia, northern China, and the Tibetan Plateau, and their wintering area in India, crossing the Himalayan mountains (along the southern border of the Tibetan plateau) during the journey. This species is recognised for its extraordinary high-altitude migrations, frequently soaring to elevations beyond 8000 m (Dorst 1963). Upon their arrival in India for the winter, the geese occupy diverse wetland regions, such as the Upper Wardha Reservoir in Maharashtra, where they remain for almost four months before migrating north in March (Wang *et al.* 2016a). The projected migration path and associated use of Bar-Headed Geese offer essential understanding of their dependence on certain stopover and wintering habitats, underscoring the necessity of preserving these vital regions (Figure 1) (Miyabayashi Y, and Mundkur T, 1999; Palm EC *et al.*, 2015).

The migratory existence of the Bar-Headed Goose exposes it to unique environmental and dietary conditions, which are thought to considerably influence the composition of its gut microbiota. The gut microbiota are essential for host health, facilitating nutrition absorption, immunological regulation, and reproductive success (Zhang *et al.* 2021; El Aidy *et al.* 2014; Dong *et al.* 2022; Wang *et al.* 2017; Wang *et al.* 2019). The gut microbiota of migratory species can fluctuate periodically, according to changes in nutrition and environmental stressors experienced in various environments. Research indicates that modifications in diet and rearing conditions might influence the prevalence of essential microbial phyla, including Firmicutes, Proteobacteria, and Bacteroidetes, which are vital to nutrient processing and immunological defence (Wang *et al.*, 2016a). While

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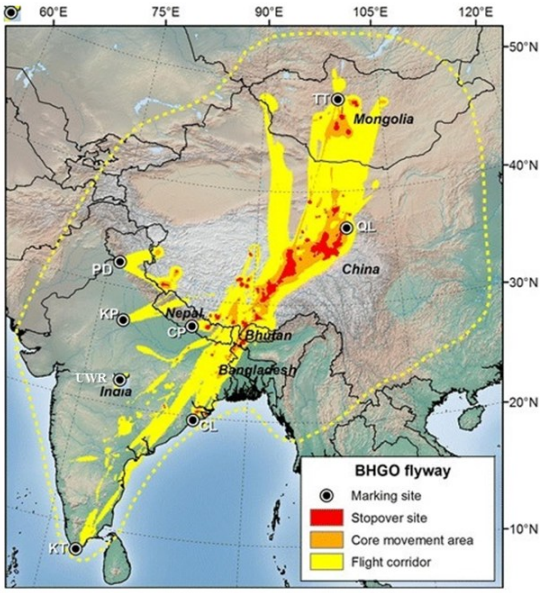

Figure 1. Estimated migration route and relative use of bar-headed geese (BHGO) in the CAF. From darkest to lightest, colors represent 50%, 75% and 99% cumulative probability contours. Marking sites include Terkiin Tsagaan Lake, Mongolia (TT), Qinghai Lake, China (QL), Chitwan National Park, Nepal (CP), Pong Dam, India (PD), Keoladeo National Park, India (KP), **Upper Upper Wardha Reservoir, Maharashtra, India (UWR),** Chilika Lake, India (CL) and Koonthankulam, India (KT). The dotted line represents the BHGO range outlines depicted in Miyabayashi and Mundkur (Palm EC *et al.*, 2015).

the microbes help with digestion and nutrient absorption (Kohl and Dearing, 2016), prevent feather breakdown (Kohl *et al.*, 2017), and regulate the immune response against infections (Kreisinger *et al.*, 2015), the host offers the microbes a suitable environment and nutrients. Recent studies on Bar-Headed Geese wintering in Tibet and other regions have identified core microbial communities that adjust to these changes, supporting physiological resilience during migratory flights (*Ran et al. 2021*; Rytkönen *et al.* 2019; Dong *et al.* 2022).

Studies on avian gut microbiota, particularly those focused on wild populations, are limited, with most research emphasizing domesticated or economically significant species, such as poultry (Waite & Taylor, 2014; Rytkönen *et al.* 2019; Wang *et al.* 2019). However, growing interest in the gut microbiota of wild birds has highlighted the importance of microbial diversity for migratory species' health, their adaptation, their endurance and disease resistance during long flights (McFall-Ngai *et al.*, 2013; Wang *et al.* 2016a; Dong *et al.* 2022). Specifically, Bar-Headed Geese's gut microbiota has shown age-related changes and regional variations, which may influence their ability to adapt to new environments encountered along migratory routes (Wang *et al.* 2016b; Wang *et al.* 2017; Dong *et al.*, 2022; Wang *et al.* 2019).

Bar-Headed Geese have been examined for their involvement in disease transmission, attributed to their broad migratory patterns, as well as for their distinctive respiratory adaptations to decreased oxygen levels (Lague et al. 1985; Ward et al. 2002). Nevertheless, there is scant knowledge regarding the function of their gut microbiota in facilitating these adaptations, particularly in relation to the dietary alterations experienced during migration. The primary determinant influencing the microbiota's composition is the host's genetic makeup. The conservation of other endangered species can significantly benefit by monitoring alterations in the gut microbiota and dietary habits of wild birds via their faeces (Wang *et al.* 2023; Wang *et al.* 2019).

This study constitutes the inaugural investigation of the gut microbiota in Bar-Headed Geese within the Upper Wardha Reservoir region of Maharashtra, India, a locale apart from prior studies undertaken in Tibetan wintering grounds and other environments. We intend to characterise the microbial communities in these geese by high-throughput metagenomic sequencing and investigate potential correlations with their dietary habits in this wintering habitat. Establishing a baseline for gut microbiota composition in this region enhances the understanding of how microbial communities facilitate migratory and high-altitude adaptations in wild birds (Scott and Milsom 2007; *Ran et al. 2021*; Wang *et al.* 2016; Wang *et al.* 2019).

MATERIALS AND METHODS

Sample Collection

Faecal specimens of Bar-Headed Geese (*Anser indicus*) were obtained from the scrubland area of the Upper Wardha Reservoir, Morshi, District Amravati, Maharashtra, India. Samples were collected during the geese's wintering season, from December to March. Recent faecal samples were meticulously gathered in sterile containers to prevent contamination and preserved at -80°C until subsequent processing.

DNA Extraction

DNA extraction was conducted utilising the Chromous DNA extraction kit according to the guidelines provided by the service provider. After cell lysis, DNA was attached to spin columns, impurities were removed, resulting in pure DNA, which was eluted in water and preserved at -20°C for later use. The concentration and quality of DNA were assessed using a Qubit fluorometer, with the sample meeting quality control standards at a concentration of 21.6 ng/μL and a DNA Integrity Number (DIN) of 5.8.

Library Preparation

Library preparation was carried out using the Kapa HyperPlus kit. 100 ng of DNA was subjected to enzymatic fragmentation to produce dsDNA fragments followed by end repair and A-tailing to produce end-repaired 5'-phosphorylated, 3'-dA-tailed dsDNA fragments. This was followed by adapter ligation where dsDNA adapters with 3'-dTMP overhangs were ligated to the 3'-dA-tailed molecules. The library generated was then amplified with minimal PCR cycles to avoid any bias. The amplified library was subjected to clean up using magnetic beads. Adapter ligation and minimal PCR amplification followed to avoid bias, and libraries were purified with magnetic beads. TapeStation analysis confirmed the expected library size of ~350 bp, with a concentration of 14.3 ng/µL for sequencing.

Sequencing

Sequencing was conducted on an Illumina NovaSeq X Plus platform with a 10B flow cell and a 300-cycle kit, generating 5 Gb of data with 150 bp paired-end reads. Quality control metrics included demultiplexing, adapter trimming (using Trimmomatic), and quality assessment with FastQC and MultiQC, ensuring high-quality reads for analysis.

Data Processing and Bioinformatics Analysis

- **Quality Control:** FastQC and MultiQC were utilised to evaluate read quality, while Trimmomatic was employed for adapter elimination.
- **Metagenomic Assembly:** MEGAHIT v1.2.9 was employed for de novo assembly, utilising parameters refined for k-mer counting to generate contigs. The quality of the assembly was assessed with QUAST.
- **Gene Prediction and Functional Annotation:** Prodigal (the Prokaryotic Dynamic Programming Gene Finding Algorithm) was utilised for gene prediction, producing coding sequences (CDS) and associated protein sequences. Prokka annotated genes, with protein sequences aligned to databases (NCBI, UniProt, Pfam, and TIGRFAMs) enabling functional analysis.
- **Pathway Analysis:** KofamScan assigned KEGG Orthology (KO) IDs to predicted proteins, mapping pathways and generating relative pathway abundances for functional profile analysis.

Taxonomy Profiling: MetaPhlAn4 was employed for taxonomy profiling, detecting microbial species through unique clade-specific markers, resulting in microbial abundances across different taxonomic levels.

Statistical Analysis

Taxonomic and functional diversity were assessed with the R package Vegan, calculating alpha diversity (Shannon index, Chao1 richness) and beta diversity metrics. Statistical significance of community structure differences was determined using ANOSIM.

RESULTS

Quality Control and Sequence Metrics

The preliminary quality assessment of the raw sequencing data produced high-quality reads, with 89% identified as microbial and 11% unclassified. Trimmomatic was utilised to eliminate adaptor sequences, yielding clean reads for subsequent analysis. The mean read length was 150 bp, yielding a total output of 5 Gb per sample. MultiQC plots indicated elevated sequence quality across samples, as demonstrated in Figure 5 from the report.

Metagenomic Assembly

The clean reads were assembled de novo using MEGAHIT, resulting in 130,138 contigs with a N50 of 1,592 bp and a GC content of 38.47%. The evaluation of assembly quality was conducted using QUAST, with the metrics demonstrating a successful assembly of genomic fragments (Figure 2). The largest contig attained a length of 207,248 bp, indicating the variety of microbial genomes found within the samples.

Gene Prediction and Functional Annotation

The analysis of gene prediction revealed a thorough collection of protein-coding genes within the assembled contigs, utilising Prodigal, with Prokka providing additional annotation for these genes. Analysis through KofamScan indicated that the most prevalent metabolic pathways were those associated with carbohydrate metabolism, cofactor metabolism, and membrane transport (Figure 3a, b & c). The pathways play a vital role in the uptake and processing of nutrients within avian gut microbiomes.

Taxonomic Profiling

The analysis conducted through MetaPhlAn4 revealed that the predominant microbial phyla were Firmicutes, Proteobacteria, and Actinobacteria. A comprehensive examination at every taxonomic tier uncovered the subsequent findings:

• **Phylum Level:** At this level of classification, the predominant groups identified were Firmicutes, Proteobacteria, and Actinobacteria, each contributing uniquely to processes such as nutrient cycling, metabolism, and adaptation within the gut (Figure 4a).

Parameter	value
Number of contigs (>= 0 bp)	331052
Number of contigs (>= 1000 bp)	39181
Number of contigs (>= 5000 bp)	3958
Number of contigs (>= 10000 bp)	1522
Number of contigs (>= 25000 bp)	320
Number of contigs (>= 50000 bp)	86
Total length (>= 0 bp)	244306445
Total length (>= 1000 bp)	111753575
Total length (>= 5000 bp)	49083497
Total length (>= 10000 bp)	32306302
Total length (>= 25000 bp)	14599734
Total length (>= 50000 bp)	6739035
Number of contigs	130138
Largest contig	207248
Total length	173044593
GC (%)	38.47
N50	1592
N75	781
L50	18709
L75	59743
Number of N's per 100 kbp	0

Figure 2. Quast results showing the assembly statistics.

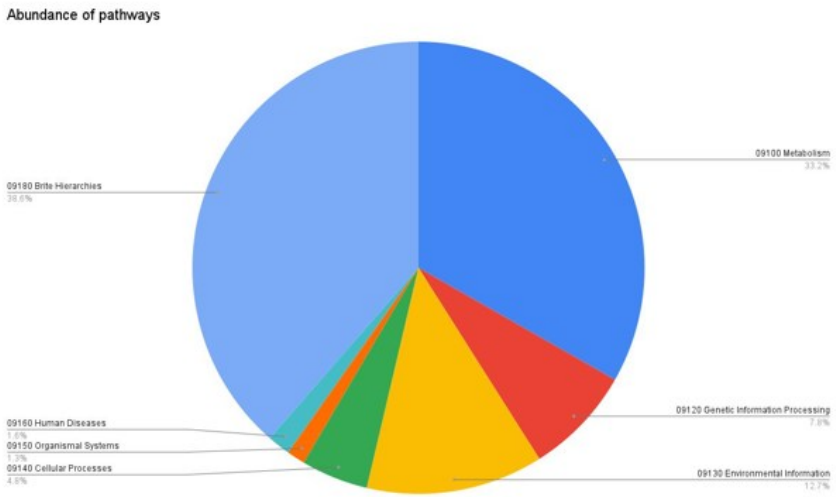

Figure 3a. Pie chart representing all the pathways annotated by KofamScan.

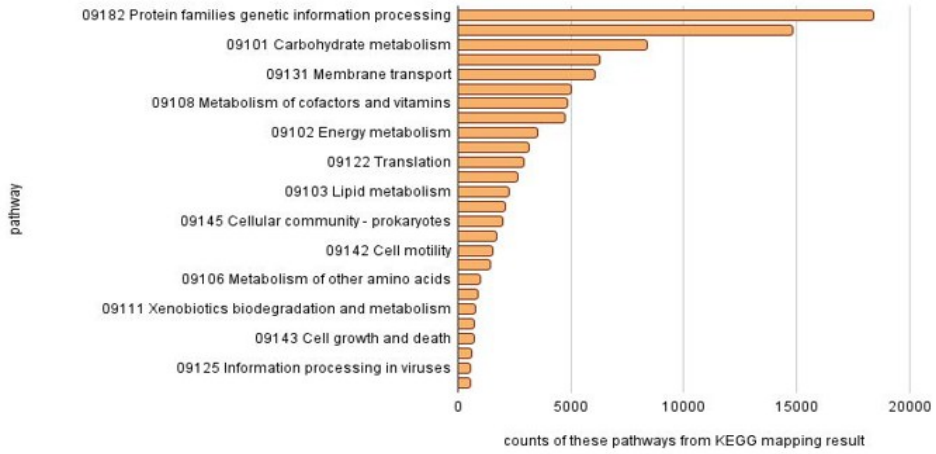

Figure 3b. Bar plot presenting the counts of the pathways resulted from KEGG mapping as a measure of pathway abundance.

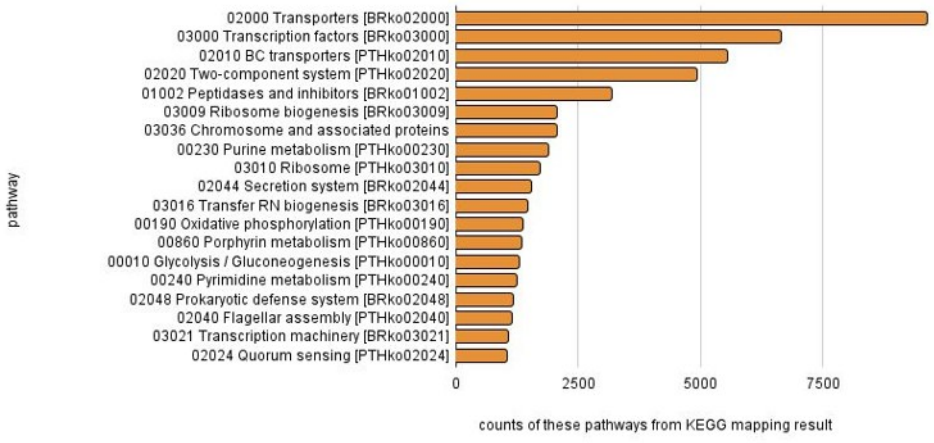

Figure 3c. Bar plot presenting the counts of the pathways resulted from KEGG mapping as a measure of pathway abundance.

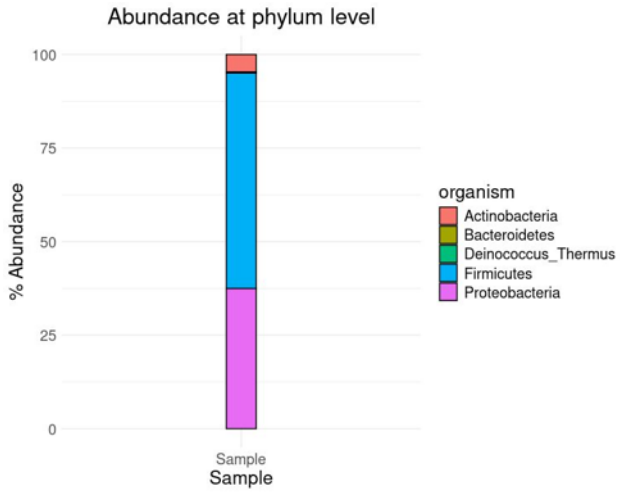

Figure 4a. Stacked bar plot representing abundance at phylum levels.

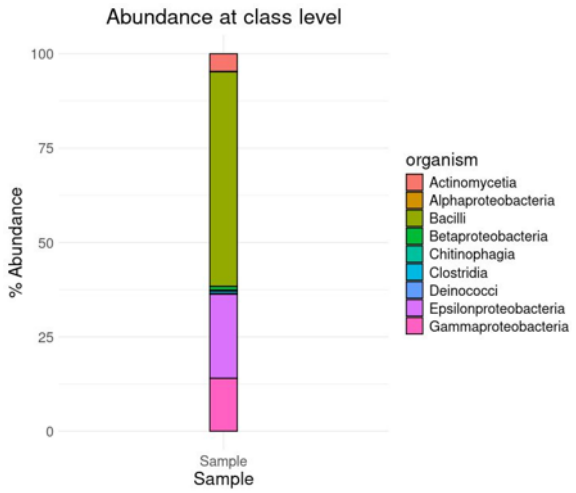

Figure 4b. Stacked bar plot representing abundance at class levels.

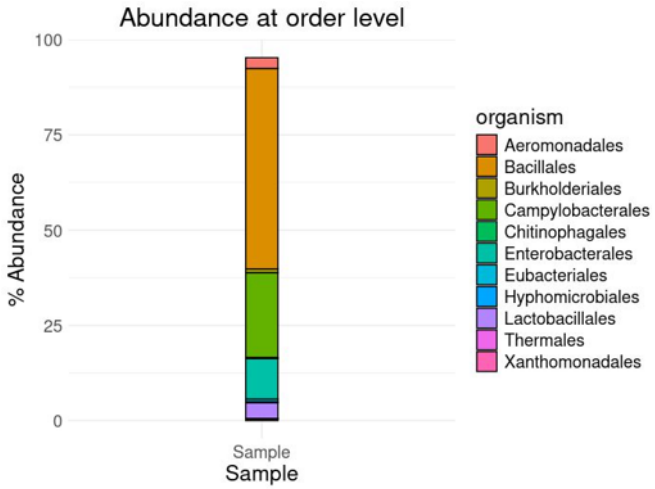

Figure 4c. Stacked bar plot representing abundance at order levels.

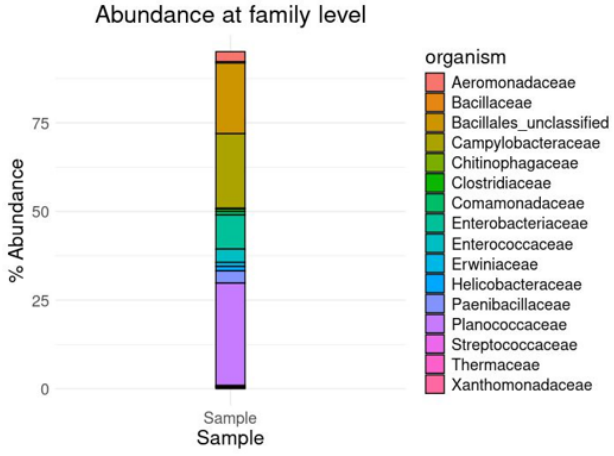

Figure 4d. Stacked bar plot representing abundance at family levels.

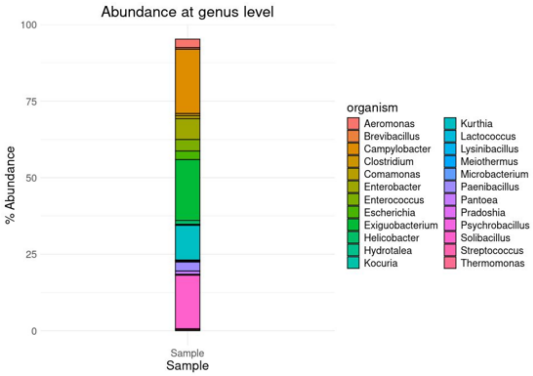

Figure 4e. Stacked bar plot representing abundance at genus levels.

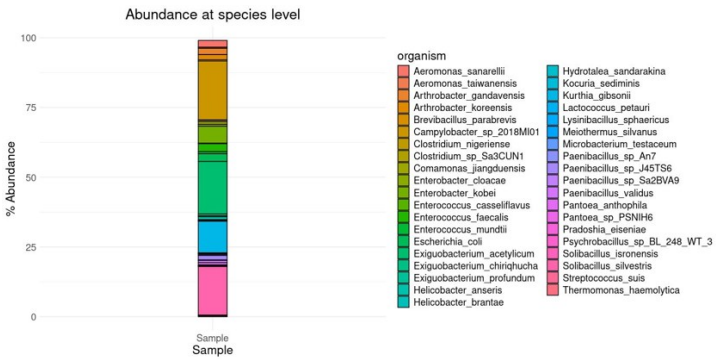

Figure 4f. Stacked bar plot representing abundance at species levels.

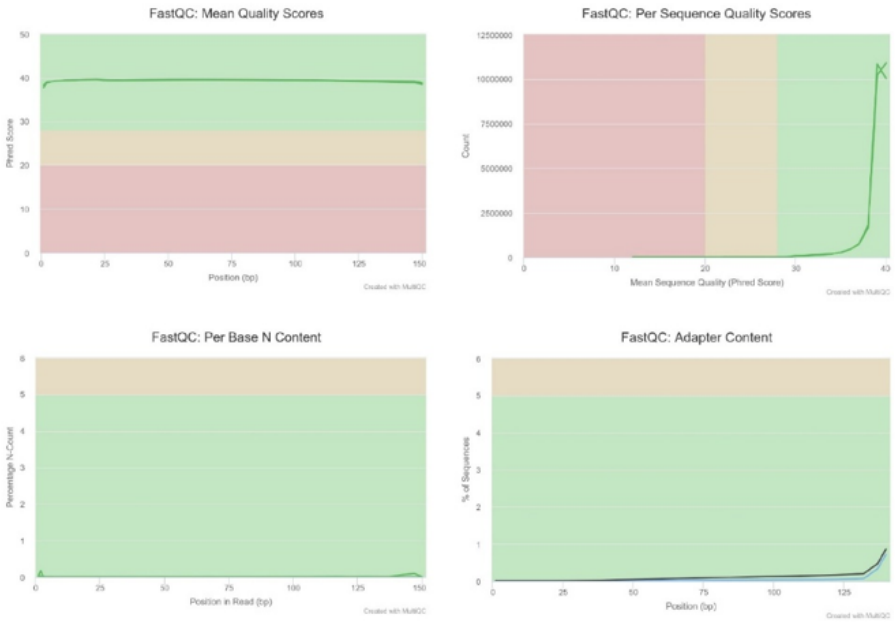

Figure 5. MultiQC plots depicting the quality of the samples (FASTQ)

♦**Class Level:** The major classes encompass Bacilli (part of Firmicutes), Gammaproteobacteria and Epsilonproteobacteria (both under Proteobacteria), and Actinomycetia (within Actinobacteria). These categories are typically linked to energy extraction and environmental resilience (Figure 4b).

The Firmicutes phylum features a predominant class known as Bacilli, recognised for its role in carbohydrate fermentation and energy production. The Proteobacteria phylum includes the classes Gammaproteobacteria and Epsilonproteobacteria. Gammaproteobacteria is involved in nitrogen cycling and gut health, whereas Epsilonproteobacteria aids in adaptation to environmental stress. The class Actinomycetia within the Actinobacteria phylum is distinguished by its capacity to decompose complex organic materials, playing a significant role in enhancing immune resilience and nutrient processing in the gut.

- **Order Level:** Significant orders identified include Bacillales, Lactobacillales, Enterobacterales, and Campylobacterales, highlighting their involvement in nutrient cycling and carbohydrate metabolism (Figure 4c).The main groups identified were Bacillales and Lactobacillales within Firmicutes, each playing a role in carbohydrate metabolism and nutrient cycling. Enterobacterales within Proteobacteria contribute to nitrogen metabolism and gut health, whereas Campylobacterales within Epsilonproteobacteria facilitate adaptation to diverse environmental conditions and enhance resilience in the gut.
- **Family Level:** Notable families comprised Bacillaceae, Campylobacteraceae, Planococcaceae, Enterobacteriaceae, Enterococcaceae, and Paenibacillaceae. The contributions of these families are vital for functions such as metabolic flexibility, adaptation to environmental stressors, and the maintenance of gut health (Figure 4d).

Here, **Bacillaceae** and **Planococcaceae** within Firmicutes are known for supporting energy metabolism and resilience in varied environments. **Campylobacteraceae** within Epsilonproteobacteria is associated with gut health and adaptation to environmental changes. **Enterobacteriaceae** within Gammaproteobacteria contributes to nitrogen metabolism and gut health, while **Enterococcaceae** and **Paenibacillaceae** play essential roles in nutrient cycling and maintaining gut stability.

- **Genus Level**: The most abundant genera were ***Campylobacter***, ***Hydrotalea***, ***Exiguobacterium, Kurthia, Paenibacillus and Psychrobacillus***, indicating a microbiota composition adapted to carbohydrate metabolism and environmental adaptation. These genera play roles in energy metabolism, important for sustaining long migratory flights (Figure 4e).

Here, **Campylobacter** (belongs to family Campylobacteraceae) is associated with gut health and environmental adaptation, while **Hydrotalea** (belongs to family Hydrotaleaceae) aids in breaking down complex carbohydrates. **Exiguobacterium** and **Kurthia,** both from the family Planococcaceae, are known for their resilience in diverse environments and roles in nutrient processing. **Paenibacillus** (belongs to family Paenibacillaceae) contributes to nitrogen fixation and soil nutrient cycling, and **Psychrobacillus** (belongs to family Bacillaceae) is adapted to cooler temperatures, supporting the geese's physiological needs during migration.

- **Species Level**: At the species level, notable species included ***Campylobacter sp. 2018MI01, Enterobacter cloacae, Enterobacter kobei, Enterococcus casseliflavus, Enterococcus faecalis, Enterococcus mundtii, Meiothermus silvanus,*** and ***Psychrobacillus sp. BL-248-WT-3***. These species highlight the microbial community's role in supporting the geese's dietary and environmental adaptations during migration (Figure 4f).

Here, *Campylobacter sp. 2018MI01* and species of *Enterobacter* play essential roles in gut health and nutrient absorption. *Enterococcus* species, such as *E. casseliflavus, E. faecalis,* and *E. mundtii,* are involved in maintaining gut balance and immunity. *Meiothermus silvanus* is known for its thermophilic properties, indicating adaptability to diverse environmental conditions, and *Psychrobacillus sp. BL-248-WT-3* is adapted to cooler temperatures, supporting resilience in varied climates during migration.

Eukaryotic Species Detection

The efforts to pinpoint eukaryotic sequences in relation to the diet yielded inconclusive results, as only a small fraction of reads corresponded with plant or insect markers. Despite thorough searches using markers, the low alignment rates indicated possible degradation of these markers in the faecal matter, which restricted the identification of plant and insect species (Table 1).

Table 1. Marker genes used for identification.

Sl. no.	Name of the gene	Origin
1	ITS	Nuclear
2	Maturase K gene (matK)	Chloroplast
3	rbcL	Chloroplast
4	psbAtrnH	Chloroplast
5	trnL–trnF	Chloroplast
6	5' region of the cytochrome oxidase subunit	Mitochondria

DISCUSSION

Comparative Analysis of Gut Microbiota

The prevalence of Firmicutes, Proteobacteria, and Actinobacteria in the gut microbiota of Bar-Headed Geese is consistent with earlier research on avian gut flora, especially in migratory and high-altitude species (Zhang *et al.* 2021; Wang *et al.* 2017; Dong *et al.* 2022; Wang *et al.* 2019). The roles of these phyla are crucial in nutrient absorption, immune response, and carbohydrate metabolism, which are essential for sustaining long migratory flights (Takekawa *et al.* 2017; Rytkönen *et al.* 2019; Wang *et al.* 2023). Recent studies in similar species have noted the significance of Firmicutes in enhancing energy extraction from complex carbohydrates, likely contributing to the geese's endurance during migration (*Ran et al. 2021*; Rytkönen *et al.* 2019). Similar microbiome patterns were observed in wild Bar-Headed Geese in the Tibetan Plateau, suggesting environmental consistency in microbial composition across different regions (McFall-Ngai *et al.* 2013; Wang *et al.* 2017; Wang *et al.* 2019; Wang *et al.* 2022).

Functional Role of Microbiota in Migration and Adaptation

The identified metabolic pathways associated with carbohydrate metabolism, cofactor metabolism, and membrane transport suggest a gut microbiota that adapts effectively to seasonal changes in diet. These findings are consistent with studies indicating that metabolic flexibility in gut microbiota supports migratory birds in fulfilling their elevated energy requirements (Wang *et al.* 2016a; Wang *et al.* 2017; Wang *et al.*, 2018; Wang *et al.* 2019). Research on Bar-Headed Geese in different wintering regions of Tibet has pinpointed essential microbial functions related to carbohydrate metabolism and nutrient absorption, highlighting the microbiota's contribution to enhancing migratory endurance (Wang *et al.* 2016a; Wang *et al.*, 2018; Wang *et al.* 2019). Figures 3a, 3b, and 3c depict the pathway abundances identified in our study, aligning with comparable findings regarding gut microbiota adaptation in high-altitude species (Rytkönen *et al.* 2019).

Environmental and Dietary Influences on Microbial Composition

The prevalence of genera such as *Campylobacter* and *Psychrobacillus* could indicate particular environmental and dietary conditions present at the Upper Wardha Reservoir. Investigations reveal the influence of migratory shifts on microbiota composition, demonstrating adaptation to environmental changes (Rytkönen *et al.* 2019). The prevalence of key microbial genera in Bar-Headed Geese across various regions corresponds with observations from other environments, indicating a consistent microbial community regardless of migratory patterns (Wang *et al.* 2016b; Dong *et al.*, 2022; Wang *et al.* 2019). The visual representation in Figures 4e and 4f illustrates the microbial diversity at both the genus and species levels within this wintering ground.

Limitations and Future Directions

The study faced challenges in accurately identifying eukaryotic species from plant or insect markers, probably resulting from the degradation of genetic markers in faecal matter. This is consistent with findings from studies on the microbiome related to age in Bar-Headed Geese, where older samples showed low alignment rates, which may influence taxonomic specificity (Waite & Taylor, 2014; Rytkönen *et al.* 2019; Dong *et al.* 2022). Enhanced preservation techniques may lead to greater marker stability, facilitating comprehensive diet profiling and more precise dietary analysis (*Ran et al. 2021*; Wang *et al.* 2017).

Implications for Conservation and Health

This investigation provides important findings for conservation by emphasising the significance of gut microbiota in enhancing the resilience and health of migratory birds. Monitoring the microbiome, illustrated by research on Bar-Headed Geese across various wintering regions, can offer indirect insights into physiological health and adaptability in migratory species (McFall-Ngai *et al.* 2013; Wang *et al.* 2016a; Wang *et al.* 2019). Exploring the role of microbiota in adaptation and immune support could enhance conservation strategies, particularly for high-altitude migratory species that are at risk due to environmental changes (Scott *et al.* 2007; Sun *et al.* 2022; Rytkönen *et al.* 2019; Wang *et al.* 2019).

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Conflict of Interest

The authors declare no conflicts of interest.

Funding statement

The study was carried out as self-funding research. There was no any external funding source.

Authors' Contributions

Ashwin G. Lunge collected faecal samples from the field and helped in manuscript writing; Bipinchandra B. Kalbande analysed the data & also led the writing of the manuscript; Aparna M. Yadav helps in manuscript writing & analysis, Gajanan A. Wagh helps in manuscript writing & saple collection.

Data Availability Statement

The metagenome sequence data generated in this study have been deposited in the GenBank repository under the reference PRJNA1119098.

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