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# Comparative Analysis of Gut Microbiota Composition in Yaks Across Different Geographical Region

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## ABSTRACT

**Background:** Despite the significance of gut microbiota in in determining the health and adaptability of the host, limited information exists regarding the influence of diverse geographic conditions on the intestinal microbiota of yaks. The study aimed at undertaking comprehensive comparison of microbiota composition in yaks originating from distinct geographic regions.

**Methods:** 15 fecal samples were collected from yaks inhabiting 5 regions in China: Madoi, Nagqu and Deqin Shangri-la, Lhasa and Yushu. Through rigorous high-throughput sequencing analysis, the alpha diversity using metrics such as Shannon, ACE and Chao indices were assessed.

**Result:** Notably, the Shangri-la samples exhibited the highest alpha diversity. Principal coordinate analysis revealed substantial variations in the composition of the intestinal microbiota across different regions. Firmicutes and Bacteroidetes emerged as the dominant phyla, with Ruminococcaceae being the most abundant family. Particularly, the genus Ruminococcaceae_UCG-005 demonstrated prominence, exhibiting higher abundance in Yushu samples compared to others. However, intriguingly, the predicted functional gene composition of the gut microbiota exhibited remarkable similarity across diverse geographical regions.

**Key words:** Antibiotic-resistance genes, Drug resistance, Metagenomics, Microbial diversity, Yak gut microbiota.

## INTRODUCTION

The yak (*Bos grunniens*) is resilient indigenous ruminant of the Qinghai-Tibetan Plateau, demonstrating remarkable adaptation to challenging environments characterized by severe cold, limited foraging resources, high ultraviolet radiation and low oxygen levels (Huang *et al.,* 2012). In varying regions, yaks face distinct food availability challenges, relying on a variety of sources such as native grass, manually cultivated forage and artificial feed supplies. Consequently, yaks harbor distinctive gut microbiota, essential for their health and survival in demanding environments (Jeevan *et al*., 2024; Singh *et al*., 2023). Extensive research has demonstrated the pivotal role of microbiota, comprising trillions of microbial cells in the gastrointestinal tract (GIT), in facilitating host adaptation to these challenging conditions (Xin *et al.*, 2019).

Recent studies on yaks have highlighted significant differences in the diversity of intestinal microbiota across various segments of the rumen, as well as variations linked to different feeding methods (Ren *et al.,* 2020; Zhang *et al.,* 2020). However, as of our knowledge, a few research has delved into understanding the impact of diverse geographical conditions on yak gut microbiota. Recognizing the pivotal role of the gut microbiome, the study undertook a comprehensive comparison of microbiota composition in yaks originating from distinct regions. Leveraging High-Throughput Sequencing (HTS) technology, microbial diversity in yaks from diverse geographical locations and predicted microbial functions based on gene composition was characterized. The outcomes of the study not only underscore the significant influence of varied geographical regions on yak gut microbiota but also advance the comprehension of species composition within the gut microbiota under different geographical circumstances. Furthermore, this research marks the inaugural identification of differences in the gut microbiota of yaks inhabiting dissimilar geographical regions, each subjected to unique environmental conditions.

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## MATERIALS AND METHODS

### Animals and sample collection

In June 2023, fifteen adult yaks were sampled from five different regions in China: Lhasa, Nagqu, (Tibet Autonomous Region), Yushu, Madoi (Qinghai Province) and

Shangri-la (Yunnan Province). Based on the geographical region of animals, the samples were divided into five groups: G1 (Lhasa), G2 (Yushu) G3 (Madoi), G4 (Shangri-la) and G5 (Nagqu), G5. Three samples were collected from each group as G1 (MM2201, MM2202, MM2203), G2 (MM3101, MM3102, MM3103), G3 (MC2101, MC2102, MC2103), G4 (MC3201, MC3202, MC3203) and G5 (MM1401, MM1402, MM1403). In each of the three regions, stool samples were collected from three individual yaks belonging to a single group that engaged exclusively in summer grazing on natural pastures. Immediately after defecation, fresh fecal samples were meticulously transferred into sterile tubes, with the utmost care taken to maintain sterility through the use of sterile gloves and spoons. The collected tubes were promptly frozen using liquid nitrogen and subsequently transported to the laboratory, where they were carefully packed with dry ice. The samples were then safely stored at -80°C until they were ready for further analysis.

**Animal research approval**

This study was approved by The Institutional Animal Care and Use Committee (IACUC) of Xinjiang Academy of Agricultural Sciences under IACUC approval number 2023/1223XAAS/2111.

**DNA extraction, library preparation, sequencing and bioinformatic analysis**

DNA extraction, library preparation, sequencing and bioinformatic analysis were done as recommended (Pasquali *et al.*, 2019).

**Assembly of metagenome**

The clean data was subsequently processed using MEGAHIT software (v1.0.4-beta) for assembly analysis. The meta-large preset parameters (-end-to-end, -sensitive, -I 200,-X 400) (Karlsson *et al.*, 2013; Nielsen *et al.*, 2014) were utilized, resulting in Scaftigs devoid of 'N' bases, which were obtained by cleaving the resulting scaffolds at 'N' junctions (Li *et al.*, 2015; Qin *et al.*, 2010).

**Gene prediction and abundance analysis**

Gene prediction and abundance analysis were carried out using MetaGeneMark (V3.05, http://topaz.gatech.edu/GeneMark/) with default parameters (Karlsson *et al.*, 2012; Oh *et al.*, 2014; Qin *et al.*, 2014). Predicted information with

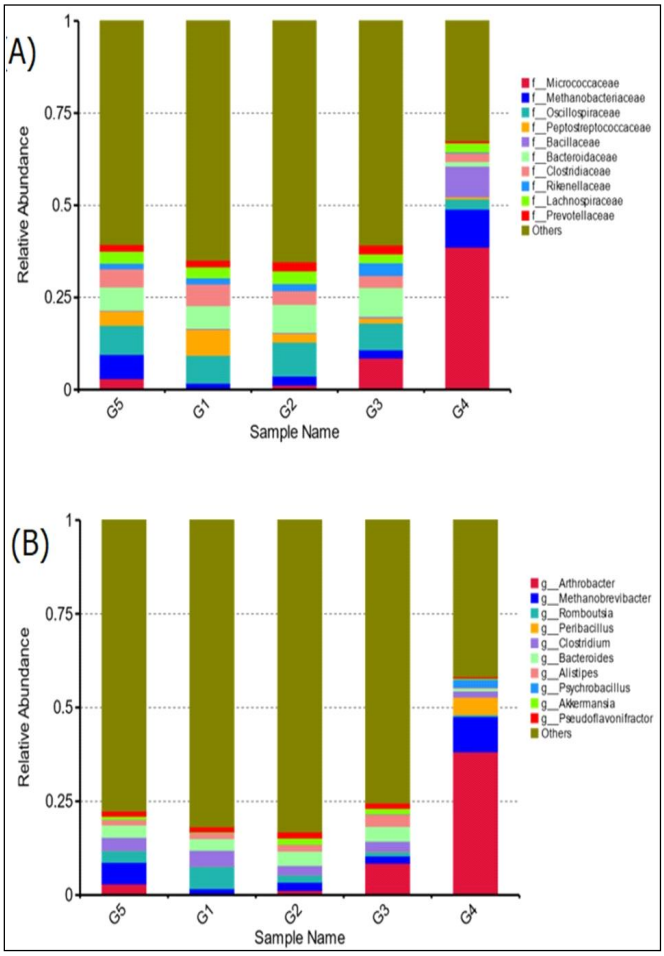



**Fig 1:** Histogram of 10 most microbial community abundance maps at the phylum (A) and genus (B) level in 5 groups.

a length less than 100 nucleotides were subsequently removed from the dataset (Qin *et al.*, 2010; Zeller *et al.*, 2014; Zhu *et al*., 2010). The ORF prediction results were then processed with CD-HIT software (Oh *et al.*, 2014). Genes with reads fewer than or equal to 2 in each sample were excluded to finalize the gene catalog (Unigenes) for subsequent analysis. The abundance of each gene in each sample was determined based on the number of aligned reads and gene length. This was then followed by species and common functional databases annotations according to recommended procedures (Zeller *et al*., 2014).

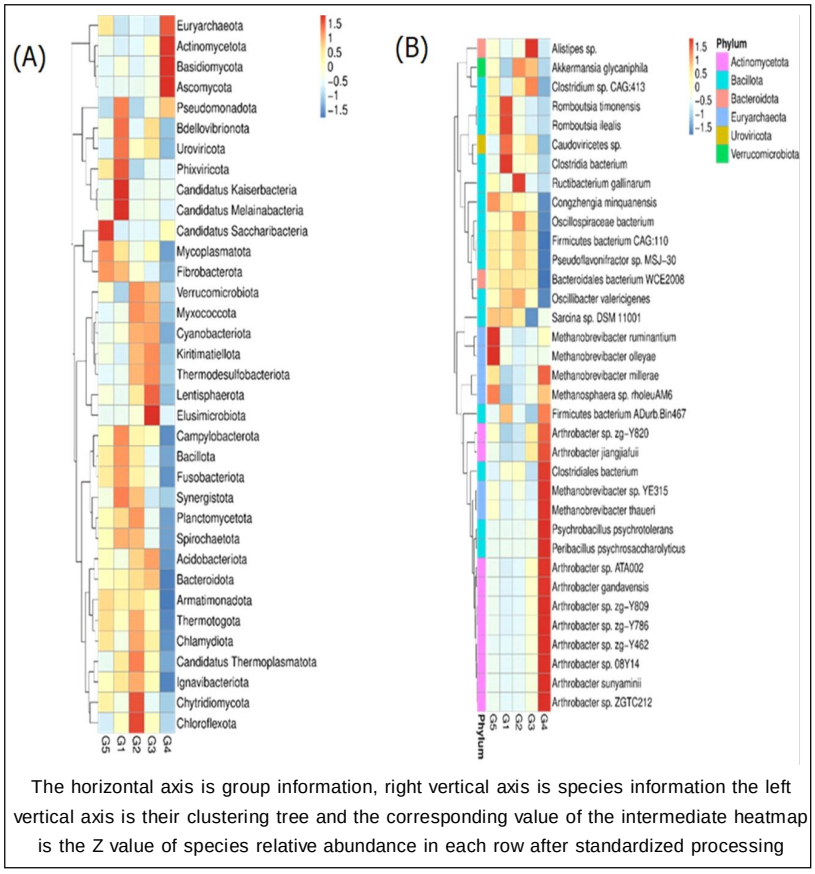
## RESULTS AND DISCUSSION

### Taxonomic analysis

The unigene sequences were aligned using DIAMOND software. To visualize the species annotation results and effectively present the relative abundance of species at various taxonomic levels in each sample, we employed Krona. The results indicated that in all 15 samples MC2101, MC2102, MC2103, MC32013, MC3201, MC3202, MM1401, MM1402, MM1403, MM2201, MM2202, MM2203, MM3101, MM3102 and MM3103, bacteria were the most abundant microorganisms at 72-82%. They were followed by unknown microorganisms, archaea, viruses and eukaryota, regardless of the yak's region.

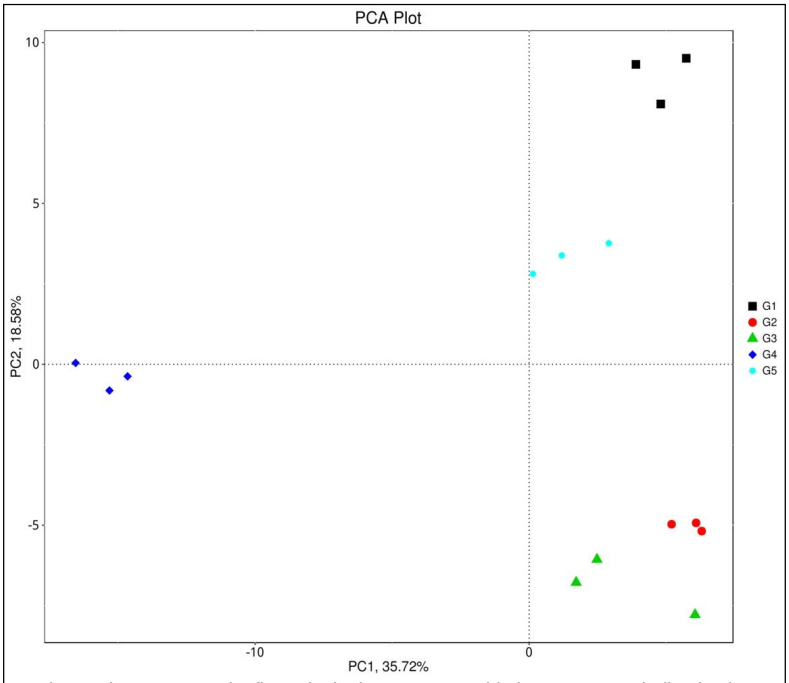
### Relative abundance of most abundant species at different taxonomic level

Bacillota, Bacteroidota, Actinomycetota and Euryarchaeota were more abundant phyla in all 5 groups G1, G2, G3, G4 and G5 (Fig 1A). Bacillota were more abundant in group G1, G2, G3 followed by Bacteroidota and Actinomycetota and Euryarchaeota, whereas in group G4 Actinomycetota were more abundant followed by Bacillota, Euryarchaeota and Bacteroidota and in group G5 Bacillota were more abundant followed by Bacteroidota, Euryarchaeota, Actinomycetota. At genus level, in group G4 Arthrobacter were more abundant followed by Methanobrevibacter, Peribacillus, Psychrobacillus, clostridium and Bacteroides. Whereas in group G5 Methanobrevibacter were more abundant followed by clostridium, Bacteroides, Arthrobacter and Romboutsia and in group G3 Arthrobacter were more abundant followed by Bacteroides, Alistipes, Clostridium, Methanobrevibacter, Akkermansia, Pseudoflavonifractor, in G1 Romboutsia were more abundant followed by clostridium, Bacteroidota, Alistipes, in group G2 Bacteroides were more abundant followed by Clostridium, Methanobrevibacter, Alistipes (Fig 1B). The heat map generated based on the abundance information of the top 35 genera revealed significant differences in the composition of the gut microbiota among different region



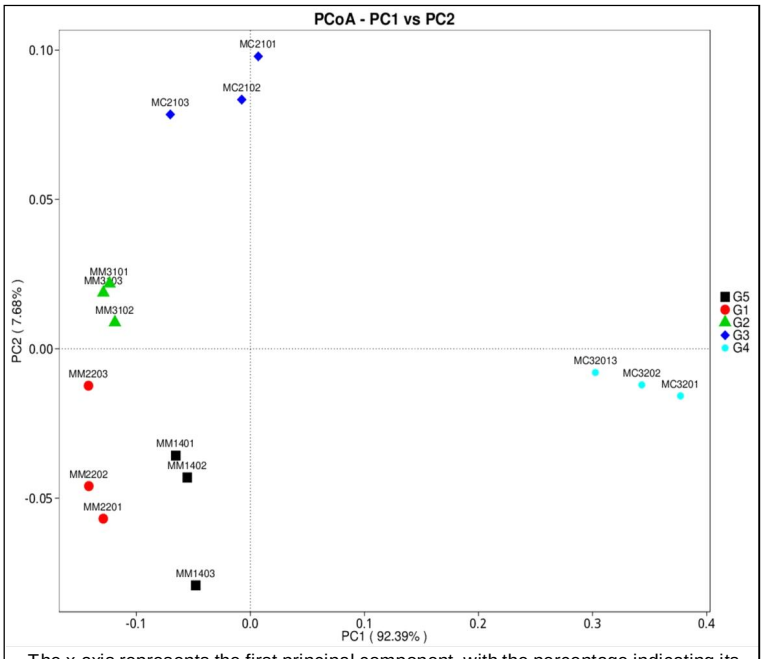

The horizontal axis is group information, right vertical axis is species information the left vertical axis is their clustering tree and the corresponding value of the intermediate heatmap is the Z value of species relative abundance in each row after standardized processing

**Fig 2:** Relative abundances of the top 30 bacterial sequence variants at different taxonomic level: genus (A), species (B), order (C).




The x-axis represents the first principal component, with the percentage indicating its contribution to the sample difference. The y-axis represents the second principal component, with the percentage indicating its contribution to the sample difference. Each data point in the figure represents a sample and samples from the same group are represented by the same color.

**Fig 3:** Principal Component Analysis (PCA) results at phylum.




The x-axis represents the first principal component, with the percentage indicating its contribution to the sample difference. The y-axis represents the second principal component, with the percentage indicating its contribution to the sample difference. Each data point in the figure represents a sample and samples from the same group are represented by the same color.

**Fig 4:** Principal coordinate analysis (PCoA) results at phylum level.

groups of yaks. Each group from different was distinctly clustered from phylum to species level, suggesting region specific gut microbiota signatures (Fig 2).

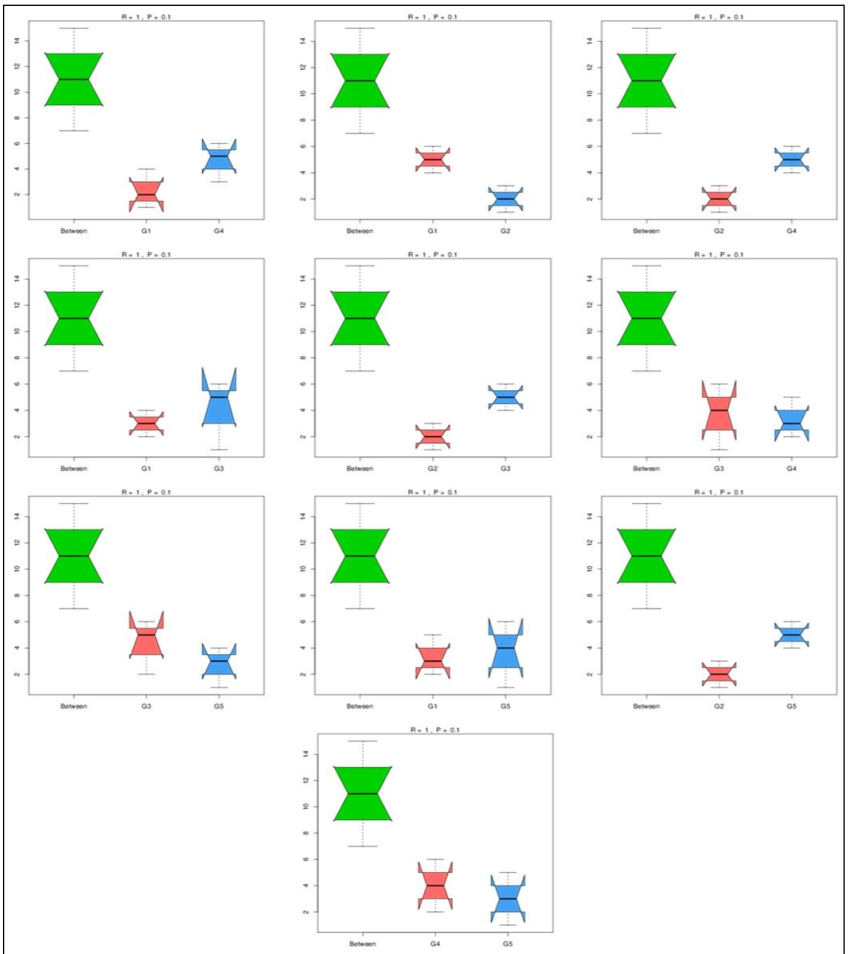
The PCA results indicate that microbiota composition between different region group is different from phylum to species level (Fig 3). Further the results of principal co-ordinates analysis based on the Bray-Curtis distance also shows that microbiota community structure and composition was different between groups and was more similar within the groups from phylum to species level (Fig 4). These findings were verified by ANOSIM analysis which indicates that microbiota composition difference at different taxonomic level between groups of different Yak region was significantly greater than within group (Fig 5).

**Common functional database analysis**

The metagenomic sequencing profiling data of samples were annotated to the KEGG database to evaluate the functional diversity. Fig 6 and 7 show the differences in the potential functional abundance of the bacterial community between the groups. The results of the pathway annotation are provided in Fig 8 and 9. Moreover, the CAZy database analysis displayed highest abundances of glycoside hydrolases (124491) followed by glycosyl transferases (70228), carbohydrate-binding modules (31399), carbohydrate esterases (11836) and polysaccharide lyase (2366), (Fig 10 and 11).

**Antibiotics resistance gene analysis**

Based on the abundance table resistance genes, the content and percentage (Fig 12) of antibiotic resistance ontology (ARO) in each sample and screen out the top 20 AROs with the highest abundance was determoneed. It has seen that there was a difference in the relative abundance and percentage of ARO among different yak age groups. Among selected top 20 AROs, the relative



The horizontal axis represents grouping information, while the vertical axis represents distance information. The 'Between' category displays the combined information of the two groups. The R-value ranges from -1 to 1, with R-values greater than 0 indicating a significant difference between groups. R-values less than 0 suggest that the difference within groups is greater than the difference between the groups.

**Fig 5:** ANOSIM analysis results at phylum level.

abundance of vancomycin resistant genes was found to be highest in all groups followed by macrolide, tetracycline, oxazolidinone, bacitracin, aminoglycoside. However, vanW_gene_in_vanI_cluster was found to be highest in G1 and lowest in G4 which contains highest relative abundance of vanY_gene_in_vanB_cluster. Further

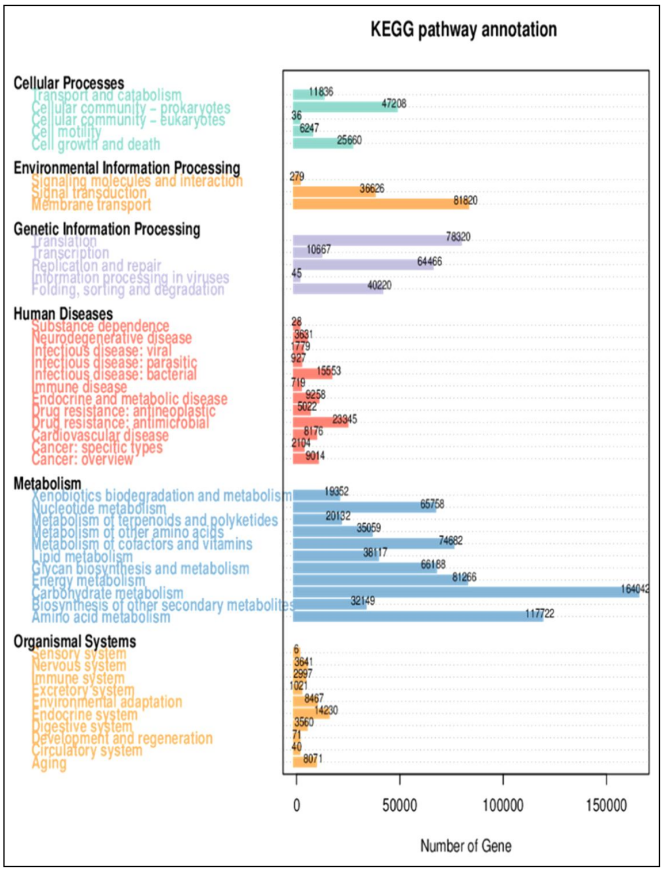



**Fig 6:** Functional prediction of the KEGG metabolic pathway.

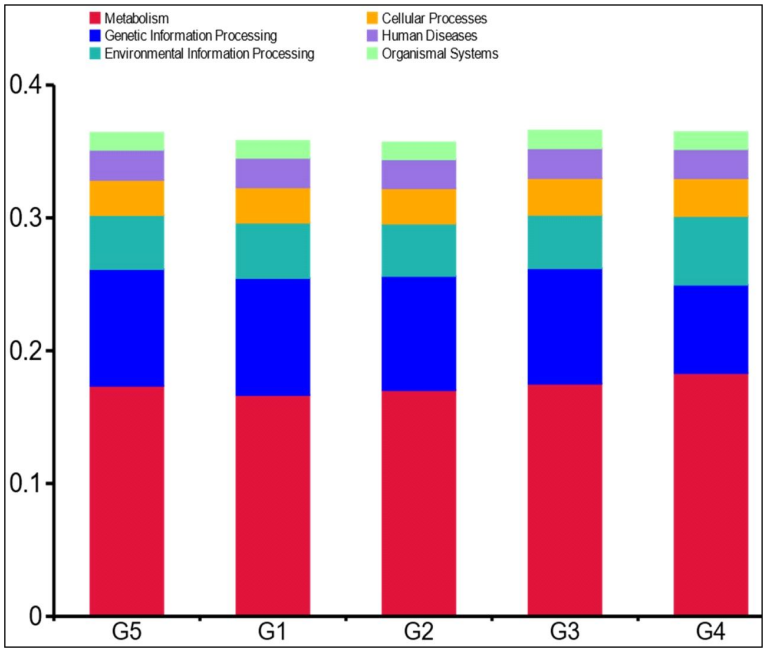



**Fig 7:** Histogram of relative abundance of functional annotations on level 1.

clustered heat map results showed that different age yak groups samples were clustered individually and ANOSIM analysis based on the Bray-Curtis distance also showed that difference between groups was significantly higher than within groups of different age.

The observed high prevalence of unknown microorganisms in samples underlines the necessity of enhancing current microbial databases to comprehensively cover global microbial diversity (Almeida *et al.*, 2019). The results also revealed a varied representation of different

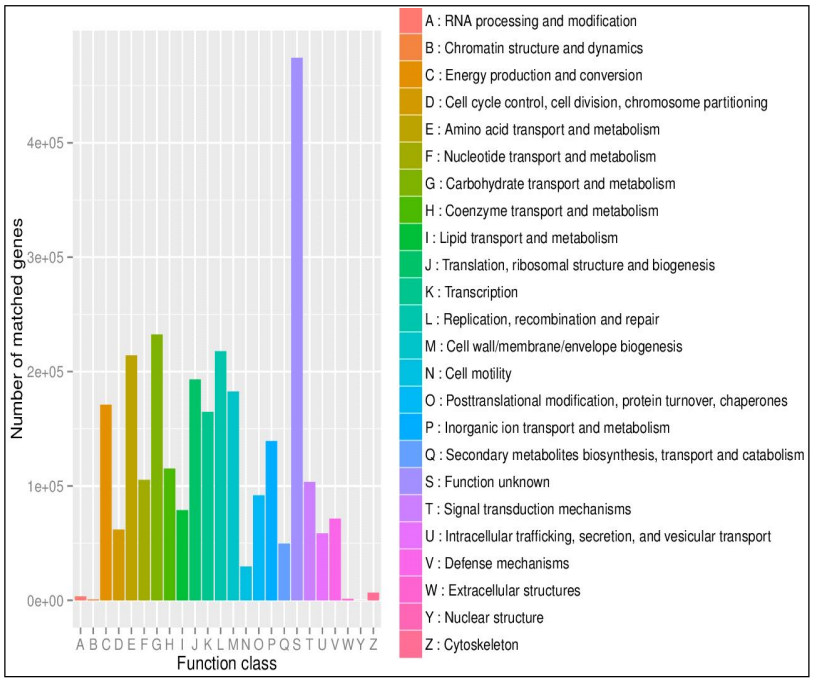



**Fig 8:** eggNOG analysis showing the number of unigenes.

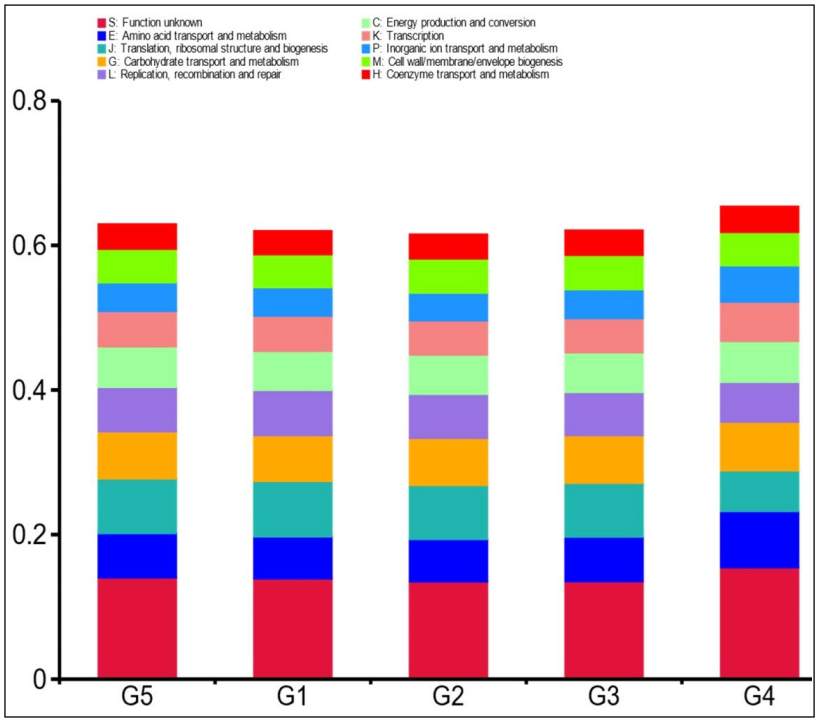



**Fig 9:** eggNOG analysis showing level 1 relative abundance histogram.

phyla across different sample groups, with Bacillota, Bacteroidota, Actinomycetota and Euryarchaeota being the most abundant. Interestingly, these phyla's representation did not remain consistent across all groups. Such variations in microbial compositions across different sample groups have been reported in other studies and could be attributed to various factors like diet, environment, host genetics and age (Zhao *et al.*, 2015). At a deeper taxonomic level, the genus Arthrobacter was found to be more abundant in groups G4 and G3, a finding that has been mirrored in previous works (Mhuireach *et al*., 2016). Arthrobacter spp. are known for their remarkable metabolic

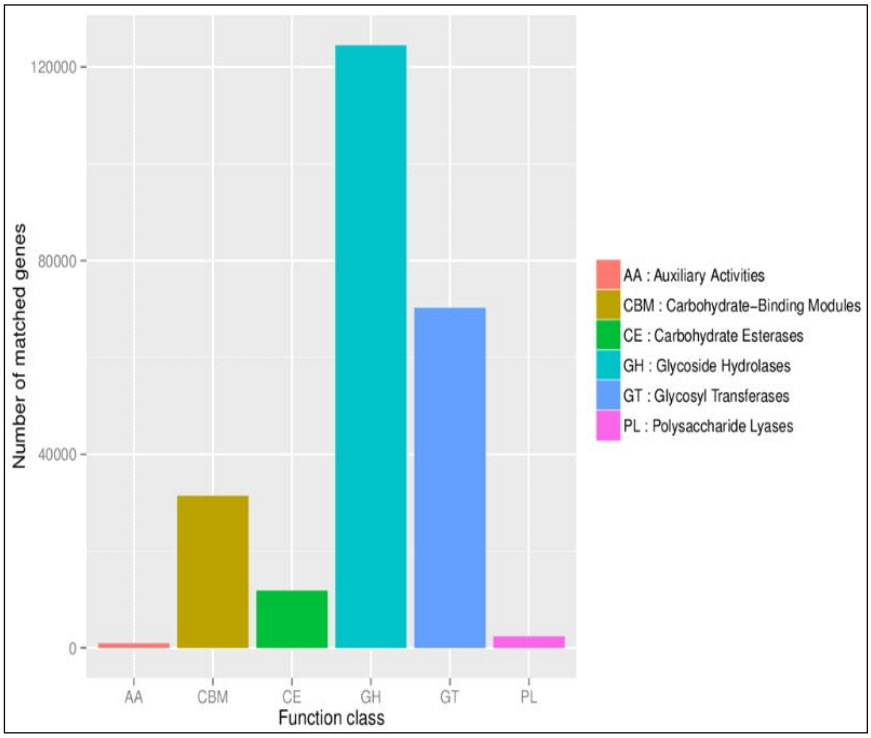



**Fig 10:** CAZy analysis for number of unigenes.

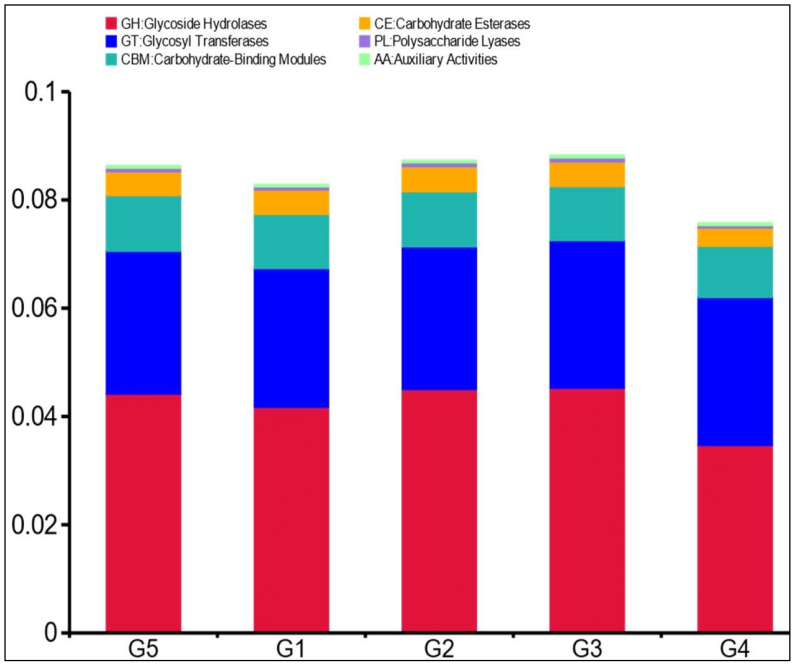



**Fig 11:** CAZy analysis for CAZy level 1 relative abundance histogram.

versatility and resilience to environmental stressors, which might explain their abundant representation in male of older ages (Mongodin *et al*., 2006). In contrast, in group G5, Methanobrevibacter was found to be more abundant. Methanobrevibacter, a well-known genus within the phylum Euryarchaeota, is a dominant archaeal group in the ruminant gut and plays a crucial role in methane production, affecting the host's energy metabolism and contributing to greenhouse gas emissions (Henderson *et al.*, 2015; Janssen and Kirs, 2008). This finding underscores the need for strategies to manipulate gut microbiota to mitigate methane emissions from ruminants. However, the varying abundance of Micrococcaceae;g__Arthrobacter, Methanobacteriaceae; g__Methanobrevibacter, Clostridiaceae;g__Clostridium and Bacteroidaceae; g__Bacteroides across different samples also highlight the intricate, dynamic and personalized nature of the microbiome (Lloyd-Price *et al.*, 2017). This study provides a detailed snapshot of the taxonomic composition of the yak microbiome, but further studies involving larger sample sizes and various environmental conditions are required to fully understand the factors that govern this diversity.

Kyoto encyclopedia of genes and genomes (KEGG), evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) and Carbohydrate-Active enZymes (CAZy) databases provide invaluable resources for the functional annotation of metagenomic sequences, which contribute significantly to the understanding of the functional potential and metabolic capacity of microbiomes. The functional analysis of unigenes in the yak microbiota using the KEGG database reveals an intriguing picture of the microbial functions in the gastrointestinal system of these animals. A high number of unigenes were linked to cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems. These findings reflect the profound influence of gut microbiota on host health and physiology, substantiated by earlier research (Nicholson *et al.*, 2012; Turnbaugh *et al*., 2006). The KEGG analysis delineated noticeable differences among the five study groups, particularly group G4, which displayed more metabolic genes and fewer disease-related genes (Fig 9). This result corroborates the widely held view that a balanced gut microbiota with high metabolic capability can contribute to host health and may confer protection against certain diseases (Honda and Littman, 2016). G4's increased metabolic functions could be driven by several factors, including diet, host genetics, or environmental factors, more energy for muscle activity associated with male, all of which have been shown to influence the microbial community structure and function (Lozupone *et al.*, 2012). Our statistical analysis, including PCA, PoCA and ANOSIM, further emphasized the functional differences among the groups (Fig 10). These results align with previous work demonstrating substantial inter-individual and intra-group variability in microbiome functions (Falony *et al.*, 2016). However, the specific factors leading to these differences in our study are not clear and would require further investigation. The minor differences in our study compared to some other published works may be due to several reasons, such as variations in sampling and data analysis methods, differences in host genetics and environmental factors (Arumugam *et al.*, 2011). Moreover, the specificity of yak gut microbiota, shaped by unique dietary and environmental pressures, could also lead to functional profiles that differ from those found in other animals or human studies (Ley *et al.*, 2008).

Further the present study also conducted an analysis of unigenes from the yak microbiota using the eggNOG and CAZy databases that provides an insightful view of the

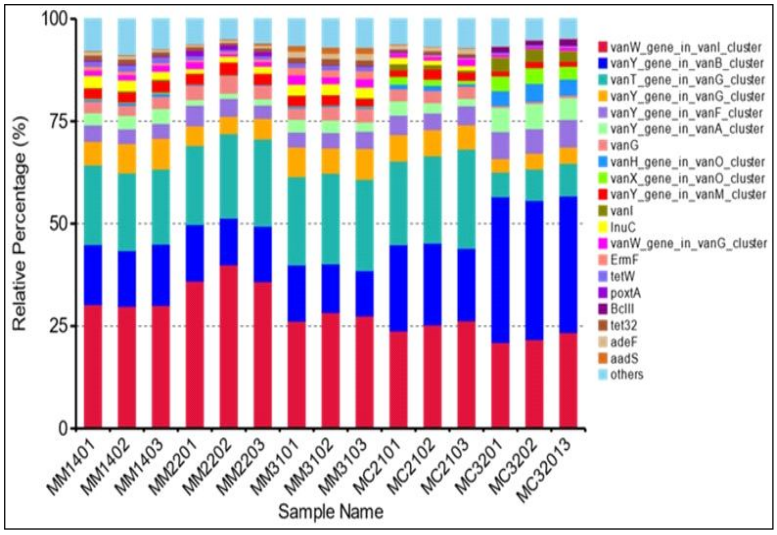



**Fig 12:** The relative abundance of top20 AROs in all AROs and others is the sum of relative abundances of non-top 20 AROs.

functional attributes of the yak microbiota, focusing on various aspects of metabolism and carbohydrate processing. The eggNOG database revealed a significant presence of genes linked to various metabolic pathways such as energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism, among others. This aligns with previous research, which indicates that gut microbiota plays a crucial role in the host's metabolic processes (Bäckhed *et al*., 2005). Notably, Group G4 exhibited higher abundance of amino acid transport and metabolic genes. It's well established that gut microbes can contribute to the host's amino acid metabolism (Neis *et al*., 2015). However, the precise factors driving the higher abundance of these genes in Group G4 warrant further investigation. Our analysis with the CAZy database showed a significant number of unigenes involved in various carbohydrate processing functions such as glycoside hydrolases, carbohydrate"binding modules and carbohydrate esterases. Glycoside hydrolases, especially, play an important role in the breakdown of dietary fibers in the gut (Flint *et al*., 2008). Interestingly, group 4 had fewer glycoside hydrolases genes, while group G3 had the most. This may suggest differences in dietary fiber processing abilities between these groups. Further, these findings could also indicate differences in diet or host-specific factors that affect the composition of the microbiota (David *et al*., 2014). Our PCA, PoCA and ANOSIM analysis demonstrated functional and metabolic differences between the groups, resonating with prior studies indicating considerable variability in microbial functions across individuals and groups (Falony *et al*., 2016). Comparatively, some differences between this and other studies could be due to diverse factors such as methodology, host genetics, environmental factors and the specific diet and lifestyle of yaks. Future investigations might provide more context to these results and contribute to a better understanding of the yak microbiota.

The study of antibiotic resistance genes (ARGs) in the microbiome of yaks of different ages bears significant implications for public and veterinary health and for the broader understanding of the resistome - the collective reservoir of antibiotic resistance in microbial communities. It permits the tracking of the development and spread of antibiotic resistance over time and changes in ARG composition can serve as a bellwether for future trends in resistance (O'Toole and Jeffery, 2015; Van Boeckel *et al*., 2015). Furthermore, this research can guide the practice of antibiotic stewardship in veterinary medicine, helping to slow the advent of further resistance (Lhermie *et al*., 2017). we investigating the distribution of antibiotic resistance genes (ARGs) within the microbiome of yaks at various ages, our findings illustrate the dynamic and complex nature of ARGs within microbial communities. In G1, Bacillota accounted for 79% of ARGs, while Bacteroidota and Actinomycetota accounted for 5% and 3%, respectively. The relative abundance analysis results in the present study suggest that there are clear differences in the antibiotic resistance ontology (ARO) among different yak groups. In terms of the top 20 AROs, the relative abundance of vancomycin resistance genes was found to be the highest in all groups, followed by resistance genes for macrolides, tetracycline, oxazolidinones, bacitracin and aminoglycosides. It is interesting to note that the vanW_gene_in_vanI_cluster was found to be highest in the G1 group and lowest in the G4 group. This difference could be influenced by several factors including environmental factors that could influence ARGs (Shenhav *et al.,* 2019). In contrast, the G4 group showed the highest relative abundance of vanY_gene_in_vanB_cluster, suggesting differential antibiotic pressures or environmental exposures for this group. Heat map results and ANOSIM analysis based on Bray-Curtis distance further underline these area-related differences. The clear clustering of different area groups in the heat map reflects distinct differences in their ARG profiles. ANOSIM analysis, a non-parametric test used to compare the similarity of microbial communities, further confirms these findings, showing that the between-group differences are significantly higher than the within-group differences. These results underline the complexity and heterogeneity of ARGs in different groups of yaks and highlight the influence of multiple factors, including age, sex and possibly antibiotic exposure in specific areas (Kim *et al.,* 2011).

The dissemination of antibiotic resistance genes (ARGs) in the environment and among different species is indeed a critical public health concern. Birds, as well as companion animals, can act as vectors for the transmission of ARGs due to their ability to migrate or through close contact with humans and other animals, respectively (Allen *et al*., 2010; Cormier *et al*., 2019). Environmental sources like soil, rivers and sediments serve as reservoirs for ARGs. The horizontal gene transfer, mainly via mobile genetic elements like plasmids, allows ARGs to move between bacteria in these environments. Therefore, animals living in such environments can acquire these ARGs, even in the absence of direct antibiotic exposure (Bengtsson-Palme *et al.,* 2018). Yaks, being grazing animals, continuously interact with the soil ecosystem while foraging. They can thereby ingest soil bacteria carrying ARGs, which then become incorporated into their gut microbiota. Moreover, as yaks drink water from rivers and other freshwater sources, they could be ingesting water-borne bacteria containing ARGs. These environmental influences could explain why the intestinal microbiome of yaks harbors ARGs despite not being directly treated with antibiotics. The finding underscores the importance of holistic, One Health approaches to tackling antibiotic resistance that consider not just medical and agricultural antibiotic use, but also environmental reservoirs of resistance.

The gastrointestinal tract of animals, including yaks, harbors a complex ecosystem of microorganisms known as the gut microbiota. These microorganisms play a crucial role in host health, including nutrient metabolism, immune

system development and protection against pathogens. Understanding the composition and functional potentials of the gut microbiota in yaks is essential for gaining insights into their digestive physiology, overall well-being and performance. A metagenomic sequencing approach was employed to investigate the gut microbial diversity, drug resistance, composition and functional potentials of the gut microbiota. The results from the metagenomic analysis showed a diverse range of microorganisms present in the yak gut microbiota that varied among different yak age groups. The predominant phyla were Bacillota, Bacteroidetes, Proteobacteria and Actinobacteria. The KEGG metabolic pathway prediction shows that amino acid metabolism and carbohydrate metabolism were abundant. The study also revealed an alarming abundance of antibiotic-resistance genes, suggesting that the gut microbiota of yaks could potentially serve as a reservoir for antibiotic resistance, which has significant implications for public health. By applying this approach to fecal samples from yaks of different age groups, this study aimed to unravel the microbial diversity, functional potential and drug resistance in the yak gut microbiome. The identified targets can be used as prebiotics and/or probiotics to improve the overall well-being and production by manipulating the dairy feed with desired microbes (El-Hentati *et al*., 2023; Gangil *et al*., 2021; Maftei *et al*., 2022).

## CONCLUSION

In conclusion, the metagenomic study of yak microbiota revealed bacteria as the most abundant microorganisms across all samples, while unknown microorganisms were also prevalent. Different area groups exhibited varying representation of phyla and genera, suggesting influences of diet, environment and age. Functional analysis highlighted the importance of gut microbiota in host health and metabolism. The study also identified distinct antibiotic resistance gene profiles among age and sex groups. Environmental reservoirs contributed to antibiotic resistance genes even without direct antibiotic exposure. Overall, the study provides valuable insights into the complex nature of the yak microbiome, with implications for understanding host-microbe interactions and managing antibiotic resistance.

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**Conflict of interest**

The researchers affirm that there are no conflicts of interest associated with this study.

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