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RESEARCH ARTICLE

Comparative Analysis of Gut Microbiota Between Healthy and Diarrheic Tibetan Pigs Using 16S rRNA Sequencing

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Abstract

Diarrhea is a common gastrointestinal symptom in Tibetan pigs, generally considered to be related to gut microbiota imbalance. However, studies comparing the gut microbiota of healthy and diarrheal Tibetan pigs are relatively few. This study used high-throughput 16S rRNA gene sequencing to analyze the fecal microbiota of diarrheal Tibetan pigs. Diversity analysis and functional prediction results showed that the microbiota richness of healthy Tibetan pigs was significantly higher than that of diarrheal Tibetan pigs, and the clustering of gut microbiota between the two groups was more obvious. The gut microbiota of healthy Tibetan pigs was mainly composed of *Firmicutes* and *Lactobacillus*, while the abundance of *Proteobacteria* and *Eubacterium* increased in diarrheal Tibetan pigs. In particular, the abundance of *Lactobacillus* was significantly different between the two groups in the Tibetan pig gut, demonstrating the role of probiotics in gut health. In addition, the complexity of the gut microbiota of diarrheal Tibetan pigs was reduced, and the stability of the gut microbiota was disrupted. Metabolic analysis showed significant differences between the different groups: the intestinal fermentation and nitrate reduction pathways were increased in diarrheal Tibetan pigs, while healthy Tibetan pigs showed enhanced amino acid metabolism and a more pronounced anaerobic phenotype. These findings elucidate the structural and functional alterations in the gut microbiota associated with diarrhea in Tibetan pigs, offering new insights for potential interventions.

Keywords: Diarrhea, Gut microbiota, Tibetan pigs, 16S rRNA sequencing

INTRODUCTION

Tibetan pigs are a unique domesticated animal breed native to the Qinghai-Tibet Plateau region of China. They possess a strong adaptability to harsh, cold environments and can survive even in conditions of extreme cold, hypoxia, and feed scarcity [1-3]. Previously, Tibetan pigs were raised through grazing, freely foraging on grasslands. However, increasing research indicates that this method makes them more susceptible to various pathogens (such as bacteria, viruses, and parasites) [4-6]. These pathogens pose a threat to the health of the herd, preventing large-scale farming of Tibetan pigs.

Diarrhea is one of the most common and harmful clinical syndromes in Tibetan pig farming, particularly affecting weaned piglets [7-9]. Its etiology involves multiple interacting factors, including pathogen infection, nutritional imbalance, environmental stress, and host immune status [10,11]. Studies have confirmed that the gut microbiota plays a crucial role in nutrient metabolism, barrier protection, and immune regulation [12-14]. In healthy pigs, dominant beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, maintain intestinal homeostasis by producing short-chain fatty acids (SCFAs), enhancing the mucosal barrier, and competitively inhibiting pathogen colonization [15-18]. However, factors such as weaning stress, dietary changes, or environmental stress can easily disrupt the balance of the gut microbiota [19]. This dysbiosis typically manifests as a reduction in symbiotic beneficial bacteria and an overgrowth of opportunistic or pathogenic bacteria (e.g., *Escherichia coli, Clostridium* spp., and *Fusobacterium* spp.), leading to diarrhea in animals [20,21]. Bacterial pathogens, the primary causative agents of diarrhea in Tibetan pigs, include enterotoxigenic *Escherichia coli* (ETEC), *Salmonella* spp., and *Shigella* spp. ETEC frequently causes

watery diarrhea and severe dehydration in newborn piglets [22], while *Salmonella* infection can lead to acute enteritis and systemic disease with a high mortality rate [23].

Studies have found that changes in gut microbiota structure are closely related to the occurrence of diarrhea in hosts [24,25]. For example, diarrheal piglets typically exhibit reduced microbial diversity and loss of functional groups associated with carbohydrate fermentation and short-chain fatty acid production. Furthermore, early stressors such as group housing, low temperatures, and sudden changes in diet exacerbate these microbiota alterations, impairing intestinal barrier function and triggering inflammatory responses. With advancements in high-throughput sequencing technology, microbial community analysis using 16S rRNA gene sequencing has become an important method for exploring gut microbiota and its disease-related microbial changes. Despite progress in related research, studies on local pig breeds raised under unique ecological conditions (such as Tibetan pigs endemic to the Qinghai-Tibet Plateau) remain limited. Tibetan pigs are frequently subjected to factors such as cold stress, hypoxia, and dietary changes, which may affect the composition and resilience of their gut microbiota. Elucidating the differences in gut microbiota between diarrheal and healthy Tibetan pigs is crucial for revealing the microbial mechanisms of diarrhea in this unique genetic and environmental context.

This study analyzed the composition and diversity of the gut microbiota in diarrheal Tibetan pigs using high-throughput 16S rRNA gene sequencing technology. By comparing the gut microbiota of individuals with diarrhea and healthy individuals, we can identify key bacterial communities that may be associated with the occurrence of diarrhea, as well as the dominant bacterial species in the gut environment of healthy individuals. This reveals the microbial mechanisms of diarrhea in Tibetan pigs and the protective role of the microbial community in the gut environment, thus providing a basis for developing effective diagnostic and preventive strategies.

Material and Methods

Ethical Approval

All animal experiments were reviewed and approved by the Animal Ethics Committee of South China Agricultural University (approval number: 2024A637), and conducted in accordance with the institutional guidelines for the care and use of laboratory animals.

Animal Management and Clinical History

All Tibetan pigs were housed under standard commercial farming conditions. The animals were kept in naturally ventilated pens with a relatively constant temperature (20-25°C) and natural light cycles. All pigs had ad libitum access to a standard commercial basal diet and clean drinking water throughout the study period. Crucially, regarding their clinical history and medication, none of the selected Tibetan pigs (both healthy and diarrheal groups) had received any antibiotic therapies, probiotic supplements, or vaccinations for at least one month prior to fecal sample collection. This strict selection criterion was implemented to ensure that the baseline gut microbiota composition was not artificially perturbed by external pharmacological or immunological interventions.

Experimental Animals and Sample Collection

This study collected fecal samples from six healthy Tibetan pigs (healthy group, C: C1-C6) and six clinically diagnosed Tibetan pigs exhibiting diarrhea symptoms (diarrhea group, F: F1-F6) at a commercial Tibetan pig farm in Linzhi, Xizang, Tibet, China. All samples were immediately frozen and transported to the laboratory under cold chain conditions to prevent degradation.

To minimize environmental contamination and maintain the integrity of microbial DNA, all sample processing was performed under sterile conditions. The core portion of each fecal sample was aseptically collected using sterile swabs to avoid contact with potentially oxidized or contaminated surface materials. Approximately 0.5-2 g of material from each sample was transferred to sterile cryopreservation tubes and aliquoted into 2-3 equal portions for later use. Processed samples were immediately flash-frozen in liquid nitrogen and subsequently transported via cold chain logistics to a commercial sequencing provider for further analysis.

DNA Extraction, 16S rRNA Gene Sequencing, and Bioinformatics Analysis

Microbial DNA extraction, PCR amplification of the V3-V4 hypervariable region of the bacterial 16S rRNA gene, library preparation, high-throughput sequencing, and downstream data analysis were performed by Beijing Tsingke Biotechnology Co., Ltd. (Guangzhou, China). Sequencing utilized the Illumina NovaSeq 6000 platform with a paired-end strategy (2 × 250 bp).

Raw reads underwent demultiplexing, quality filtering, merging, and denoising using the DADA2 algorithm integrated in QIIME2 version 2020.6 to generate high-quality amplicon sequence variants (ASVs). Chimeric sequences were removed during this process. Representative sequences were classified and annotated using a pre-trained classifier trained against the SILVA database.

Diversity analysis was conducted as follows: α-diversity metrics (Chao1, Shannon, Simpson, etc.) were compared using biological t-tests. β-diversity (Bray-Curtis dissimilarity) was assessed via principal coordinate analysis (PCoA),

non-metric multidimensional scaling (NMDS), and UPGMA clustering, with statistical testing performed using PERMANOVA and ANOSIM.

Additionally, LEfSe, STAMP, and Metastats were employed to identify differentially abundant taxa. PICRUSt2, BugBase, and FAPROTAX were utilized to infer microbial metabolic functions, phenotypes, and ecological roles. In all comparative analyses, P-values <0.05 were considered statistically significant.

Results

Sequencing Quality and ASV Statistics

16S rRNA gene sequencing was performed using the Illumina NovaSeq platform with a paired-end sequencing strategy. In total, 930.725 raw reads were generated across the 12 samples. After quality filtering, noise reduction, chimera removal, and sequence merging using the DADA2 plugin in QIIME2 (version 2020.6), a total of 871.049 clean reads were retained, ranging from 47.685 to 75.511 reads per sample. The final results showed that 4.683 non-chimeric amplicon sequence variants (ASVs) were identified in the samples *(Fig. 1-a)*. The number of ASVs per sample ranged from 352 to 938, and the average number of ASVs in diarrheal samples was noticeably lower than in healthy samples.

Prior to the calculation of alpha and beta diversity metrics, the ASV table was rarefied to an even depth of 47.685 reads per sample (based on the sample with the minimum number of clean sequences) to correct for differences in sequencing depth. Furthermore, the dilution curves at both the sample and population levels eventually approached saturation after a certain fold of dilution, indicating that the sequencing depth was sufficient for assessing microbial diversity *(Fig. 1-c, d)*. Venn diagram analysis showed that the healthy group (C) and the diarrheal group (F) shared 480 ASVs, while the healthy group and the diarrheal group had 2.700 and 1.503 unique ASVs, respectively *(Fig. 1-b)*. This indicates that diarrhea strongly alters the gut microbiota composition, resulting in a pronounced reduction in the gut microbiota of diarrheal Tibetan pigs. Simultaneously, similar to the previous results, the species richness in the diarrhea group was consistently lower than that in the healthy group, suggesting that the gut microbiota of diarrheal Tibetan pigs was more impoverished and unstable.

Alpha Diversity Analysis

Based on the ASV abundance matrix, we calculated four indices for α-diversity: ACE, Chao1, Shannon, and Simpson. The results showed that the ACE and Chao1 indices of the gut microbiota in diarrheal pigs were significantly lower than those in healthy pigs *(Fig. 2-a, b)*. The Shannon and Simpson indices also showed a decreasing trend in diversity in the diarrheal group *(Fig. 2-c, d)*, indicating that after diarrhea symptoms appeared in Tibetan pigs, community complexity and evenness decreased, and the abundance of related microorganisms significantly declined. The Shannon abundance curve further supported these findings, showing a lower abundance level in the diarrheal group compared to the healthy group *(Fig. 2-e)*, clearly demonstrating the reduced gut microbiota diversity under diarrheal conditions. The

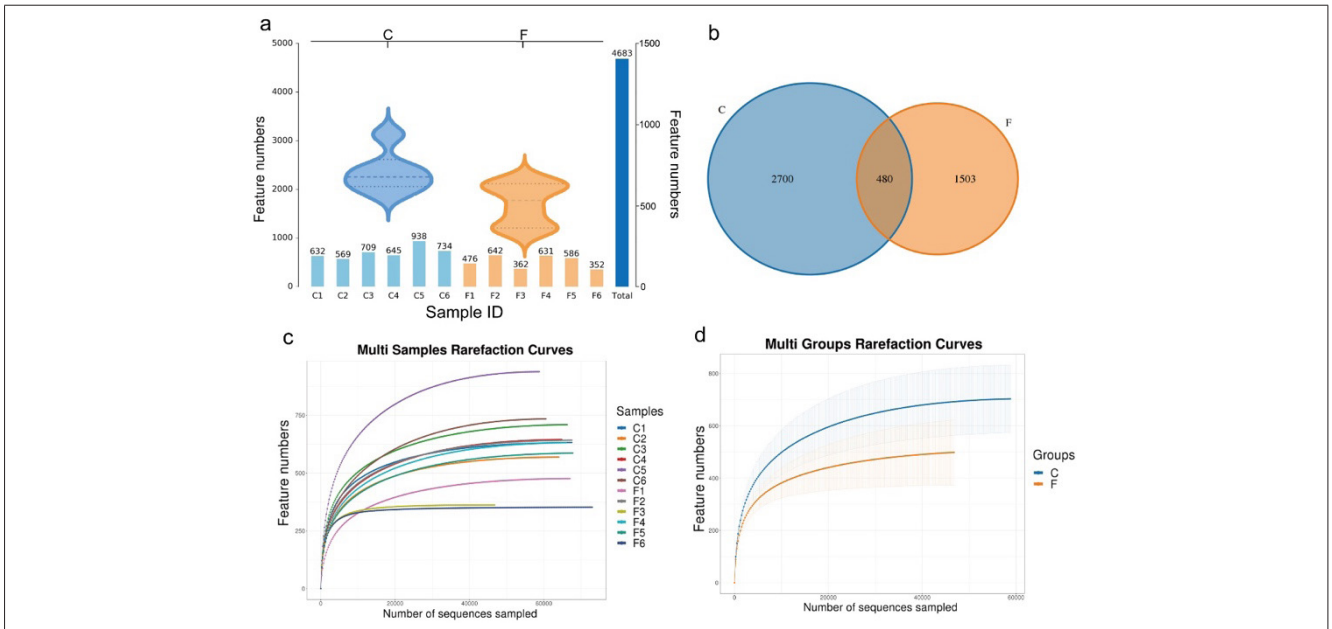

Fig 1. Sequence data quality assessment and diversity saturation analysis. **a)** Bar charts display the number of observed features (ASVs) per sample, with violin plots illustrating distributions across both datasets, **b)** Venn diagram. **c)** Dilution curves for individual samples. **d)** Sparsity curves for group samples

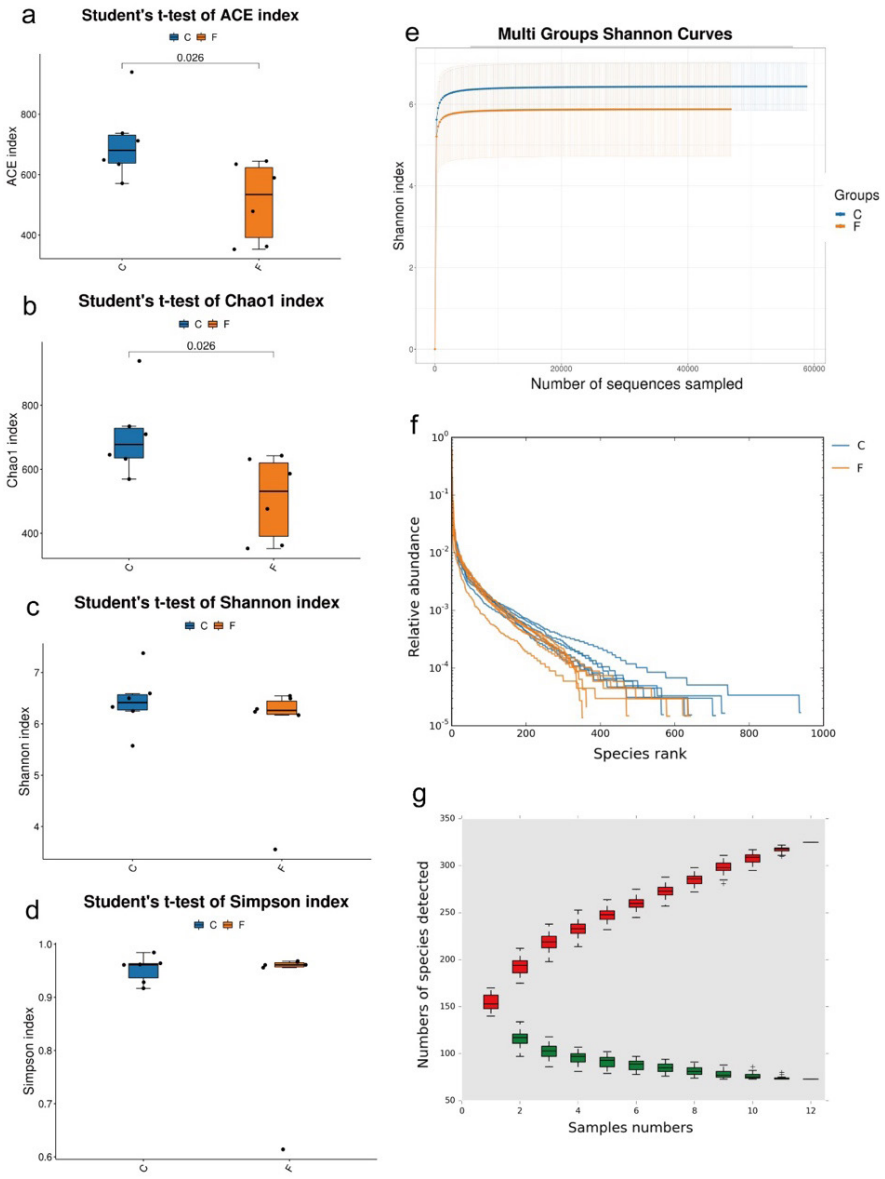

Fig 2. Analysis of gut microbiota α diversity. **a, b, c, d**) Box plots of α diversity indices between groups, including: ACE index, Chao1 index, Shannon index, Simpson index, **e)** Shannon rarity curve, **f)** Abundance-richness curve. **g)** Shared species accumulation curve

abundance ranking curves also illustrate this: the curve for the healthy group was wider and flatter, indicating higher species richness and more even distribution; while the curve for the diarrheal group was steeper, reflecting the absence of some species *(Fig. 2-f)*.

Furthermore, the shared species accumulation curve *(Fig. 2-g)* showed a rapid upward trend, indicating that the current sample size was sufficiently large and that most microbial taxa were successfully detected.

Beta Diversity Analysis

Beta diversity analysis was performed based on Bray-Curtis similarity. The results of principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) are shown in *Fig. 3-a, b, c*. The first two principal coordinates of PCA explained 72.60% and 10.71% of the variance, respectively, while PCoA explained 25.75% and 23.08% of the variance, respectively. A clear separation trend was observed between the healthy and diarrheal groups in the ordination plots, indicating a distinct structural shift in the gut microbiota composition associated with diarrhea. To rigorously evaluate this divergence, PERMANOVA and ANOSIM were conducted. PERMANOVA indicated that the disease status explained 17.5% of the variation in microbiota composition (R^2=0.175, P=0.023]). Consistent with the visual separation, the ANOSIM test supported a strong trend of community divergence (R=0.152, P=0.069). These results further suggest a strong correlation between diarrheal status and altered gut microbiota composition

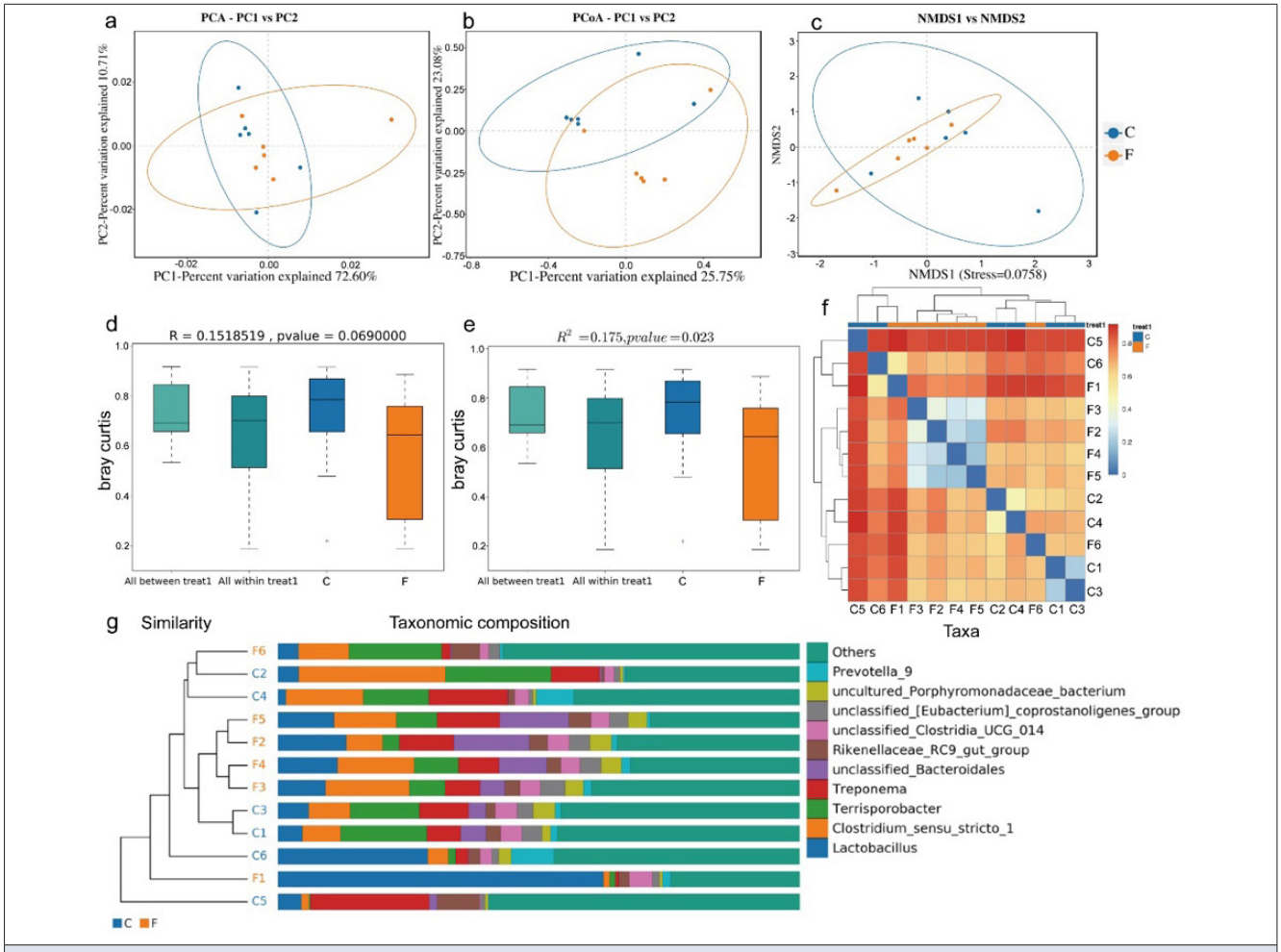

Fig 3. β-diversity analysis of the gut microbiota. **a, b, c)** Ordination plots of Bray-Curtis distances. Including: Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA), Non-metric Multidimensional Scaling (NMDS), **d)** ANOSIM results, **e)** PERMANOVA analysis, **f)** Heatmap of Bray-Curtis distances among all samples, **g)** UPGMA clustering tree and genus-level bar chart

in Tibetan pigs, albeit with some inter-individual variations.

A heatmap based on Bray-Curtis distance further confirmed this difference in microbiota composition, with most samples clustering according to group *(Fig. 3-f)*. Genus-level UPGMA clustering analysis also revealed different microbiota patterns within specific groups, with samples from the same cohort tending to cluster together *(Fig. 3-g)*. This indicates that the changes in the gut microbiota caused by diarrhea are quite similar, with a more significant impact on specific bacterial groups.

Taxonomic Composition and Relative Abundance Analysis

Further hierarchical clustering analysis based on the heatmap of the top 50 genera *(Fig. 4-a)* revealed significant differences in the gut microbiota between healthy and diarrheal pigs. Samples from the healthy group clustered together, rich in various beneficial or symbiotic bacteria, such as *Lactobacillus, Bacteroides,* and the [*Eubacterium*] *eligens* group; while samples from the diarrheal group clustered together, rich in the *NK4A214* group, *Colidextribacter,* and other unclassified bacteria that may be associated with dysbiosis or inflammation.

To further investigate the taxonomic composition of the Tibetan pig gut microbiota, we performed visualization analysis of the microbial community structure at the phylum and genus levels for both the healthy and diarrheal groups. At the phylum level *(Fig. 4-b)*, the dominant phyla in all samples were *Firmicutes, Bacteroidota,* and *Spirochaetota*. Notably, *Firmicutes* were dominant in the healthy group, while the relative abundance of *Spirochaetota* decreased in the diarrheal group, suggesting that diarrhea symptoms may be related to changes in the gut microbiota structure.

At the genus level *(Fig. 4-c)*, the composition of the gut microbiota became more diverse. The healthy group showed a relatively even distribution of microbial species, with *Terrisporobacter mayombei* and *Clostridium disporicum* being particularly abundant. In contrast, the diarrheal group showed an increased proportion of *Lactobacillus*

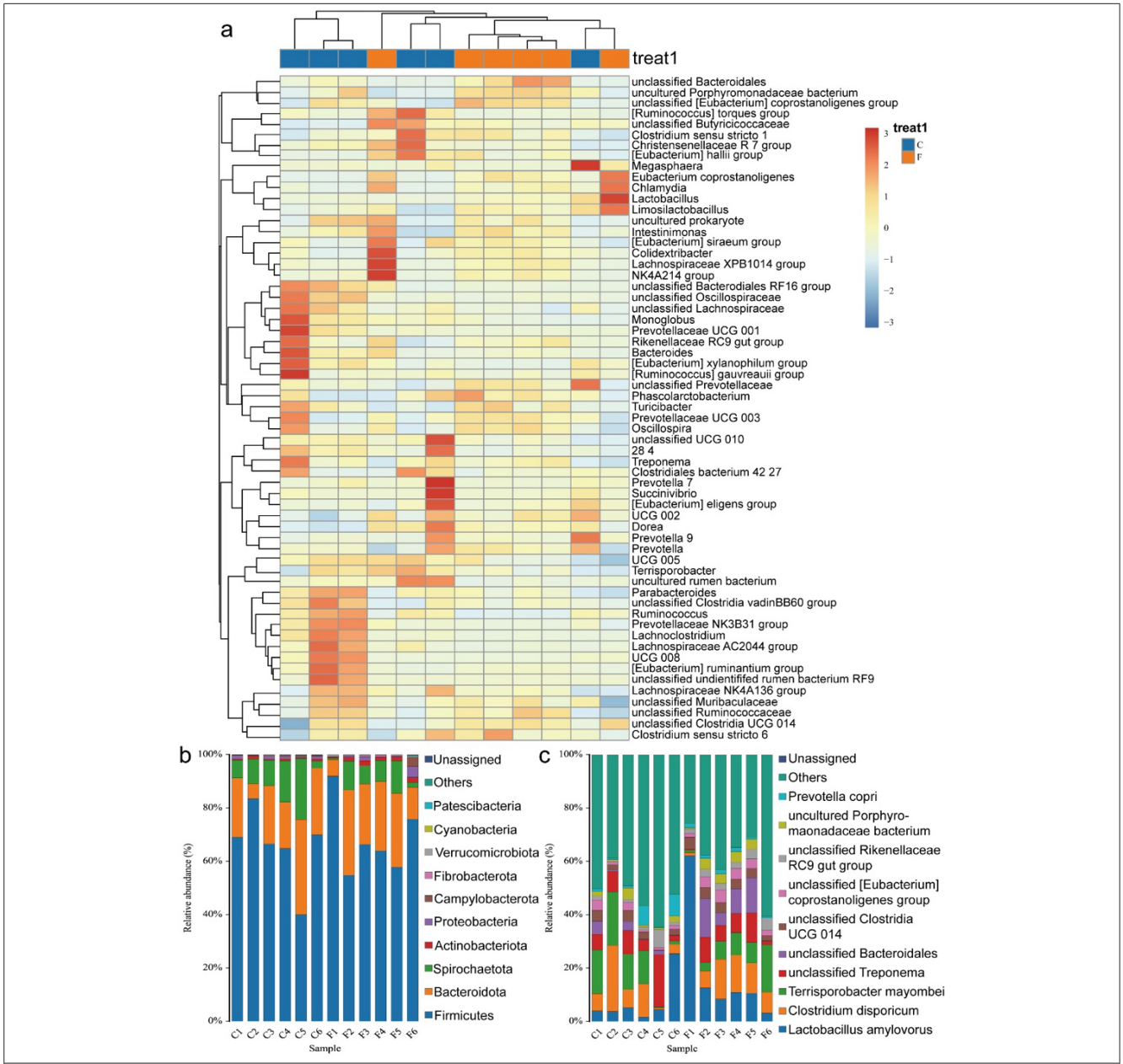

Fig 4. Taxonomic composition and heatmap analysis of the gut microbiota. **a)** Hierarchical clustering heatmap of genus-level abundance based on Z-score standardization, **b)** Relative abundance composition at the bacterial phylum level, **c)** Relative abundance composition at the bacterial genus level

amylovorus, uncultured *Porphyromonadaceae,* unclassified *Eubacterium coprostanoligenes* group, and unclassified *Bacteroidales*. These changes in the microbiota may reflect changes in the dominant bacterial species and their resistance to pathogenicity during diarrhea in Tibetan pigs.

Furthermore, a circular phylogenetic tree was constructed to explore the phylogenetic relationships of the dominant groups in the Tibetan pig gut microbiota *(Fig. 5)*. The results showed different clustering patterns between the healthy and diarrheal groups, with significant segregation of dominant bacterial groups. In the healthy group, members of the phylum *Firmicutes* (yellow portion), especially *Lactobacillus* and *Clostridium*, were significantly enriched and showed close phylogenetic relationships. Conversely, the abundance of *Bacteroides* was significantly increased in the diarrhea group, including *Treponema* and the *NK4A214* group. These bacteria are located on different branches and are phylogenetically distant from the core symbiotic flora of the healthy group.

These results indicate that the composition of the gut microbiota in Tibetan pigs undergoes significant changes after diarrhea occurs. These changes suggest that the cause of diarrhea in Tibetan pigs is a reduction or disappearance of beneficial microorganisms and an increase or appearance of potentially pathogenic or opportunistic taxa.

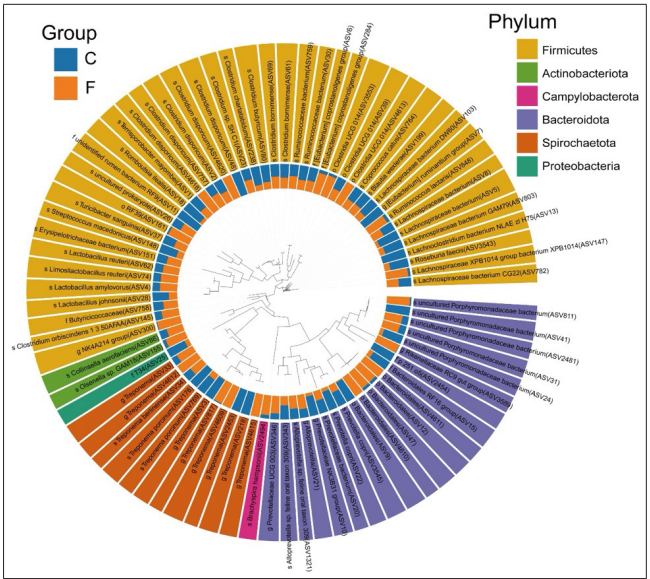

Fig 5. Phylogenetic tree and taxonomic distribution of dominant bacterial communities. The inner circle indicates the relative abundance of corresponding genera across samples. The outer circle displays phylum-level taxonomy, with branches colour-coded. Blue and orange distributions within each segment of the inner circle represent Groups C and F respectively

Abundance Difference Analysis Between Healthy and Diarrhea Groups

To further identify the key taxa that differentiate between diarrheal and healthy Tibetan pigs, species-level abundance difference analyses were performed using LEfSe, Metastats, and univariate statistical methods *(Fig. 6)*. LEfSe analysis *(Fig. 6-a, b)* showed that members of the *Lachnospiraceae* and *Lactobacillus* families were significantly enriched in the healthy group. Conversely, the *Eubacterium coprostanoligenes* group was significantly enriched in the diarrheal group, with a linear discriminant analysis (LDA) score exceeding 4.0, indicating a greater degree of difference. Relative abundance histograms further illustrated these differences. *Lactobacillus* consistently showed high abundance in healthy samples, with significantly increased proportions of *Lachnoclostridium* and *Ruminococcus,* suggesting a potential role in preventing diarrhea-related dysbiosis. In contrast, unclassified *RF39* and *Eubacterium coprostanoligenes* were significantly increased in diarrheal samples *(Fig. 6-c, d, e)*, and the occurrence of intestinal inflammation is likely related to this dysbiosis.

Transposon analysis *(Fig. 6-f)* confirmed the above results, revealing statistically significant differences in the bacterial communities of multiple genera between the two groups (P<0.05). Box plots *(Fig. 6-g)* further illustrated the different distribution patterns of key taxa. Notably, in the healthy group, several species, including *Terrisporobacter*, *Treponema*, and *Lachnoclostridium,* showed significant increases, suggesting that these bacteria play an important role in maintaining the health of Tibetan pigs. In contrast, the increased taxa such as *Subdoligranulum* in the diarrhea group may indicate inflammatory damage to the intestines, leading to diarrhea symptoms in Tibetan pigs.

Co-occurrence Network and Microbial Interaction Analysis

To investigate the ecological interactions among the gut microbiota of Tibetan pigs, co-occurrence network analysis was performed based on Spearman's rank correlation coefficient, yielding the network diagram *(Fig. 7-a)*: a complex interaction network with 46 nodes and multiple edges was constructed, indicating the complexity and diversity of the microbial structure in the gut micro-ecosystem. Most correlations were positive (red lines), suggesting synergistic effects among most bacterial groups; while a few negative correlations (green lines) indicated potential competition. Bacteria belonging to the phyla *Firmicutes* and *Bacteroidota* were located in the central region of the network and had high node degrees, indicating their important role in stabilizing the gut micro-ecological environment as major gut microbes. Clearly, *Lactobacillus* (node 33), *Terrisporobacter* (node 35), and unclassified *Bacteroidales* (node 36) had the highest connectivity, suggesting they may be key species in the gut micro-ecological environment of Tibetan pigs.

Further ecological analysis using Zi-Pi topology analysis confirmed the ecological roles of these groups *(Fig. 7-b)*. The results showed that most groups were classified as marginal groups, i.e., Zi <2.4 and Pi <0.62, indicating that the role of most gut microbiota communities was limited to their respective low-connectivity groups. However, a node located in the peripheral region, with Pi >0.62, was marked in red, indicating that this group played a unique role in the connectivity network.

Functional Prediction of Microbial Communities

Metagenomic maps were inferred from 16S rRNA data using PICRUSt2, and pathway annotation was performed using the KEGG, BugBase, and FAPROTAX databases to investigate the functional potential of the gut microbiota. The results are shown in *Fig. 8*. KEGG pathway prediction results showed a significant difference in microbial metabolic capacity between the healthy and diarrheal groups *(Fig. 8-a)*. Specifically, the healthy group showed significant enrichment in pathways such as global and overview maps, amino acid metabolism, and metabolism of cofactors and vitamins, indicating that their gut microbiota is better suited to regulating overall metabolism and nutrient transformation. In contrast, the diarrheal group showed upregulated expression in pathways such as membrane transport, translation, nucleotide metabolism, and replication and repair. This result demonstrates the adaptive response of the Tibetan

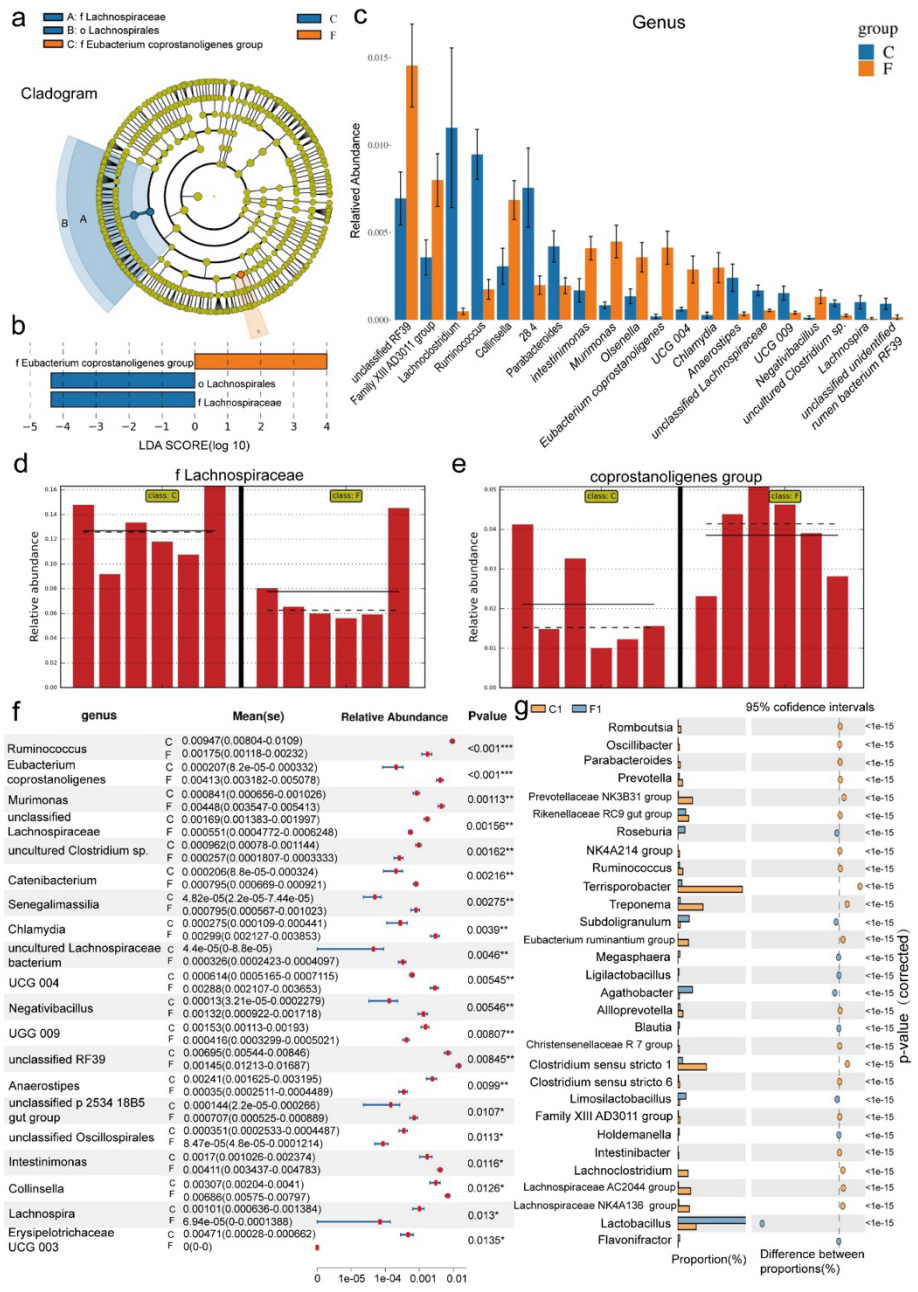

Fig 6. Analysis of differential bacterial genus abundance. **a)** LEfSe analysis results and corresponding phylogenetic tree, **b)** Histogram of LDA scores (log10) for taxonomic units with significant intergroup abundance differences, **c)** Relative abundance bar chart of the top 20 genera with significant differences between groups C and F, **d, e)** Inter-group relative abundance comparisons for *Lactobacillus* (d) and *Coprosterolus* (e), **f)** Metastats analysis results, **g)** STAMP analysis

pig's gut microbiota to intestinal environmental dysbiosis and inflammation after the onset of diarrheal symptoms. Furthermore, the diarrheal group showed upregulated abundance of functional genes related to carbohydrate metabolism, suggesting significant changes in the intestinal environment and that the microbiota influenced changes in carbohydrate metabolism. Compositional and functional analysis of the dominant phyla indicated that these changes primarily originated from *Acidobaceriota*, *Actinobacteriota*, and *Bacteroidota* (*Fig. 8-b*).

FAPROTAX functional annotation further confirmed these findings. Fermentation and chemoheterophy-related processes were significantly enhanced in the diarrhea group. In contrast, the healthy group exhibited more stable functions, but showed stronger expression in xylanolysis-related processes (*Fig. 8-c*). BugBase phenotypic prediction revealed an increase in aerobic and gram-negative bacteria in the diarrhea group, particularly potentially pathogenic bacteria, indicating a significant increase. This suggests that the hypoxic environment of the gut alters after diarrhea, affecting gut microbiota

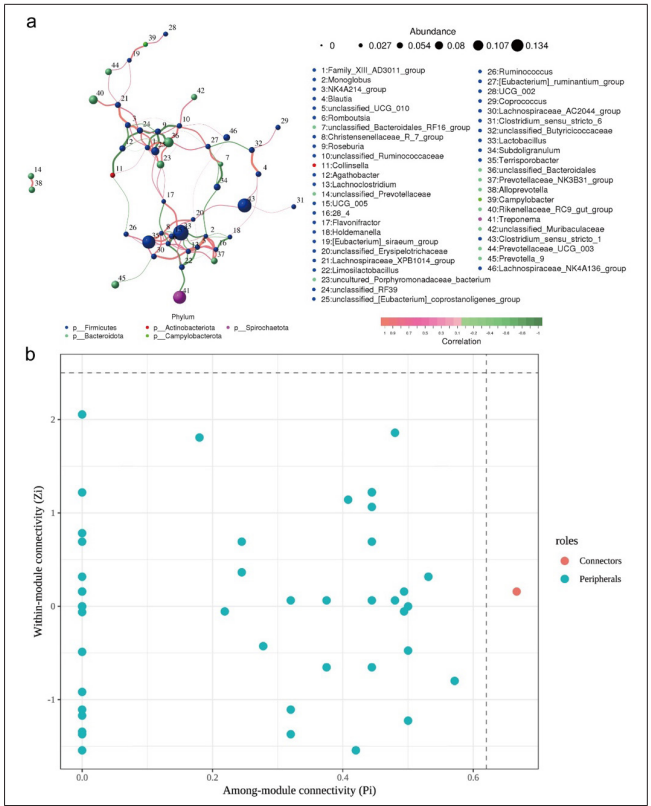

Fig 7. Co-occurrence network analysis and topological role classification of gut microbial species. **a)** Microbial co-occurrence network derived from Spearman's correlation analysis. Nodes represent microbial genera, with size indicating average abundance. Edges denote significant correlations ($|\rho| > 0.6$, $P < 0.05$), **b)** Z-P plot classifying topological roles based on intra-module connectivity (Zi) and inter-module connectivity (Pi). Nodes are categorised as connectors (high Pi, red) and peripheral nodes (low Pi, blue)

metabolism. Conversely, the healthy group showed a higher abundance of anaerobic bacteria, which can indicate the homeostatic balance of the gut microbiota *(Fig. 8-d, e)*.

Discussion

This study used 16S rRNA gene sequencing technology to comprehensively characterize the changes in the gut microbiota of healthy and diarrheal Tibetan pigs. The results showed significant differences in the composition, diversity, and functional potential of the gut microbiota between the two groups, indicating that diarrhea significantly remodels the gut microbiota. These findings are consistent with previous reports on the association between intestinal diseases and dysbiosis in pigs and other mammals [26-30].

Analysis of α- and β-diversity of the gut microbiota in Tibetan pigs revealed a significant decrease in the number of gut microbiota communities and a decreasing trend in community complexity, which were significantly different from healthy pigs. Decreased α-diversity is typically associated with gut ecosystem dysbiosis, decreased resistance to beneficial bacteria colonization, and increased susceptibility to pathogens [26,31]. While previous studies suggest external factors may perturb the microenvironment and trigger dysbiosis [32], it is highly plausible that this relationship is bidirectional. The rapid intestinal transit and excessive fluid secretion during diarrheal episodes can mechanically flush out commensal bacteria, further driving the loss of diversity. Compared to the diarrhea group, the healthy group exhibited a richer and more homogeneous gut microbiota composition, likely due to its larger and more stable population, stronger resilience to external factors, and greater capacity to repair the effects of external factors on the gut [33].

Taxonomic analysis showed that both groups were dominated by *Firmicutes* and *Bacteroidetes,* consistent with previous findings on porcine gut microbiota [34-36]. However, *Firmicutes* abundance was higher in the healthy group, while *Spirochetes* abundance was lower in the diarrhea group. At the genus level, *Terrisporobacter mayombei* and other bacteria were dominant in the healthy group, while *Porphyromonadaceae* and *Bacteroidales* showed significant aggregation in the gut of diarrheal pigs. Interestingly, *Lactobacillus amylovorus* also showed an unexpected increasing trend in diarrheal pigs. Rather than a simple "compensatory increase", this proliferation can be explained by its strong amylolytic capacity. During diarrhea, the impaired absorptive function of the small intestine often allows undigested carbohydrates, such as starches, to reach the hindgut. This nutrient influx provides an ideal substrate for *L. amylovorus*, driving its opportunistic overgrowth. Consequently, excessive fermentation by this species can lead to the accumulation of lactic acid, potentially causing hindgut acidosis and exacerbating osmotic diarrhea [37,38]. These changes in gut microbiota may suggest that diarrhea is related to the proliferation of harmful bacteria that cause inflammation or the reduction of beneficial bacteria such as *Lactobacillus* that can maintain gut microbiota balance. This may lead to damage to intestinal epithelial tissue and immune system dysregulation [39,40], resulting in the invasion and colonization of opportunistic pathogens. Therefore, specific gut microbiota, especially *Lactobacillus* and related bacteria, can serve as biomarkers of gut health in Tibetan pigs, and the increase of *Subdoligranulum* and related bacteria may indicate the occurrence of inflammation, possibly reflecting gut microbiota dysregulation associated with diarrhea [41]. Co-occurrence network analysis supports this explanation. Some important nodes appeared in the microbial network, with closer connections and more obvious interactions with other microbiota. Among them, *Lactobacillus*, as mentioned above, had the highest connectivity in the shared network, indicating that it is a key species in the gut of Tibetan pigs. This species may serve as an indicator of gut health and homeostasis in Tibetan pigs, and it may also exchange metabolites with other

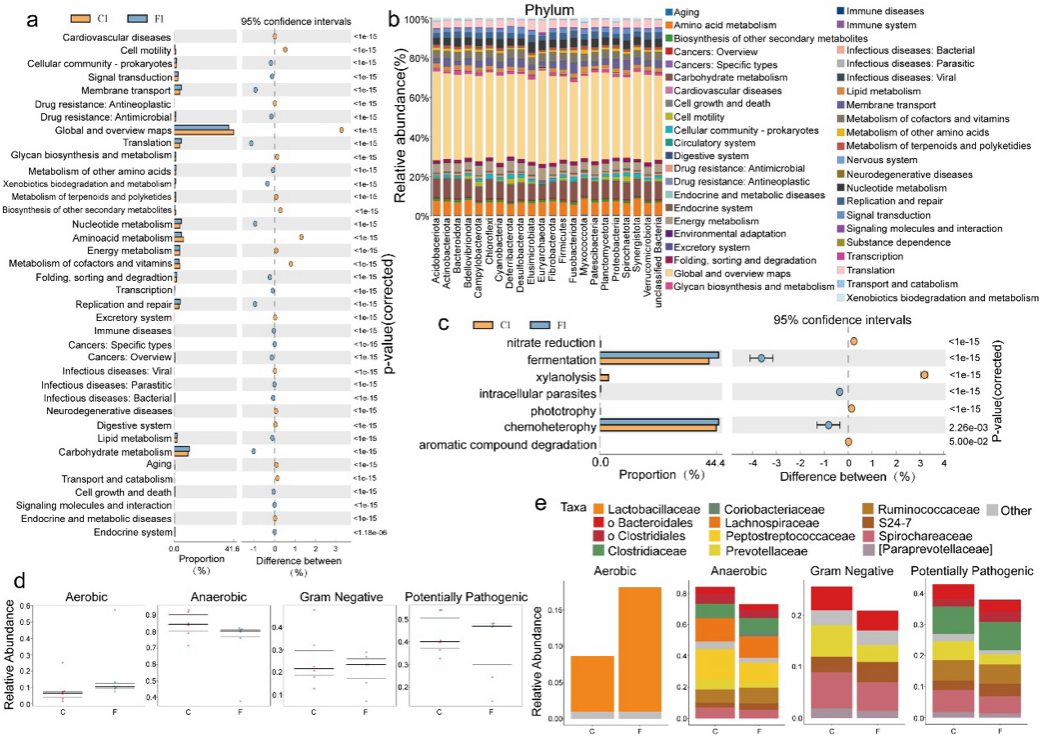

Fig 8. Gut microbiota functional prediction by PICRUSt2 and FAPROTAX. **a)** Differences in abundance of PICRUSt2-predicted KEGG level-2 functional pathways, **b)** PICRUSt2 KEGG level-2 functional composition profile, **c)** FAPROTAX-predicted functional features, **d)** BugBase predictions, **e)** Comparison of BugBase-predicted microbial phenotypes across samples using bar charts and differential plots

microbial communities in the gut, potentially reflecting the synergistic effect of *Lactobacillus* in the Tibetan pig gut and its resistance to external interference[26].

Differential abundance analysis using LEfSe and Metastats further identified several key taxa associated with specific groups. We found that the enrichment of *Lachnospiracea, Lactobacillus,* and *Ruminococcus* in healthy pigs indicated a functional protective role of the microbiome [42-44], while the significantly increased *Eubacterium coprostanoligenes* in the gut of diarrheal Tibetan pigs suggested a close relationship with diarrhea. Notably, *Eubacterium coprostanoligenes* has been found to be associated with lipid metabolism and pro-inflammatory states [45,46], and other elevated proportions of certain microbial communities in the gut of diarrheal Tibetan pigs have also been associated with intestinal barrier dysfunction and intestinal inflammation [47]. These results suggest that key taxa, such as *Lactobacillus,* may serve as microbial biomarkers for the intestinal health or disease progression of Tibetan pigs in future research.

Functional predictions further confirmed the biological significance of these taxonomic changes. Pathway enrichment in diarrheal Tibetan pigs was associated with membrane transport, translation, nucleotide metabolism, and replication and repair processes, indicating that Tibetan pigs have begun to develop repair mechanisms to combat the disordered intestinal environment under diarrheal conditions [48,49]. Furthermore, the fermentation potential in the intestines of diarrheal Tibetan pigs was also increased, possibly related to a significant increase in fermentative microbiota. This enhanced fermentation capacity may lead to excessive production of organic acids or gases, thereby exacerbating diarrhea symptoms [50]. In contrast, healthy Tibetan pigs exhibited enhanced pathways related to amino acid and vitamin metabolism, suggesting that the gut microbiota of healthy Tibetan pigs plays an indispensable role in maintaining intestinal microecological homeostasis [40]. BugBase predictions highlighted an increase in Gram-negative bacteria and potentially pathogenic bacterial phenotypes in diarrheal pigs, which may exacerbate fluctuations in the intestinal microbiota, potentially exacerbating intestinal inflammation and promoting diarrhea [51]. In summary, these results indicate that diarrhea in Tibetan pigs is characterized not only by altered gut microbiota composition but also by significant metabolic and phenotypic changes. These alterations suggest an intricate interplay where dysbiosis and inflammation may mutually exacerbate diarrheal symptoms. Identified functional pathways, particularly those involved in membrane transport, fermentation, and processes related to potential pathogens, may serve as key indicators or therapeutic targets for managing gut microbiota dysbiosis in high-altitude local pig breeds.

In summary, the findings indicate multifaceted dysbiosis in the gut microbiota of diarrhea-prone Tibetan pigs, characterized by reduced ecological diversity and stability, decreased probiotic counts, increased potential pathogenic bacteria, and a shift towards intestinal barrier disruption and inflammatory damage. While such changes have been observed in other species [52,53], Tibetan pigs living at high altitudes require further investigation due to their unique environmental adaptability and disease resistance. Importantly, given the cross-sectional design of this study, we cannot definitively establish a causal direction between gut dysbiosis and diarrhea. It is highly likely that the observed microbial alterations are, at least in part, a consequence of the diarrheal environment (e.g., increased gut motility and luminal wash-out) rather than the sole primary cause. Therefore, longitudinal studies or fecal microbiota transplantation (FMT) models are needed to track microbial succession and determine causality. Additionally, the specific duration and severity of diarrhea prior to sampling were not incorporated as continuous variables in the current evaluation criteria, which limits our understanding of the temporal dynamics of dysbiosis during disease progression. Furthermore, the lack of measurement of host physiological parameters limits our ability to investigate the mechanisms of host-microbiota interactions. Finally, the limited capabilities of 16S rRNA sequencing suggest that functional inferences may require further validation through metabolomics analysis. Despite these limitations, this study comprehensively explores the associations between gut microbiota changes and diarrhea in Tibetan pigs, focusing on candidate biomarkers and therapeutic targets. These findings lay the foundation for future microbial therapies such as probiotic supplementation or fecal microbiota transplantation, which could help reduce the incidence of digestive diseases in high-altitude livestock farming and thus improve animal health.

This study employed 16S rRNA gene sequencing to comprehensively characterize the gut microbiota of healthy and diarrheal Tibetan pigs. Diarrhea was associated with significantly reduced microbial richness and diversity, altered community structure, and an increased abundance of potentially pathogenic and pro-inflammatory bacteria, particularly *Eubacterium coprostanoligenes*. In contrast, healthy pigs harbored a higher proportion of beneficial genera such as *Lactobacillus*, which contribute to gut microbiota balance and intestinal barrier integrity. Functionally, the diarrheal microbiota exhibited enhanced fermentation capacity and elevated pathogenicity potential. These findings demonstrate that gut microbiota dysbiosis plays a critical role in the pathogenesis of diarrhea in Tibetan pigs and identify potential microbial biomarkers for diagnosis and intervention. This study provides a foundation for developing microbiome-based strategies to improve gut health in Tibetan pigs, particularly in high-altitude pastoral environments.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding authors (ZH, LY) on reasonable request.

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Conflict of Interest: The authors have no relevant financial or non-financial interests to disclose.

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