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Original Article

Duodenal intestinal flora diversity of Tibetan pigs is lower than that of York pigs based on 16S rRNA analysis

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Abstract

Tibetan pigs exhibit remarkable characteristics, particularly their heightened tendency for fat deposition and increased resistance to diseases in comparison to Yorkshire pigs. Surprisingly, there has been a noticeable scarcity of research delving into the underlying mechanisms responsible for these advantageous traits, especially from the perspective of intestinal microorganisms, particularly those inhabiting the duodenum. To bridge this research gap, the study harnessed 16S rRNA sequencing to conduct a comprehensive examination of the duodenal microbiota in Tibetan and Yorkshire pigs. The results stemming from amplicon sequencing of duodenal contents unveiled that at the phylum level, Firmicutes dominated the duodenal microbiota in both Tibetan and Yorkshire pigs. Intriguingly, Tibetan pigs showcased a significantly reduced proportion of Bacteroidetes in comparison to Yorkshire pigs ($P < 0.05$), leading to a substantially higher Firmicutes to Bacteroidetes ratio in Tibetan pigs (55.95) as opposed to Yorkshire pigs (3.86) ($P < 0.05$). Of remarkable note, at the genus level, Tibetan pigs displayed a significantly elevated relative abundance of Lactobacillus spp. when compared to Yorkshire pigs ($P < 0.01$). Functional predictions pertaining to the duodenal microbiota in both pig breeds primarily revolved around amino acid metabolism, cofactor and vitamin metabolism, terpene and polyketide metabolism, amino acid derivative metabolism, and lipid metabolism. This study underscores the intricate and interdependent relationship between the composition and abundance of the duodenal microbiota and the unique characteristics of fat deposition and disease resistance in Tibetan pigs. It suggests Lactobacillus spp. as significant contributors to fat deposition, the development of the duodenal intestinal barrier, and immune function. Additionally, the Firmicutes-to-Bacteroidetes ratio appears to be associated with fat deposition. These findings provide valuable insights that can serve as a valuable reference for future endeavors related to the development and utilization of Tibetan pigs.

Keywords: Tibetan pigs, Duodenum, Intestinal microorganisms, Fat deposition, Disease resistance

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Introduction

The study of gut microorganisms has made significant strides thanks to advancements in sequencing technology (Langille et al., 2013). The intestine, being the largest digestive organ in both humans and animals, plays a crucial role in the digestion and absorption of nutrients, particularly in the duodenum. Its intricate structure, characterized by numerous folds and villi, provides an extensive surface area, fostering efficient absorption. Additionally, the intestine harbors a diverse population of microorganisms that actively contribute to the absorption and utilization of all nutrients (Gao et al., 2000). These gut microorganisms engage in a symbiotic relationship with the host, collectively forming a dynamic ecosystem within the gut that works tirelessly to maintain homeostasis (Ornelas et al., 2022). Modifications in the composition of gut microorganisms have been well-established as having a profound impact on host health. These effects encompass metabolic processes, chronic inflammation, the occurrence of intestinal ulcers, and the development of obesity (Round and Mazmanian, 2009). Symbiotic microorganisms play a pivotal role by generating metabolic substances, such as short-chain fatty acids, that significantly influence host health and the absorption of nutrients within the intestine, ultimately contributing to host obesity (Zhou et al., 2021). Furthermore, it has been documented that the intestinal flora is responsible for secreting a wide array of metabolites that actively promote the development of the intestinal immune system. These microbiota and their metabolic products emerge as critical factors that exert a substantial influence on intestinal immune function, thereby indirectly impacting the progression of various diseases

The Tibetan pig is an indigenous pig breed inhabiting high-altitude plateau regions, and it boasts a remarkable set of attributes honed through long-term natural selection in challenging environments characterized by high altitudes, low oxygen levels, and cold temperatures. These characteristics include outstanding adaptations to low-oxygen conditions, robust disease resistance, tolerance for roughage in their diet, and a heightened propensity for fat deposition. (Ma et al., 2022; Chen et al., 2022). Yorkshire pigs, which belong to a lean breed introduced from outside, are recognized for their rapid growth, exceptional feed digestibility, and a notable propensity for high ketogenic leanness (Duan et al., 2021).

The research conducted by Shang et al. in 2022 has shed light on the significant impact of the microbial composition found in the colon and cecum of Tibetan pigs. This impact extends to both the functioning of the intestinal immune system and the development of the obesity phenotype (Shang et al., 2022). It's worth noting that the establishment of the intestinal flora in pigs begins early in life, reaches maturity at approximately 80 days of age, and stabilizes when the pigs are around six months old. With the intention of validating the composition of duodenal microorganisms and investigating potential correlations between intestinal colonization, fat deposition, and disease resistance, the study conducted an examination of the duodenal intestinal flora in two groups: Tibetan pigs, renowned for their propensity for fat deposition, and lean Yorkshire pigs. Both groups were aged nine months and raised under the same feeding management conditions, despite their differing characteristics. This comprehensive study involved histological analysis of samples and the use of 16S rRNA sequencing to evaluate the diversity and functional attributes of the duodenal microbiota in these two pig breeds. The expectation is that the knowledge gained from this research will provide valuable contributions to future studies in this field.

Material and Methods

Samples collection

The materials used in this study were obtained from the teaching practice pasture of the Tibetan Agricultural and Animal Husbandry College in Linzhi, Tibet, situated at an average altitude of 3,000 meters above sea level. At the age of 9 months, twelve pigs were selected for the study, with an equal representation of six Tibetan pigs (TP) and six Yorkshire pigs (YY), all of which were non-producers. These pigs were randomly divided into two groups, labeled as T and Y, resulting in a total of twelve distinct samples identified as T1, T2, T3, T4, T5, T6, Y7, Y8, Y9, Y10, Y11, and Y12. Both male and female. Both the Yorkshire and Tibetan pigs were maintained on an identical dietary regimen for the entire duration of the study. To conclude the experiment, humane euthanasia was administered to the test pigs through bleeding from the anterior vena cava. Following this, a series of carefully executed

procedures were undertaken, beginning with the meticulous dissection of the abdominal cavity and the subsequent extraction of the intestines. Approximately 20 cm of the intestinal canal, located at the midpoint of the duodenum, was ligated. Operational procedures were conducted under strict sterile conditions. A precise, small incision was methodically fashioned in the middle of the duodenal intestinal segment, employing ophthalmic scissors. The chyme, situated within the duodenum of the test pigs, was gently expressed into sterile lyophilization tubes. These collected samples were rapidly subjected to snap-freezing through the use of liquid nitrogen and were subsequently transferred to a storage facility maintained at -80°C to ensure their long-term preservation. Additionally, a segment of roughly 5 cm from the duodenum was surgically excised, and the contents were meticulously cleansed with saline before being promptly immersed in pre-prepared formalin for the purposes of preservation.

Histological analysis

The collected duodenal intestinal segments were initially stored at room temperature and subjected to a fixation period of 24 hours. Following fixation, the duodenum underwent a dehydration process involving ascending ethanol concentrations, followed by cleaning in xylene. Subsequently, the specimens were embedded in paraffin and sectioned into approximately 5 mm thick slices. These sections were subjected to staining with hematoxylin for 3 minutes, followed by eosin staining for 20 seconds, all conducted at room temperature. To assess the histological characteristics of the duodenum, the sections were examined utilizing an inverted microscope (Olympus BX51, Japan). Various parameters, including intestinal villi dimensions, muscle layer thickness, and cell density, were quantitatively measured in both Tibetan and Yorkshire pigs. This quantification was carried out using ImageJ software, and random sampling methods were employed. Statistical differences were subsequently analyzed using IBM SPSS Statistics version 26.0.

DNA extraction, amplification, sequencing, quality control and clustering

Microbial DNA was extracted using a HiPure Fecal DNA Kit (Magen, Guangzhou, China) according to the manufacturer's protocol. We used primers 519F: CAGCMGCCGCGGTAA and 915R: GTGCTCCCCCGCCAATTCCT for PCR amplification of the 16S rRNA V4 region (extension at 95℃ for 2 min, then at 98℃ for 10 s, 62℃ for 30 s, 68℃ for 30 s, and finally at 68℃ for 10 min). The PCR reaction system was 50 μL, which contained 5 μL 10×KOD Buffer, 5 μL 2.5 mM dNTPs, 1.5 μL each of upper and lower primers (5 μM), 1 μL KOD polymerase, and 100 ng template DNA; the rest of the system was made up with ddH2O. Amplicons were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.). Quantification was performed using an ABI StepOnePlus real-time fluorescence quantitative PCR instrument (Thermo Fisher Scientific) according to the manufacturer's instructions. Purified amplicons were subjected to equimolar sequencing and paired-end sequencing (2 × 250) on an Illumina platform, according to standard protocols.

Bioinformatics analysis

The presence of adapters or raw data of low-quality reads has an impact on assembly and analysis. After the sequencing data were available, to obtain high-quality clean reads, the raw data were filtered using FASTP (https://github.com/OpenGene/fastp) following the rules. Reads containing more than 10% of unknown nucleotides (N) and those with less than 80% base mass greater than 20 were removed. Clean paired-end reads were merged into the original tags using FLASH (Magoč and Salzberg, 2011) (version 1.2.11) with a minimum overlap of 10 bp and a mismatch error rate of 2%. Raw tags were filtered using the QIIME pipeline (Caporaso et al., 2010) (version 1.9.1) under specific filtering conditions (Bokulich et al., 2013) to obtain high-quality clean tags. Clean tags were searched against a reference database (http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html). All chimeric labels were removed, and valid labels were obtained for further analysis. The valid labels were clustered into operational classification units (OTUs) using the le></titles>< periodic UPARSE (Edgar, 2013) pipeline. The most abundant labeled sequences in each cluster were selected as representative sequences. Intergroup Venn analysis was performed using R (version 3.4.1) to identify unique and common OTUs. The RDP classifier (Wang et al., 2007) (version 2.2) based on the SILVA (Pruesse et al., 2007) database (https://www.arb-

silva.de/) was used to bioclassify representative sequences using the naïve Bayes classifier with a confidence threshold range of 0.8–1.

Alpha diversity and beta diversity analysis

The abundance statistics for each taxon were visualized using Krona (Ondov et al., 2011) (version 2.6). A meter (White et al., 2009) (version 20090414) and LEfSse software (Segata et al., 2011) (version 1.0) were used to screen the biomarker features of each group. Chao1, Simpson, and other alpha diversity indices were calculated using QIIME. The OTU sparsity and abundance curves were ranked. Alpha index comparisons between R project groups were calculated using the Welch t-test and Wilcoxon rank-sum test. Principal Coordinate Analysis (PCoA) was used to compare the differences in gut microbiota between samples.

Community function prediction and microbial ecological function prediction

The KEGG pathway analysis of the OTUs was performed using Tax4Fun (Aßhauer et al., 2015) (version 1.0). Prediction of FAPROTAX ecological functions was based on SILVA-annotated abundance tables and counting the distribution of the abundance of various functions in the sample.

Results

Histological analysis of duodenum from Tibetan and Yorkshire pigs

The results from the hematoxylin and eosin (HE) staining unveiled that the intestinal tracts of both Tibetan and Yorkshire pigs exhibited structurally intact characteristics with well-defined boundaries. Additionally, cup-shaped cells were uniformly distributed across the intestinal mucosa. Upon closer examination at the same magnification, a comparison of duodenal morphology between Tibetan and Yorkshire pigs revealed that the thickness of the duodenal muscle layer (Fig. 1\Sch. 1) was slightly greater in Tibetan pigs when compared to Yorkshire pigs. However, this difference was not found to be statistically significant ($P > 0.05$).Furthermore, the density of absorptive cells, cup cells, and small intestinal glands did not display any significant variations ($P > 0.05$) between the two groups. However, it's noteworthy that the height of the duodenal villi was significantly greater in Tibetan pigs as opposed to Yorkshire pigs ($P < 0.01$).

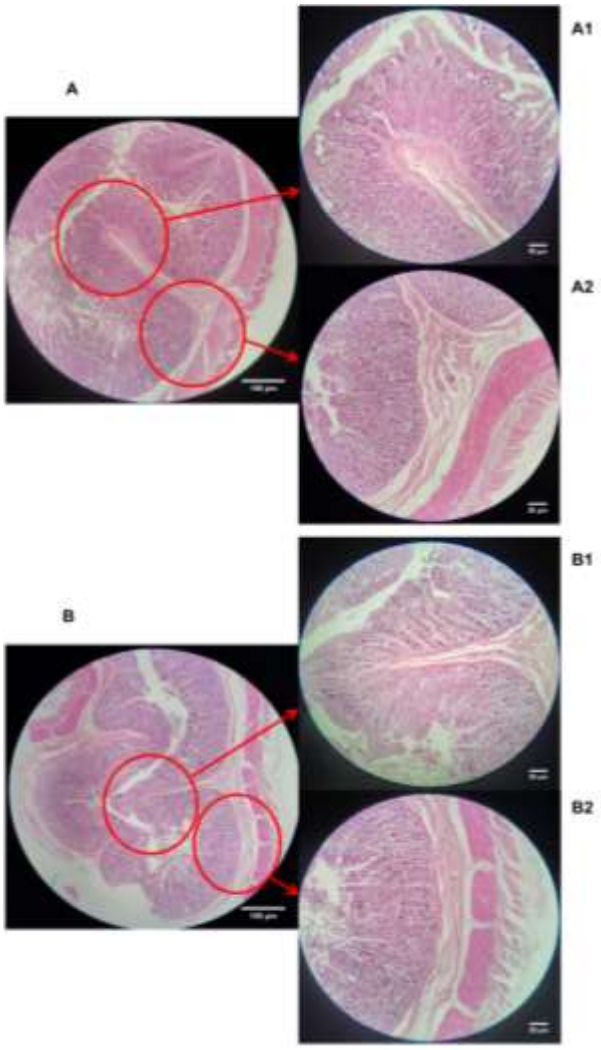


Figure-1. Morphological observation of duodenal in Tibetan pigs and Yorkshire pigs under different magnifications.

A) A1, and A2 show sections of duodenal intestine of Tibetan pigs; B) B1, and B2 show sections of duodenal intestine of Yorkshire pigs.

Schedule-1. The statistical results of duodenal villus length and muscle layer thickness in Tibetan pigs and Yorkshire pigs. [* Indicates significant difference ($P < 0.05$), ** indicates highly significant difference ($P < 0.01$)].

	TP	YY	F
Villus length (μm)	305.47± 31.62	246.30± 43.18	9.779**
Muscle layer thickness (μm)	183.40± 34.72	154.78± 17.60	4.325
Cell density	99.00± 53.56	76.00± 20.08	0.485

Sequencing data and OTU clustering results

A total of 1,309,665 reads were initially acquired. Following the elimination of low-quality reads, the dataset was refined to 1,184,614 reads. The clustering process identified a total of 9,379 operational taxonomic units (OTUs), with 5,326 and 4,053 OTUs attributed to Tibetan and Yorkshire pigs, respectively (refer to Schedule 2). Sparse and Shannon curves were constructed utilizing these OTUs and microbial diversity indices. The sequencing depth was deemed adequate, suggesting comprehensive coverage of all species within the samples. Notably, the Shannon index and Chao1 index exhibited significant disparities between the two groups, with the Shannon index demonstrating the most substantial variability across the treatments. The Shannon index serves as an indicator of species diversity, revealing that both diversity and evenness were markedly higher in group Y when compared to group T.

Analysis of the composition and structure of bacterial diversity

Based on the annotated abundance maps of species at each level of the duodenal gut microbes of Tibetan and Yorkshire pigs (Fig.2), which showed rich diversity, we specifically analyzed colonization at the phylum and genus levels. All duodenal bacteria were clustered into 27 phyla and 269 genera. At the phylum level, the ten most abundant phyla were Firmicutes (68%), Actinomycetes (15%), Bacteroides (9%), Proteobacteria (6%), Streptococcus, Epsilonbacteraeota, Euryarchaeota, Cyanobacteria, Fusobacteria, and Chlamydia (Fig.1A). At the genus level, the ten most abundant genera were *Clostridium spp.* (14%), *Lactobacillus spp.* (11%), *Bifidobacterium spp.* (8%), *Streptococcus spp.* (8%), *Trichosporon spp.* (4%), *Danserella spp.* _UCG-009 (3%), *Rhodobacter spp.* (3%), *Pseudonococcus spp.* (2%), *Bacillus subtilis* (2%), and *Streptococcus digestiveis* (3%) [Fig.1B]. A total of 134 species from Tibetan and Yorkshire pigs were clustered at the genus level, and the distribution of bacterial phyla and genera across the samples was consistent with the abundance stacking plots (Fig.2C–D).

Schedule-2. Statistics of OTU numbers (97% similarity threshold) and Alpha diversity indices (photo, ace, Shannon)

Sample Name	Effective Tags	OTUs	chao	ace	Shannon	Simpson
T1	120229	646	9.14E+02	9.53E+02	5.50E+00	9.33E-01
T2	98195	644	9.06E+02	9.45E+02	5.07E+00	8.83E-01
T3	65058	880	7.98E+02	8.16E+02	6.73E+00	9.72E-01
T4	105721	868	1.22E+03	1.27E+03	6.23E+00	9.43E-01
T5	94184	781	9.01E+02	9.49E+02	6.26E+00	9.59E-01
T6	114803	1187	8.06E+02	8.45E+02	5.90E+00	9.54E-01
Y7	118083	867	6.20E+02	6.65E+02	5.32E+00	9.30E-01
Y8	102046	743	7.63E+02	8.19E+02	4.15E+00	8.83E-01
Y9	75053	781	7.01E+02	7.37E+02	4.47E+00	8.66E-01
Y10	111539	886	7.07E+02	7.36E+02	4.02E+00	8.72E-01
Y11	112416	553	7.17E+02	7.59E+02	4.68E+00	9.15E-01
Y12	67287	691	9.43E+02	9.99E+02	4.88E+00	9.17E-01

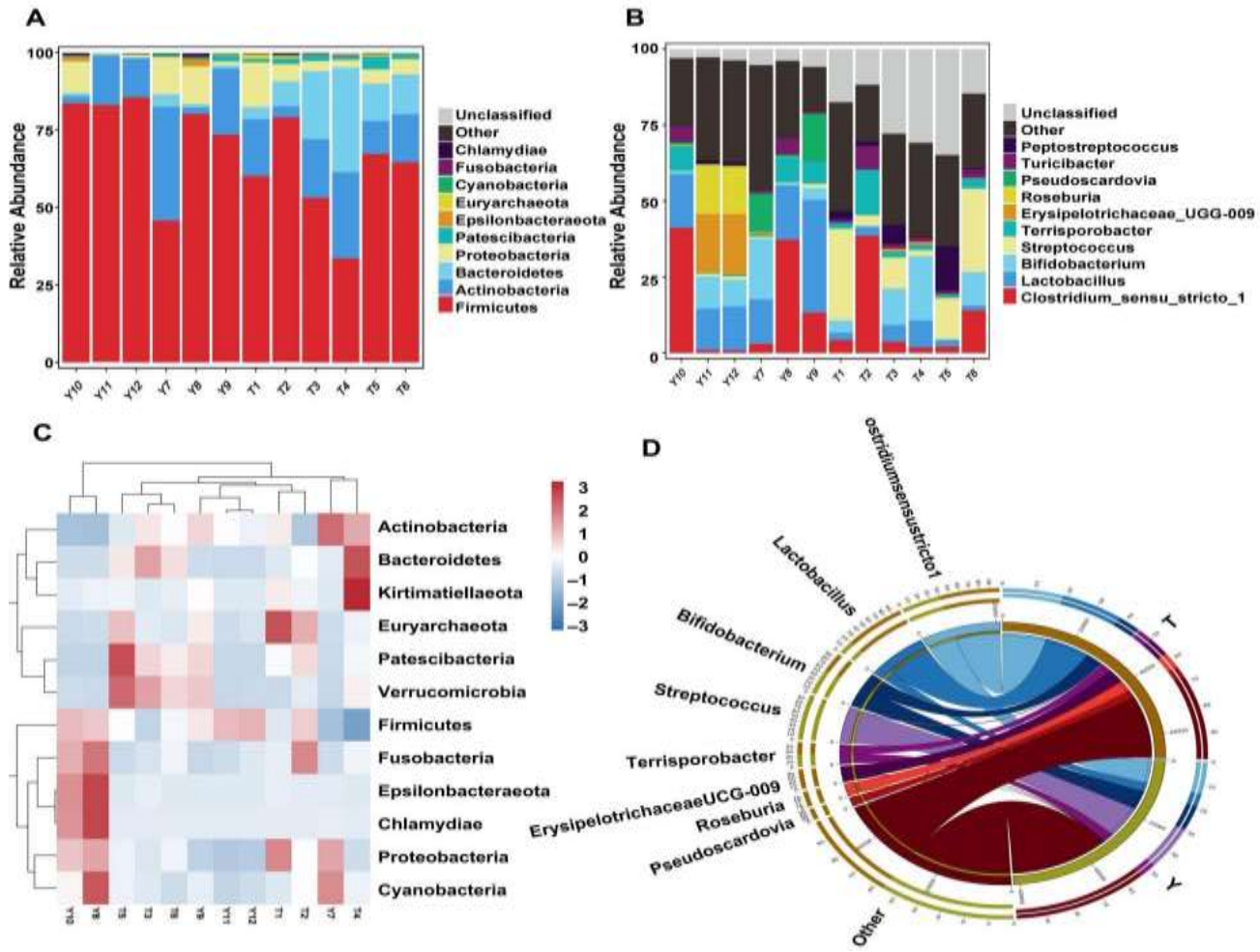


Figure-2 Heat map showing the relative abundance of intestinal microflora in Tibetan and Yorkshire pigs at phylum and genus levels.

(A–D) Horizontal coordinates: T (1–6) are duodenal content samples from the Tibetan pig group; Y (7–12) are duodenal content samples from the Yorkshire pig group. Abundance stacking plots for which the vertical coordinate is (A) the species abundance TOP10 at the phylum level and (B) species abundance TOP10 at the genus level. (C) Species distribution heat map, for which the vertical coordinate is the 12 common phyla in different colonies. (D) Interspecies microbial composition map at the genus level. Each color block in the heat map represents the relative abundance of a genus in the sample. Clustering can distinguish taxon with different abundance, and color gradient and similarity can reflect the similarities and differences of multiple samples at different classification levels. The blue-red gradient shows the change of abundance from low to high.

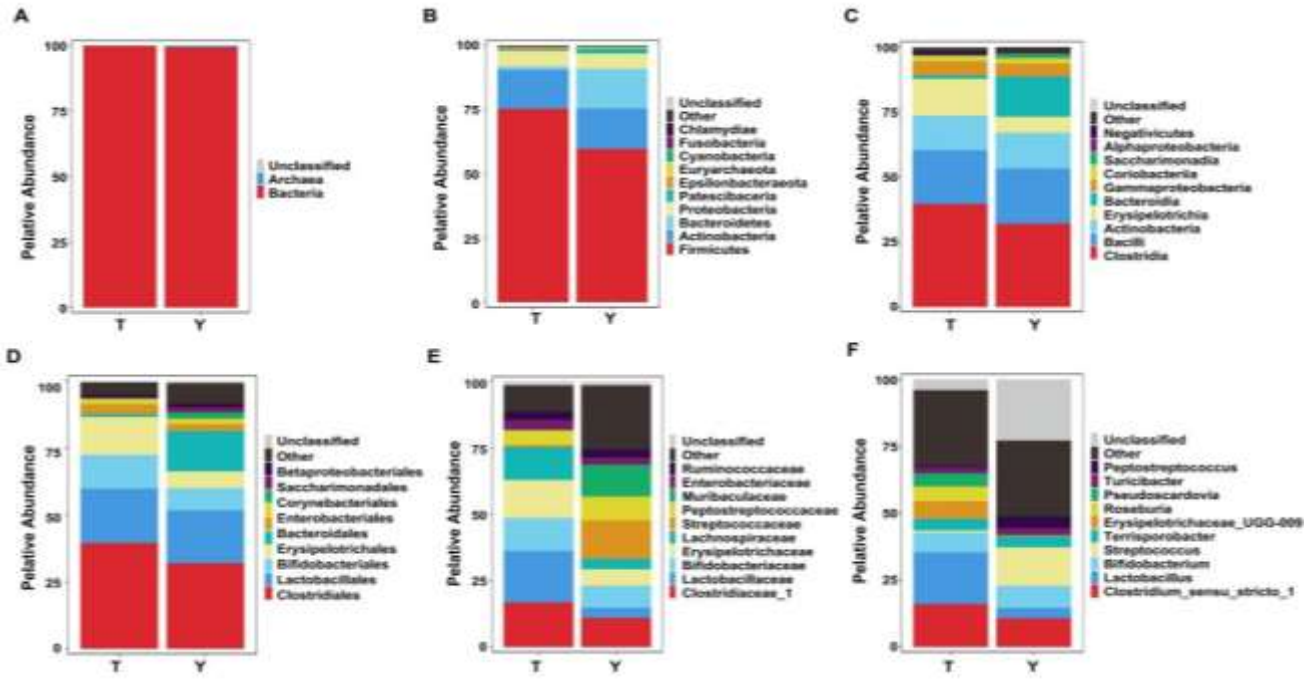


Figure-3. Ten most abundant taxa of duodenal intestinal microflora in Tibetan (T) and Yorkshire (Y) pigs at boundary.

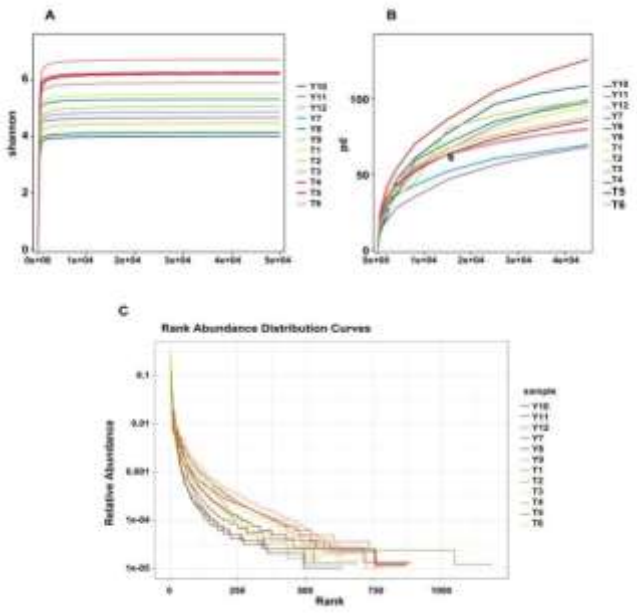
(A) kingdom, (B) phylum, (C) class, (D) order, (E) family and (F) genus.

By classifying duodenal colonies in groups T and Y, the relative percentages of dominant species in taxa of the kingdom, phylum, order, family, and genus taxa were analyzed. As shown in Fig.3**A**, more than 99% belonged to the bacterial kingdom, (99.89% and 99.42% for Tibetan and Yorkshire pigs, respectively). As shown in Fig.3**B**, Firmicutes was the dominant phylum in the duodenum of Tibetan and Yorkshire pigs, and the relative abundances of phylum Firmicutes were 75.28% and 59.63% in Tibetan and Yorkshire pigs, respectively; the relative abundance of the phylum Bacteroidetes in the duodenum of Tibetan pigs (1.35%) was significantly lower than that of Yorkshire pigs (15.44%) [$P < 0.05$]. As shown in Fig.3**C**, the ten most abundant microorganisms in the duodenal flora hierarchy of Tibetan and Yorkshire pigs were Clostridia (39.48% and 32.01%), Bacilli (20.67% and 21.04%), Actinobacteria (13.22% and 13.67%), Erysipelotrichia (14.13% and 6.31%), Bacteroidia (1.34% and 15.44%), Gammaproteobacteria (5.48% and 4.93%), Coriobacteriia (1.84% and 1.96%), Saccharimonadia (0.37% and 1.86%), Alphaproteobacteria (0.58% and 0.62%), and Negativicutes (0.73% and 0.16%), among the duodenal microflora in group T. Negativicutes in the duodenal microbial colonies of group T was significantly higher than that of group Y ($P < 0.05$). As shown in Fig.3**D**, the relative abundance of Selenomonadales purpose was significantly higher in Tibetan pigs (0.73%) than in the Y group (0.16%) [$P < 0.05$]. As shown in Fig.2**E**, the relative abundances of Lactobacillaceae, Muribaculaceae, and Actinomycetaceae were significantly different between Tibetan and Yorkshire pigs ($P < 0.05$), with Tibetan pigs having a significantly higher relative abundance of Lactobacillaceae. The relative abundance of Lactobacillaceae in Tibetan pigs was significantly higher than that in Yorkshire pigs ($P < 0.01$). Fig.3**F** shows the top ten stacks of duodenal colonies ranked in terms of abundance at the genus level: Clostridium_sensu_stricto_1, Lactobacillus, Bifidobacterium, Streptococcus, Terrisporobacter, Erysipelotrichaceae_UCG-009, Roseburia, Pseudoscardovia, Turicibacter, and Peptostreptococcus. The relative abundance of Lactobacillaceae in Tibetan pigs was significantly higher than that in Yorkshire pigs ($P < 0.01$).

Structural diversity of duodenal colonies in Tibetan and Yorkshire pigs

The Simpson indices of Tibetan and Yorkshire pigs were 0.90 and 0.94, respectively, and there was a

significant difference between the two groups ($P < 0.05$). The Shannon index of the two groups was 4.58 and 5.95, respectively, with highly significant differences between the two groups ($P < 0.01$). This indicates that the intestinal colony diversity in the duodenum of Tibetan pigs is significantly lower than that of Yorkshire pigs. Simpson and Shannon indices showed highly significant differences between all samples ($P < 0.01$). The Chao1 and Sob indices of the Tibetan and Yorkshire groups were 741.72,923.79 and 675.50,887.67, respectively. The Chao1 and Sob indices showed significant differences ($P < 0.05$) in the homogeneity of fungal microorganisms among different groups (Supplementary Fig.1).



Supplementary Figure-1. Duodenal microbial diversity of Tibetan and York pigs. (A): Shannon diversity index curve; (B): PD diversity index curve; (C): Rank abundance curve.

Tibetan and Yorkshire pigs indicator species

According to the above analysis, there are significant differences between Tibetan pigs and Yorkshire pigs in terms of class, family, and genus. Next, we analyzed the differences in community composition at these levels.

As shown in Fig.4A, the top ten richnesses at the phylum level were Clostridia, Bacilli, Actinobacteria, Erysipelotrichia, Bacteroidia, Gammaproteobacteria, Coriobacterii, Saccharimonadia, Alphaproteobacteria, and Negativicutes. Clostridia were the dominant colonies in the duodenum of Tibetan and Yorkshire pigs, accounting for 39.48% and 32.01%, respectively. The abundance of Clostridia (Negativicutes) in the duodenum was significantly higher in Tibetan pigs (0.73%) than in Yorkshire pigs (0.16%) [$P < 0.05$]. As shown in Fig.4**B**, Clostridiales, Lactobacillales, Bifidobacteriales, Erysipelotrichales, Bacteroidales, Enterobacteriales, Coriobacteriales, Corynebacteriales, Saccharimonadales, and Betaproteobacteria were present. For Enterobacteriales, Coriobacteriales, Betaproteobacteriales, and the duodenal flora of Tibetan pigs (0.73%), the abundance of Selenomonadales was significantly higher than that of Yorkshire pigs (0.16%) [$P < 0.05$]. In addition, the relative abundances of Bacteroidales (1.08%) and Saccharimonadales (0.37%) in group T were significantly lower than those in Yorkshire pigs (15.1 and 1.86%, respectively) [$P < 0.05$]. As shown in Fig.4**C**, the top ten in terms of family level richness were Clostridiaceae_1, Lactobacillaceae, Bifidobacteriaceae, Erysipelotrichaceae, Lachnospiraceae, Streptococcaceae, Peptostreptococcaceae, Muribaculaceae, Enterobacteriaceae, and Ruminococcaceae. Among them, Tibetan pig Lactobacillaceae abundance was extremely significantly higher than that of Yorkshire pigs ($P < 0.01$). As shown in Fig.4**D**, the top ten in abundance at the genus level were Clostridiumsensustricto1, Lactobacillus, Bifidobacterium, Streptococcus, Terrisporobacter, ErysipelotrichaceaeUCG-009, Roseburia, Pseudoscardovia, Turicibacter, and Peptostreptococcus. The relative abundance of the genus Lactobacillus was significantly higher than that in Tibetan pigs ($P < 0.01$).

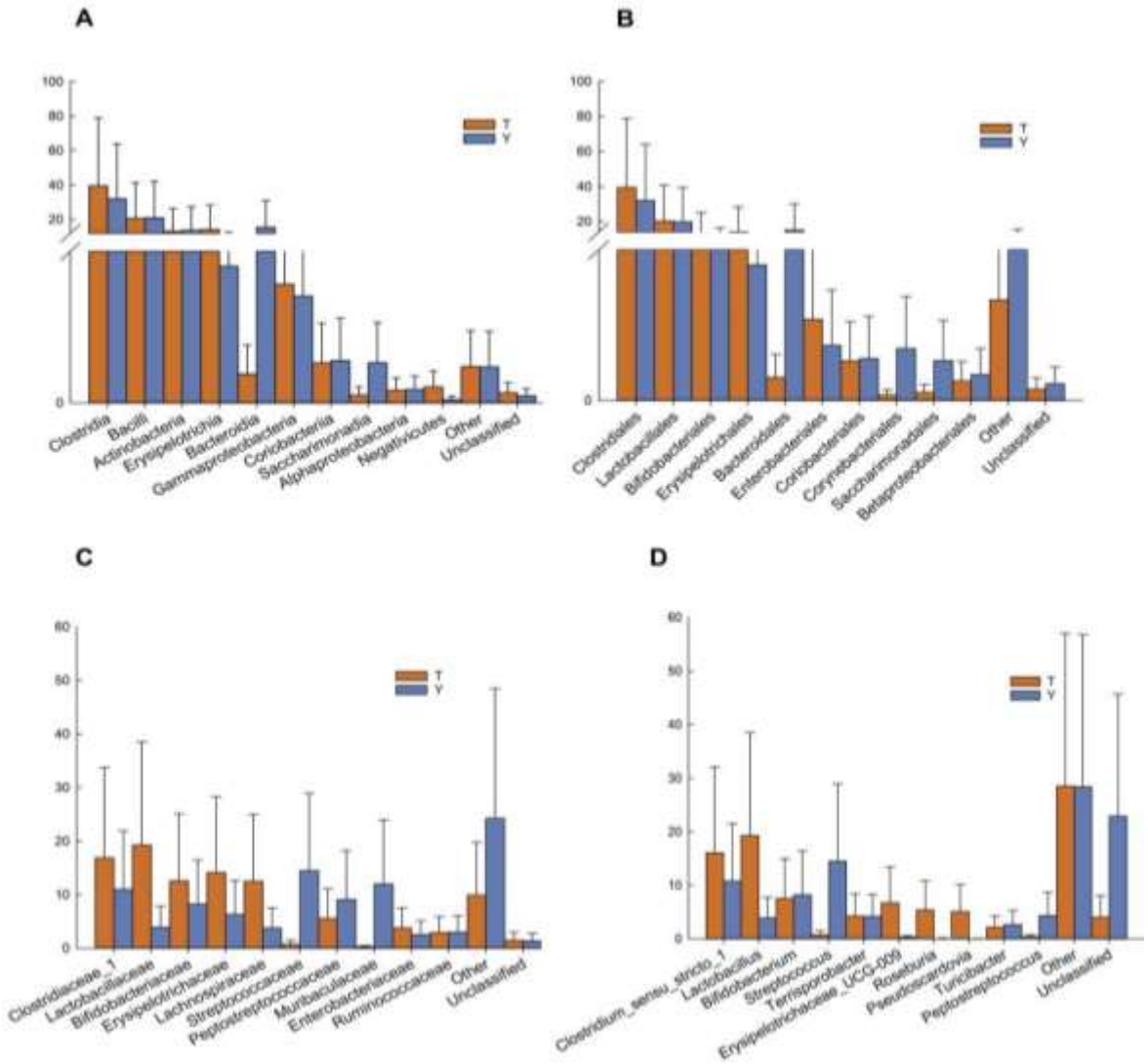
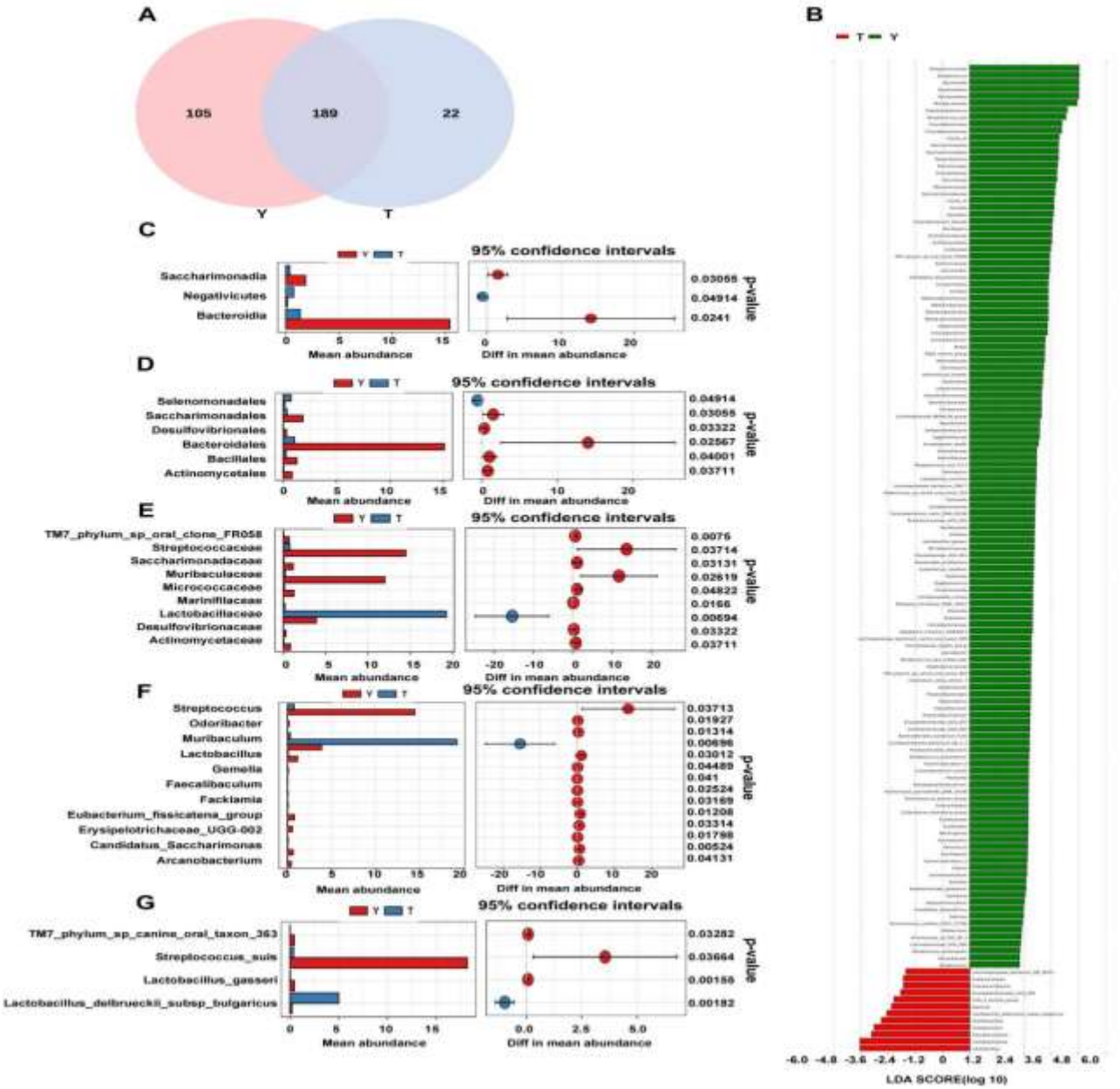


Figure-4. Plot of community differences in duodenal gut microbial composition between Tibetan and Yorkshire pigs at the phylum (A), order (B), family (C), and genus (D) levels. All data represent mean values.

The above analysis showed that there were significant differences in the diversity of the duodenal microflora between Tibetan and Yorkshire pigs. In this study, the intersection of Venn diagrams of duodenal intestinal microorganisms at the genus level was performed specifically for the Tibetan and Yorkshire pig groups. The results showed (Suppl. Fig.2**A**) that there were 189 genera in these two groups, and 22 species of endemic bacteria were detected in the duodenal contents of Tibetan pigs. To identify the bacterial species specific to the duodenal microbes of Tibetan and Yorkshire pigs, we performed LEFSE analysis of the LDA>2 community to further determine the differences in duodenal microbial composition between Tibetan and Yorkshire pigs (Suppl. Fig.2**B**). The results showed that 130 of the duodenum in the Tibetan group were higher and 12 were lower than that in the Yorkshire group. As shown in Suppl. Fig.2**C**, the relative abundance of the duodenal Negativicutes phylum was significantly higher in the Tibetan pig group than in the Yorkshire pig group ($P < 0.05$). As shown in Suppl. Fig.2**D**, the relative abundance of duodenal Selenomonadales in the Tibetan pig group was significantly higher than that in the Yoke pig group ($P < 0.05$). As shown in Suppl. Fig.2**E**, the relative abundance of the duodenal Lactobacillaceae family in the Tibetan pig group was significantly higher than that in the Yorkshire pig group ($P < 0.01$).



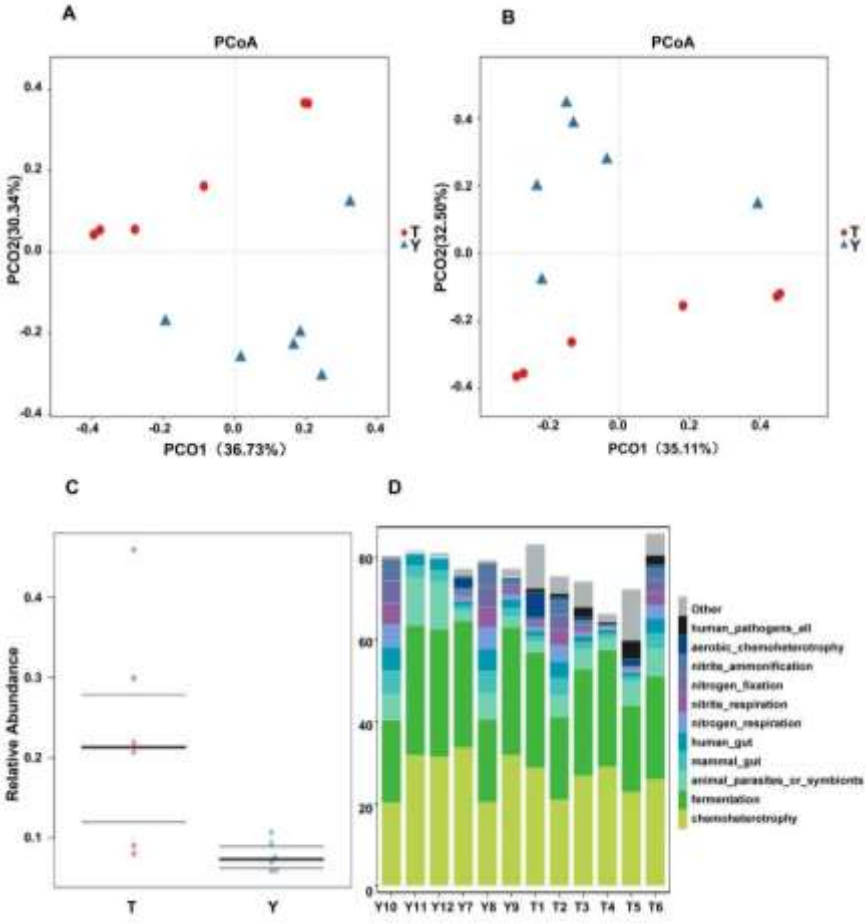
Supplementary Figure-2. Differences in intestinal microbial composition between Tibetan (T) and Yorkshire (Y) pigs. (A) Venn diagram analysis of duodenal gut microbiota at the genus level in groups T and Y. (B) Differences in species abundances in groups T and Y, (LDA > 2). (C–G) Significant differences in duodenal gut microbiota at the phylum (C), order (D), family (E), genus (F) and species (G) levels in groups T and Y.

As shown in Suppl. Fig.2F, the relative abundance of *Lactobacillus spp.* in the duodenum of the Tibetan pig group was significantly higher than that in the Yorkshire pig group ($P < 0.01$). Suppl. Fig.2**G** shows that the relative abundance of duodenal L. delbrueckiisubspbulgaricus species in the Tibetan pig group was significantly higher than that in the Yorkshire pig group ($P < 0.01$); Lactobacillus delbrueckiisubspbulgaricus belongs to *Lactobacillus* genus Lactobacillaceae family.

Prediction of duodenal intestinal microbial ecological function in Tibetan and Yorkshire pigs

In the PCoA results, each point represents a sample, different colors indicate different groups, and the distance between points reflects the difference in gut microbiota between samples. The distance between points reflects the difference in intestinal microbiota

between samples, and the two maxima found by principal component analysis were used as the horizontal and vertical coordinates, respectively. The results showed that the differences in diversity between the duodenal bacteria of the two groups of Tibetan and Yorkshire pigs at the family (PCo1:36.73%; PCo2:30.34%; Suppl. Fig.3A) and genus (PCo1:35.11%; PCo2:32.50%; Suppl. Fig.3B) levels were statistically significant; Adonis statistics showed that the differences between the two groups of samples were statistically significant ($P < 0.05$). Differences in the distribution of duodenal bacteria between Tibetan and Yorkshire pigs were observed at the family (Suppl. Fig.3A) and genus levels (Suppl. Fig.3B). This is consistent with the results of previous analyses. The duodenal microbial community in the Tibetan pig group was significantly higher ($P < 0.05$) than that in the Yorkshire pigs in the aerobic activity functional group (Suppl. Fig.3C). As predicted in Suppl. Fig.3D, for the top 11 ecological functions, the horizontal coordinates indicate one sample each, and the bars of different colors in the vertical coordinates indicate the relative abundance of different ecological functions. The results showed that microbial colonization of duodenal species in Tibetan and Yorkshire pigs was mainly associated with metabolism, genetic information processing, cellular processes, environmental information processing, organismal systems, and human diseases. Its main functions are concentrated in the metabolism of carbohydrates, amino acids, cofactors and vitamins, terpenes and polyketides, and other amino acids and lipid-like compounds (Table 1).



Supplementary Figure-3. Prediction of ecological functions performed by intestinal microbiota of Tibetan (T) and Yorkshire (Y) pigs. Principal component analysis of group T and group Y at family (A) and genus (B) levels. Each point represents a sample and the distance between two points indicates the difference in duodenal microbiota. Aerobic activity of gut microbes(C), stacked map of ecological functional abundance (D).

Table-1. Functional prediction information for the Tibetan Yorkshire pig groups

Level_1	Level_2	Tibetan pigs	Yorkshire pigs
Metabolism	Carbohydrate metabolism	338499.5796	368644.3537
Metabolism	Amino acid metabolism	292028.0393	329056.235
Metabolism	Metabolism of cofactors and vitamins	266025.4581	313482.2877
Metabolism	Metabolism of terpenoids and polyketides	234328.3683	275866.9088
Metabolism	Metabolism of other amino acids	198662.0731	221177.1373
Metabolism	Lipid metabolism	174792.3149	176280.9853
Metabolism	Energy metabolism	126410.3918	140062.4206
Metabolism	Xenobiotics biodegradation and metabolism	120269.6648	134237.6747
Metabolism	Glycan biosynthesis and metabolism	62433.55438	78808.0639
Metabolism	Nucleotide metabolism	53195.18322	58908.84977
Metabolism	Biosynthesis of other secondary metabolites	47419.59302	58605.61033
Genetic Information Processing	Replication and repair	161042.1792	173161.0483
Genetic Information Processing	Translation	86294.8859	95944.3516
Genetic Information Processing	Folding, sorting, and degradation	76716.49835	85958.952
Genetic Information Processing	Transcription	21322.01348	21760.24848
Cellular Processes	Cell growth and death	37922.45938	40518.77073
Cellular Processes	Cell motility	47246.44333	27759.48697
Cellular Processes	Transport and catabolism	4966.08288	6489.29593
Cellular Processes	Cellular community—prokaryotes	3960.00285	3989.26315
Environmental Information Processing	Membrane transport	59917.97735	60927.05433
Environmental Information Processing	Signal transduction	9189.15268	8691.4912
Environmental Information Processing	Signaling molecules and interaction	0.9003	60.92855
Organismal Systems	Environmental adaptation	5252.2083	4563.79937
Organismal Systems	Endocrine system	2126.88993	2505.78248
Organismal Systems	Immune system	1367.05707	1241.17788
Organismal Systems	Digestive system	256.6062	543.81353
Organismal Systems	Excretory system	0	0.04412
Human Diseases	Infectious diseases	7812.4797	7396.09153
Human Diseases	Neurodegenerative diseases	708.98887	398.16392
Human Diseases	Cardiovascular diseases	3.67285	80.81795
Human Diseases	Immune diseases	0.88833	0.08697

Discussion

Pigs, being one of the most extensively utilized animals in the field of livestock production, serve as a vital source of high-quality meat products for human consumption. Additionally, due to their remarkable anatomical and physiological resemblances to humans, pigs find extensive application as valuable animal models in medical research, especially in the study of diseases and drug development. Consequently, research on the microbial communities residing within the gastrointestinal tracts of pigs has been both comprehensive and far-reaching (Patil et al., 2020; Guo et al., 2022). Microorganisms constitute a vast group of organisms with significant implications for both human and animal health (Belvoncikova et al., 2022; Chen et al., 2021; Malik et al., 2022). Tibetan pigs possess outstanding characteristics, including their ability to adapt to low-oxygen environments in high-altitude plateau regions, their robust disease resistance, high capacity for fat deposition, and tolerance to roughage (Shang et al., 2022). The Yorkshire pig is an exceptional lean breed known for its remarkable attributes, including efficient feed utilization, rapid growth rate, and a high carcass yield (Shang et al., 2019). Moreover, research has revealed that individuals with obesity tend to exhibit reduced microbial diversity within their gut compared to those with a lean physique (Cheng et al., 2022). The results indicated substantial disparities in both the diversity and abundance of duodenal microorganisms between

Tibetan and Yorkshire pigs. Tibetan pigs exhibited notably greater richness and evenness in duodenal microbial species compared to their Yorkshire counterparts. These findings align consistently with prior research outcomes.

The intestine stands as a pivotal organ in the process of digestion. Within the porcine gut resides a profusion of microorganisms, forming an intricate microbial ecosystem that dynamically interacts with the host (Hooper and Gordon, 2001; Patil et al., 2020). Of particular significance is the small intestine, renowned as the primary site for the digestion and absorption of essential nutrients, encompassing carbohydrates, proteins, and lipids. The specialized physiological structure of the small intestine enhances its capability to efficiently and comprehensively absorb nutrients, especially those abundant in cupped cells. The thickness of the intestinal wall directly affects digestion and absorption. A longer villus promotes better absorption, while a thicker muscular layer indicates greater expansion and contractile ability, resulting in faster digestion and absorption. The duodenum, situated at the start of the small intestine and closely connected to the liver and pancreas, exhibits a highly specialized internal structure. This structure includes numerous folds and an extensive presence of villi and microvilli, collectively increasing the available surface area for efficient nutrient absorption. Within this intricate environment resides a diverse microbial community (Gao et al., 2000). In the typical human intestinal microbiome, the predominant taxonomic groups include Firmicutes, Bacteroides, Actinomycetes, Proteobacteria, Fusobacteria, and Verrucomycetes (Round and Mazmanian, 2009). Interestingly, prior research has proposed that the Firmicutes-to-Bacteroidetes ratio can serve as an indicator linked to obesity (Magne et al., 2020). Our study's findings lend support to this concept, as we observed a significant elevation in the Firmicutes-to-Bacteroidetes ratio in the duodenum of Tibetan pigs when compared to Yorkshire pigs ($P < 0.05$). This suggests that an elevated Firmicutes to Bacteroidetes ratio was associated with obesity in the intestinal microbial communities, consistent with the findings from the analysis of microorganisms in the duodenum of obese Tibetan pigs.

It has been shown that the probiotic *Lactobacillus* and its long-lived bacteriocins are involved in a variety of functional pathways in the intestine and are associated with obesity and diseases with species specificity (Anjana and Tiwari, 2022). The findings from our current study underscore a substantial difference in the relative abundance of the Lactobacillus family and genus levels between Tibetan pigs and their larger Yorkshire counterparts, with statistical significance ($P < 0.01$). This observation aligns with the well-documented traits of Tibetan pigs, characterized by their remarkable capacity for fat deposition and robust resistance to diseases. These results are in line with prior research, further solidifying the consistency of these findings. Indeed, prior research has highlighted the role of Lactobacillus enrichment in fostering the development of highly functional small intestinal villi, consequently facilitating the improved digestion and absorption of essential nutrients (Zhu et al., 2022). Furthermore, *Lactobacillus spp*. have been linked to fat deposition traits in the colon of Tibetan pigs, as reported by Shang et al. (2022). Oxidative stress is intricately connected with several types of cancer and is known to induce inflammation through multiple pathways, thereby contributing to the development of chronic diseases. *Lactobacillus spp.* play a pivotal role in maintaining the integrity of the gastrointestinal barrier's immune system, modulating the gastrointestinal tract's resistance to oxidative stress, and augmenting the anti-inflammatory properties of the gut microbiota, as elucidated by Kong et al. (2020). These studies suggest that a higher abundance of Lactobacillus is conducive to fat deposition and enhances host immunity. In our current investigation, we observed a significantly greater presence of Lactobacillus spp. and Bifidobacterium spp. in Tibetan pigs compared to Yorkshire pigs. This finding, when taken together with the distinctive traits of Tibetan pigs, supports the notion of a favorable duodenal microflora composition (Duarte and Kim, 2022).

Beyond its primary role in digestion, the gut microbiota plays a multifaceted role by producing bioactive compounds that exert influence over various aspects of the intestinal environment. This influence encompasses the immune system, the integrity of the barrier function, and the proliferation of cells within the mesojejunum of the small intestine. The microbiota closely associated with the mucosal lining engages in direct interactions with the intestinal epithelium. This interaction is mediated through the metabolites secreted by enterocytes, facilitated by an adhesion system. Notably, health-promoting microorganisms, such as Lactobacillus spp. and Bifidobacterium spp., exhibit a heightened presence

within the mucosal environment. This microbial community also actively regulates the production of mucus, a vital physical barrier that prevents pathogen adhesion. Furthermore, the positive role of short-chain fatty acids in preserving the stability of the intestinal barrier function cannot be overlooked (Ornelas et al., 2022).

Notably, research has shown that gut microbes and their metabolites, including SCFA (short-chain fatty acids) and bile acids, can contribute to obesity (Zhang and Dang, 2022; Zhou et al., 2021). The host's consumption of different energy diets influences the growth of intestinal stem cells and the production of microbial metabolites. These metabolites, in turn, function as signaling molecules, regulating the activity of intestinal stem cells (Liu et al., 2021), Changes in the levels of short-chain fatty acids have been linked to radiation-induced intestinal damage, with microbial abundance profiles impacting these fatty acid levels. Interestingly, the primary microorganisms responsible for producing short-chain fatty acids belong to the Thickworm and Bacillus phyla, which aligns with the findings of our study (Duarte and Kim, 2022).

The results of functional enrichment in metabolism showed a slightly higher relative abundance of duodenal microorganisms in Yorkshire pigs than in Tibetan pigs, mainly in carbohydrate metabolism, amino acid metabolism, cofactor and vitamin metabolism, metabolism of terpenoids and polyketides, and metabolism of other amino acids and lipid-like compounds. The reason for this difference could be related to the faster metabolism of Yorkshire pigs and the high conversion rate to feed, with high carcass content. Furthermore, the duodenal microbiota of Tibetan pigs exhibited a higher relative abundance of microorganisms associated with environmental adaptation and immune responses compared to Yorkshire pigs, aligning with the distinctive traits of Tibetan pigs.

Conclusion

In conclusion, the diversity of duodenal microbial colonies in Tibetan pigs was found to be lower compared to Yorkshire pigs, with Firmicutes dominating at the phylum level. The composition and abundance of duodenal intestinal microflora may have implications for fat deposition and disease resistance, particularly the presence of the Lactobacillus genus, which plays a role in fat deposition, duodenal intestinal barrier development, and immune function. This analysis enhances our knowledge of the intestinal microbial diversity in Tibetan pigs and contributes to the advancement and utilization of this breed.

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Contribution of Authors

Sun W & Wang Y: Performed literature review, analyzed data, edited and approved the final manuscript
Zhang J: Collected and analyzed the data, edited and approved the final manuscript
Yin Y: Analyzed data, edited and approved the final manuscript
Duan M: Collected and analyzed the data
Yangzom C & Shang P: Conceived idea and designed the experiments, Performed literature review, analyzed data, edited and approved the final manuscript

References

Aßhauer KP, Wemheuer B, Daniel R and Meinicke P, 2015. Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. Bioinformatics. 31(17):2882-4.

Anjana and Tiwari SK, 2022. Bacteriocin-Producing

Probiotic Lactic Acid Bacteria in Controlling Dysbiosis of the Gut Microbiota. Front Cell Infect Microbiol. 12:851140.

Belvoncikova P, Splichalova P, Videnska P and Gardlik R, 2022. The Human Mycobiome: Colonization, Composition and the Role in Health and Disease. J Fungi (Basel). 8(10):1046.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA and Caporaso JG, 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods. 10(1):57-9.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J and Knight R, 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 7(5):335-6.

Chen D, Tang Q, Su H, Zheng H, Chen K and Zhong G, 2021. Rumen microbial community and functions of rumen bacteria under different feeding regime. Pak Vet J, 41(3): 341-346.

Chen X, Saeed NM, Ding J, Dong H, Kulyar MFEA, Bhutta ZA, Mehmood K, Ali MM, Irshad I, Zeng J, Liu J, Wu Q and Li K, 2022. Molecular epidemiological investigation of Cryptosporidium sp., Giardia duodenalis, Enterocytozoon bieneusi and Blastocystis sp. infection in free-ranged yaks and tibetan pigs on the plateau. Pak Vet J. 42(4): 533-539.

Cheng Z, Zhang L, Yang L and Chu H, 2022. The critical role of gut microbiota in obesity. Front Endocrinol (Lausanne). 13:1025706.

Duan M, Wang Z, Guo X, Wang K, Liu S, Zhang B and Shang P, 2021. Integrated analysis of transcriptomic and proteomic analyses reveals different metabolic patterns in the livers of Tibetan and Yorkshire pigs. Anim Biosci. 34(5):922-930.

Duarte ME and Kim SW, 2022. Intestinal microbiota and its interaction to intestinal health in nursery pigs. Anim Nutr. 8(1):169-184.

Edgar RC, 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 10(10):996-8.

Gao C, Zhao J and Gregersen H, 2000. Histomorphometry and strain distribution in pig duodenum with reference to zero-stress state. Dig Dis Sci. 45(8):1500-8.

Guo Y, Li R, Sun X, Zhang Z, Zheng H, Han L, Cui Y, Zhang D and Liu M, 2022. In vitro antibiotic susceptibility, virulence genes profiles and integrons of streptococcus suis isolates from pig herds in Liaoning Province of China. Pak Vet J. 42(1): 117-121.

Hooper LV and Gordon JI, 2001. Commensal host-bacterial relationships in the gut. Science. 11; 292(5519):1115-1118.

Kong Y, Olejar KJ, On SLW and Chelikani V, 2020. The Potential of Lactobacillus spp. for Modulating Oxidative Stress in the Gastrointestinal Tract. Antioxidants (Basel). 9(7):610.

Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG and Huttenhower C, 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 31(9):814-21.

Liu BN, Liu XT, Liang ZH and Wang JH, 2021. Gut microbiota in obesity. World J Gastroenterol. 27(25):3837-3850.

Ma L, Wang R, Feng S, Yang X, Li J, Zhang Z, Zhan H, Wang Y, Xia Z, Wang CC and Kang L, 2022. Genomic insight into the population history and biological adaptations of high-altitude Tibetan highlanders in Nagqu. Front. Ecol. Evol. 10. https://doi.org/10.3389/fevo.2022.930840

Magne F, Gotteland M, Gauthier L, Zazueta A, Pesoa S, Navarrete P and Balamurugan R, 2020. The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? Nutrients. 12(5):1474.

Magoč T and Salzberg SL, 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 27(21):2957-2963.

Malik F, Nawaz M, Anjum AA, Firyal S, Shahid MA, Irfan S, Ahmed F and Bhatti AA, 2022. Molecular characterization of antibiotic resistance in poultry gut origin Enterococci and horizontal gene transfer of antibiotic resistance to Staphylococcus aureus. Pak Vet J. 42(3): 383-389.

Ondov BD, Bergman NH and Phillippy AM, 2011. Interactive metagenomic visualization in a Web browser. BMC Bioinform. 12(1):1–10.

Ornelas A, Dowdell AS, Lee JS and Colgan SP,

2022. Microbial Metabolite Regulation of Epithelial Cell-Cell Interactions and Barrier Function. Cells. 11(6):944.

Patil Y, Gooneratne R and Ju XH, 2020. Interactions between host and gut microbiota in domestic pigs: a review. Gut Microbe. 11(3):310–34.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J and Glöckner FO, 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35(21):7188-96.

Round JL and Mazmanian SK, 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 9(5):313–323.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS and Huttenhower C, 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12(6):R60.

Shang P, Li W, Liu G, Zhang J, Li M, Wu L, Wang K and Chamba Y, 2019. Identification of lncRNAs and Genes Responsible for Fatness and Fatty Acid Composition Traits between the Tibetan and Yorkshire Pigs. Int J Genomics. 2019:5070975.

Shang P, Wei M, Duan M, Yan F and Chamba Y, 2022. Healthy Gut Microbiome Composition Enhances Disease Resistance and Fat Deposition in Tibetan Pigs. Front Microbiol. 13:965292.

Wang Q, Garrity GM, Tiedje JM and Cole JR, 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 73(16):5261-7.

White JR, Nagarajan N and Pop M, 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Computat Biol. 5(4):e1000352.

Zhang S and Dang Y, 2022. Roles of gut microbiota and metabolites in overweight and obesity of children. Front Endocrinol (Lausanne). 13:994930.

Zhou H, Yu B, Sun J, Liu Z, Chen H, Ge L and Chen D, 2021. Short-chain fatty acids can improve lipid and glucose metabolism independently of the pig gut microbiota. J Anim Sci Biotechnol. 12(1):61.

Zhu C, Yao J, Zhu M, Zhu C, Yuan L, Li Z, Cai D, Chen S, Hu P and Liu HY, 2022. A meta-analysis of Lactobacillus-based probiotics for growth performance and intestinal morphology in piglets. Front Vet Sci. 9:1045965.