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RESEARCH ARTICLE

Protective Effects of Traditional Chinese Herbal Medicine Formulas (TCHMFs) Via Influencing Anti-Oxidative Capacity, Inflammatory Mediators, and Gut Microbiota in Weaned Yaks

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ABSTRACT

Traditional Chinese medicine is an emerging area of scientific interest due to concerns regarding antimicrobial resistance (AMR). To explore the effects of three different Traditional Chinese Herbal Medicine Formulas (TCHMFs) on weaning yaks, 24 yaks in the weaning period with similar physical conditions were randomly divided into four groups (n=6) with one control group and three TCHMFs diet groups (TCHMF1, 2, and 3). Three TCHMFs were added to the diet for one month, and blood and fecal samples were collected every 15 days. Results showed that the TCHMFs diet increased levels of total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and decreased malondialdehyde (MDA) levels. In addition, the TCHMFs diet decreased the levels of inflammatory cytokines interleukin-6 (IL-6) and IL-10. Concerning the gut microbiota, the addition of TCHMFs to the diet significantly ($P<0.05$) increased microbial diversity and richness and improved microbial stability. The relative abundance of *Firmicutes* and *Actinobacteria* increased while *Bacteroidetes* decreased, and the ratio of *Firmicutes/Bacteroidetes* increased in the experiment groups ($P<0.05$ or $P<0.01$). The abundance of genera such as *5-7N15*, *Oscillospira*, *Bacteroides*, *Prevotella*, etc. was significantly reduced, while the abundance of *Clostridium*, *Bifidobacterium*, *Blautia*, *Anaerostipes*, etc. was increased in the experimental groups ($P<0.05$ or $P<0.01$), concluding that supplementing TCHMFs along with normal diet proved beneficial by decreasing inflammatory cytokines (stimulation intestinal immunity) and increasing the antioxidant enzymes levels, and optimizing the gut microbiota in weaned yaks.

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INTRODUCTION

Yak (*Bos grunniens*) native breed in China, mainly distributed in the high-altitude areas such as Qinghai Tibet Plateau and its surroundings typically above 3000 meters. Yak has been the dominant livestock species in the Qinghai Tibet Plateau since ancient times. Its hide, meat, milk, and even manure is considered to be of great importance for the survival of the people in the Qinghai Tibet region (Lu *et al.*, 2023). Although yaks can survive in cold regions with high altitudes and low pressure, their long calving interval, slow growth rate, prolonged weaning period and high susceptibility to diarrhea especially in weaned calves are the main reasons that restrict the development of the yak breeding industry in the Tibet.

At the time of weaning, the intake of herbage and forage can stimulate the development of the gastrointestinal tract, greatly improving the acquired immunity (Deng, 2010; Fox *et al.*, 2013). However, weaning has drastic effects on young yaks such as reduced

herbage and fodder intake which can lead to oxidative stress in calves, resulting in weakened immunity and gastroenteritis. This can seriously hamper the growth of young yaks (Deng, 2010; Li *et al.*, 2023a). Therefore, research is needed to examine the anti-inflammatory, antioxidant, immunostimulatory, and anti-diarrheal properties of different TCHMFs during the weaning period.

Chinese herbal medicine is a natural nutrient cum medicine that has been inherited for thousands of years, with the advantages of low toxicity, minimal side effects, and low cost of production (Sham *et al.*, 2014; Meng *et al.*, 2024). Although Chinese herbal medicine was not classified as an orthodox medicine, due to its multi-active ingredient properties, however, it has a wide range of pharmacological effects. Regarding TCHMF constituents, the roots of Chinese pulsatilla exhibit antiparasitic, antibacterial, and antioxidant effects, treating bloody diarrhea (Lee *et al.*, 2001; Li *et al.*, 2023b). Additionally, it has been reported that TCM ameliorates intestinal inflammation by modulating gut microbiota. In livestock husbandry, Chinese pulsatilla decoction (composed of Chinese pulsatilla, *Coptis chinensis*, *Phellodendron amurense*, and ash bark) is frequently used to treat diarrhea in yaks (Liu *et al.*, 2021b; Li *et al.*, 2023b). Numerous pharmacological studies have highlighted the therapeutic potential of Dandelion, including antibacterial, antioxidant, anticancer, and anti-rheumatic activities, in addition to relieving intestinal inflammation and gastrointestinal diseases (Ding and Wen, 2018; Li *et al.*, 2022a; Fan *et al.*, 2023). Apart from these three main constituents, other Chinese herbal medicines in TCHMFs have also been proven to be nutritious and exhibit their effects in curing diarrhea, having anti-inflammatory properties, and strengthening the immune system (Leite *et al.*, 2022). From a physiological perspective, reactive oxygen species (ROS) generated can be counteracted by the redox balance system of yaks (Li *et al.*, 2022b).

Gut microbiota is composed of millions of beneficial bacteria, and other microorganisms, which play a vital role in host digestion, metabolism, development, and immune regulation (Rowland *et al.*, 2018; Li *et al.*, 2024). In contrast, gut dysbiosis may indicate the occurrence of metabolic diseases and immune dysbiosis (Xi *et al.*, 2021). In previous studies, high-throughput sequences of the 16s rRNA gene have been extensively used to plumb the relationship between the microbiome, health, and disease status (Johnson *et al.*, 2019; Chen *et al.*, 2023). However, to the best of our knowledge, very limited data is available regarding the effect of the TCHMFs diet on the gut bacteria in weaned yaks by studying the effect of TCHMFs on levels of antioxidant enzymes and interrelations to inflammatory cytokines. Hence, this study was carried out to evaluate the effects of three TCHMFs on the antioxidant capacity, inflammatory cytokines, and gut microbiota in weaned yaks, with the potential for using these herbal drugs as alternatives to antibiotics in the future to prevent and treat different diseases and conditions.

MATERIALS AND METHODS

Experimental design: The experiment was conducted at the Gesangtang Yak Breeding Base located in Linzhou County, Lhasa, Xizang. Twenty-four young yaks, (approximately 6 months old, just weaned), were randomly divided into four groups. The treatment groups were as follows: **(1)** XA: 5% dietary supplementation of TCHMF-I (*Coptis chinensis* 18.18%, *Dioscorea opposite* 13.64%, *Prunus mume* 13.64%, *Terminalia chebula* 13.64%, *Rheum officinale* 13.64%, *Plantago asiatica* 13.64% and *Glycyrrhiza uralensis* 13.64%); **(2)** XB: 5% dietary supplementation of TCHMF-II (*Dioscorea opposita* 25%, *Coptis chinensis* 12.5%, *Artemisia scoparia* 12.5%, *Inula racemosa* root 12.5%, *Paeonia lactiflora* 12.5%, *Anisodamine* 12.5% and *Glycyrrhiza uralensis* 12.5%); **(3)** XC: 5% dietary supplementation of TCHMF-III (*Taraxacum officinale* 30%, *Terminalia chebula* 25%, *Anisodamine* 15%, *Magnolia officinalis* 15% and *Pueraria lobata* 15%); **(4)** XD: the control group, fed *ad libitum*. The above additives were added based on the basic diet, the composition and nutrient levels of the forage are shown in Table 1. Experimental animals were acclimatized for 7 days and supplemented with dietary formulations for 30 days. The sample collection was performed at two-point intervals on days 15 (XZ1) and 30 (XZ2).

Table 1: Composition and nutrient levels of experimental ration

Basal diet	
Alfalfa Hay (%)	20.00
Oaten hay (%)	30.00
Corn (%)	30.00
Wheat bran (%)	11.00
Soybeans meal (%)	1.00
Cottonseed meal (%)	1.00
rapeseed oil (%)	1.50
Premix (%)	4.00
Calcium hydrogen carbonate (%)	0.57
Limestone (%)	0.30
Sodium bicarbonate (%)	0.30
Salt (%)	0.30
Choline chloride (%)	0.03
Nutrient level	
DM (%)	88.03
ME(MJ/kg)	9.85
CP (%)	11.67
NDF (%)	31.83
ADF (%)	18.50
Ca (%)	0.88
P (%)	0.60

DM: Dry matter; ME: Metabolizable Energy; CP: Crude protein; NDF: Neutral detergent fiber; ADF: Acid detergent fiber. Premix was formulated to provide the following per kg of total diet DM: 600IU of vitamin A, 275IU of vitamin D, 60IU of vitamin E, 0.1mg of Co, 10mg of Cu, 0.5mg of I, 30mg of Mn, 0.2mg of Se, 30mg of Zn, 50mg of Fe, and 20mg of monensin.

Sample collection, processing, and storage: Before offering feed, 6mL of blood was collected from the anterior vena cava of each calf in the morning, using a disposable blood collection needle. Collected blood samples were left to clot at room temperature for about 2 hours to separate serum from cellular components. Finally, serum was obtained by centrifugation at 2000 rpm for 10 minutes and stored at -80°C until further analysis. Rectal fecal samples (3-5g) were collected from each calf using sterile cotton swabs. Samples were immediately transferred to sterile centrifuge tubes and stored at -80°C until further analysis.

Estimation of serum inflammatory mediators and antioxidant enzyme activity: Serum levels of IL-6 and IL-10 were measured using the Bovine IL-6/IL-10 ELISA Kit (Shanghai Enzyme-linked Biotechnology, China)

according to the manufacturer's recommendations. Levels of GSH-Px, SOD, T-AOC, and MDA in the serum were quantified using specific assays provided by Nanjing Jiancheng Biotechnology Research Institute, China.

DNA extraction, PCR amplification, and sequencing library preparation: Genomic DNA from fecal samples was extracted using the Stool Genomic DNA Extraction Kit (Solarbio®, Beijing, China) following the recommended protocols. DNA quantification was performed utilizing a Nanodrop 2000 spectrophotometer, and DNA quality was evaluated by 1.2% agarose gel electrophoresis. Primers targeting the hypervariable regions (V3-V4) of 16s rRNA genes were designed. PCR amplification was carried out, and the resulting amplicons were purified and recovered using magnetic beads (Vazyme VAHTSTM DNA Clean Beads). Fluorescence quantification of PCR products was performed using the Quant-iT PicoGreen dsDNA Assay Kit and a Microplate reader (BioTek, FLx800). Sequencing libraries were prepared using the TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, USA). Quality inspection of the libraries was performed utilizing the Agilent High Sensitivity DNA Kit. Qualified libraries were subjected to double-ended sequencing on a NovaSeq sequencer using the NovaSeq 6000 SP Reagent Kit (500 cycles) (Rognes *et al.*, 2016).

Bioinformatics and statistical analysis: Alpha diversity of samples was evaluated according to the distribution of operational taxonomic units (OTUs) using rarefaction curves. Beta diversity analysis was conducted to compare differences among groups based on OTU levels. Species abundance composition and statistical differences between groups were determined to identify marker species. The SparCC association network was constructed to identify key species. PICRUSt2 was employed to predict functional gene abundance in the 16S rRNA gene sequence against the KEGG database. Differential pathways were identified, and the species composition of specific pathways was obtained. Statistically, significance was determined at a $P<0.05$ value.

RESULTS

Serum antioxidant enzymes and inflammatory mediators: In this study, the serum antioxidant enzymes, including SOD, glutathione peroxidase (GPx), and MDA were evaluated. It was found that the antioxidant indicators of weaned yaks (GSH-Px) fed on traditional Chinese medicine formulas in their diet were higher than control groups ($P<0.05$). While the MDA levels in control animals were significantly higher, especially in groups B and C (Fig. 1a). In addition, inflammation-related factors IL-6 levels in control yaks were remarkably higher than in yaks in the groups A, B, and C (Fig. 1b).

High throughput sequencing data analysis: High throughput sequencing generated a total of 3,684,506 raw sequences, with 767,601±6,256 raw sequences (Mean ± SEM) per sample. Finally, 1,933,107 sequences were retained, with 40,273±4,698 per sample for annotations on species classification (Table 2). The Rarefaction Curve tended to be flat as the sequencing depth increased (Fig. 2a). The rank abundance curve was wide and sloped gently, suggesting that the richness and uniformity of the samples meet the requirements for subsequent analysis (Fig. 2b). A total of 32,479 OTUs were recognized based on 97% sequence homology. Veen maps showed that 730 and 877 OTUs were established core OTUs of samples from two periods, accounting for 4.25% and 5.73% of the total OTUs in group XZ1 and group XZ2, respectively (Fig. 2c).

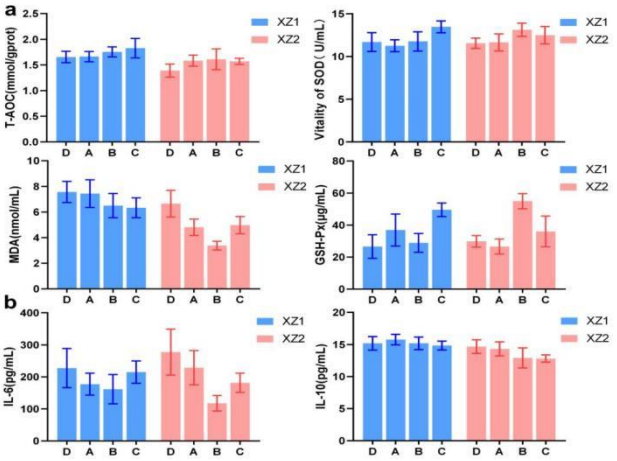

Fig. 1: Analysis of serum parameters. (a) Levels of antioxidant enzymes, including total antioxidant capacity (T-AOC), Superoxide dismutase (SOD), Malondialdehyde (MDA), and glutathione peroxidase (GSH-Px). (b) Levels of the inflammatory mediators, interleukin-6 (IL-6), and interleukin-10 (IL-10).

Gut microbial diversity and composition with traditional Chinese medicine formulas: On the 15th day of the experiment, in terms of α-diversity in the bacteria, multiple indexes displayed that the species richness, diversity, and evenness of the control group were significantly higher ($P<0.05$). However, on the 30th day of the experiment, α-diversity showed no significant difference among groups (Fig. 3a). In terms of beta diversity analysis, dots belonging to the control group gathered alone, separated from the experimental groups by a considerable distance, while the distance of dots belonging to the three experimental groups was relatively closer. Over time, dots of the control group and the experimental groups were observed gradually approaching (Fig. 3b). On the 15th day, the dominant bacterial phyla consisted of *Firmicutes* (53.85±1.55%), *Bacteroidetes* (22.16±4.82%), and *Actinobacteria* (16.96±6.17%). Notably, group XBZ1 exhibited a higher *Firmicutes/Bacteroidetes* ratio compared to the control group (Fig. 4a). The predominant bacterial genus was an unclassified genus within the family Ruminococcaceae (21.29±2.48%). Among classified genera, *Bifidobacterium* (14.43±5.67%) dominated in the experimental groups, whereas 5-7N15 (family Bacteroidaceae) (6.16±0.93%) was prevalent in the control group (Fig. 4b).

By the 30th day, the dominant bacterial phyla remained *Firmicutes* (58.09±3.01%), *Bacteroidetes* (24.21±2.09%), and *Actinobacteria* (11.67±2.50%), with *Bacteroidetes* still relatively richer in the control group. Furthermore, the *Firmicutes/Bacteroidetes* ratio remained low in XDZ2,

however, it tended to stabilize in the experimental group (Fig. 4c). The primary genus remained an unidentified genus within the Ruminococcaceae family (25.37±1.67%), followed by *Arthrobacter* (9.62±2.65%) and 5-7N15 (2.60±0.78%) (Fig. 4d).

Changes in the gut microbiota after Traditional Chinese medicine formulas to diet: Linear discriminant analysis effect size (LEfSe) (LDA Effect Size) analysis was employed to find robust differential species among groups. The analysis of gut microbiota on the 15th day of the experiment showed that, at the phylum level, *Fibrobacteres* were elevated abundantly in the control group, while the abundance of *Actinobacteria* was higher in group XAZ1, and decreased in the control group, while *Bacteroidetes* presented an opposite trend. Genera *Fibrobacter* (p-*Fibrobacteres*), *Faecalibacterium*, and *Eubacterium* (p-*Firmicutes*) were significantly enriched in the control group (P<0.05 or P<0.01).

In group XAZ1, Family Micrococcaceae and genus *Bifidobacterium*, both belonging to *Actinobacteria*, exhibited significantly higher abundance, whereas the genus *Sharpea* within *Firmicutes* was more abundant in group XBZ1. Notably, *Bifidobacterium* was markedly less abundant in group XDZ1 compared to other groups (Fig. 5a). By the end of the experimental period, genera *Veillonella* and families Veillonellaceae and Ruminococcaceae were more abundant in group XDZ2, while genera *Bacillus* and *Clostridium*, along with families Planococcaceae and Peptostreptococcaceae, were less prevalent in this group. Within the Proteobacteria phylum, the genus *Acinetobacter* was a significant enrichment in group XAZ2. Genus *Clostridium* (*Firmicutes*) was found to be enriched in group XBZ2, whereas *Bacillus*, *Sporosarcina*,

Table 2: Statistics of sequencing data of yaks in all the groups

Sample ID	Input	Filtered	Percentage of input passed filter (%)	Denoised	Percentage of input denoised (%)	Merged	Non-chimeric	Non-singleton
XA1	75775	70851	93.50181458	67307	88.82481029	46708	36107	35864
XA2	82334	77647	94.30733354	75331	91.49440086	60573	48820	48718
XA3	61764	58038	93.96735963	56119	90.86037174	46716	40524	40457
XA4	77738	73149	94.0968381	70044	90.1026525	51972	37015	36908
XA5	73972	68993	93.26907479	64764	87.55204672	43190	32630	32437
XA6	105282	98968	94.0027735	94721	89.96884558	66426	48947	48773
XB1	73035	68568	93.88375436	65500	89.68302868	51573	41727	41527
XB2	73361	69439	94.65383514	67724	92.31608075	58368	51770	51729
XB3	71888	66444	92.42710884	63656	88.54885377	47661	36367	36220
XB4	78325	73463	93.79253112	69882	89.22055538	49053	37870	37716
XB5	77232	72192	93.47420758	69057	89.41500932	49470	37414	37269
XB6	90717	85030	93.73105372	81954	90.34028903	64195	50898	50793
XC1	93464	88044	94.20097578	84521	90.43161003	64075	51406	51288
XC2	80489	74706	92.81516729	71187	88.4431413	48875	35581	35423
XC3	75897	71415	94.09462825	68117	89.74926545	48752	39038	38875
XC4	72987	68286	93.55912697	65353	89.54060312	48601	39850	39691
XC5	79655	74993	94.14726006	71134	89.30261754	48465	37547	37384
XC6	81216	76810	94.5749606	73046	89.94040583	52182	39936	39763
XD1	87950	82483	93.78396816	80370	91.38146674	70569	66897	66820
XD2	86689	80756	93.15599442	77421	89.30890886	58971	49111	48903
XD3	85311	79335	92.99504167	76249	89.37768869	56501	43391	43167
XD4	86295	81021	93.88840605	77754	90.10255519	56872	43165	42915
XD5	89812	84958	94.59537701	80899	90.0759364	58182	42558	42289
XD6	90719	84488	93.13153805	80494	88.7289322	57038	46443	46217
XA7	74568	69916	93.76139899	66130	88.68415406	44167	34880	34626
XA8	71559	67034	93.67654663	64564	90.22484942	48623	38771	38706
XA9	63358	59383	93.72612772	56451	89.09845639	42045	32885	32705
XA10	77330	72110	93.24970904	69245	89.54480797	52893	42195	42009
XA11	63697	59857	93.97145862	57012	89.50500024	41147	32288	32063
XA12	72567	68314	94.13920928	65382	90.09880524	48411	39207	39088
XB7	73584	69132	93.94977169	65944	89.61730811	45905	35261	35117
XB8	68540	63956	93.31193464	60948	88.92325649	41956	31901	31781
XB9	79087	74369	94.03441779	71271	90.11721269	51165	37747	37604
XB10	73367	68483	93.34305614	65144	88.79196369	46752	38195	38009
XB11	77585	72091	92.91873429	68511	88.30444029	47711	41063	40970
XB12	87527	81693	93.33462817	77711	88.78517486	53779	46856	46702
XC7	75958	71454	94.07040733	67458	88.80960531	43036	34262	34030
XC8	71651	67018	93.53393533	64017	89.34557787	45392	33388	33213
XC9	66723	62540	93.73079748	59681	89.4459182	43328	34777	34651
XC10	67923	63235	93.09806693	60457	89.00814157	45985	39723	39638
XC11	67889	63809	93.99018987	60901	89.70672716	43948	35554	35408
XC12	69659	65107	93.46530958	62103	89.15287328	44156	37177	37021
XD7	66219	61558	92.96123469	58591	88.48064755	42157	33087	32876
XD8	74425	69157	92.92173329	66253	89.01981861	49097	39583	39411
XD9	76161	71355	93.6896837	68456	89.88327359	51340	42878	42715
XD10	75194	69696	92.6882464	67082	89.21190521	51195	39911	39723
XD11	75302	70795	94.0147672	68657	91.17553319	57593	46200	46123
XD12	62726	58590	93.40624303	56134	89.49080126	43126	37853	37772
Total	3684506	3450729		3300707		2429895	1940654	1933107
Mean ± SEM	767601±6256	71890±5914	93.64±0.36	68765±5724	89.58±0.63	50623±4955	40430±4686	40273±4698

The data are presented as Mean±SEM.

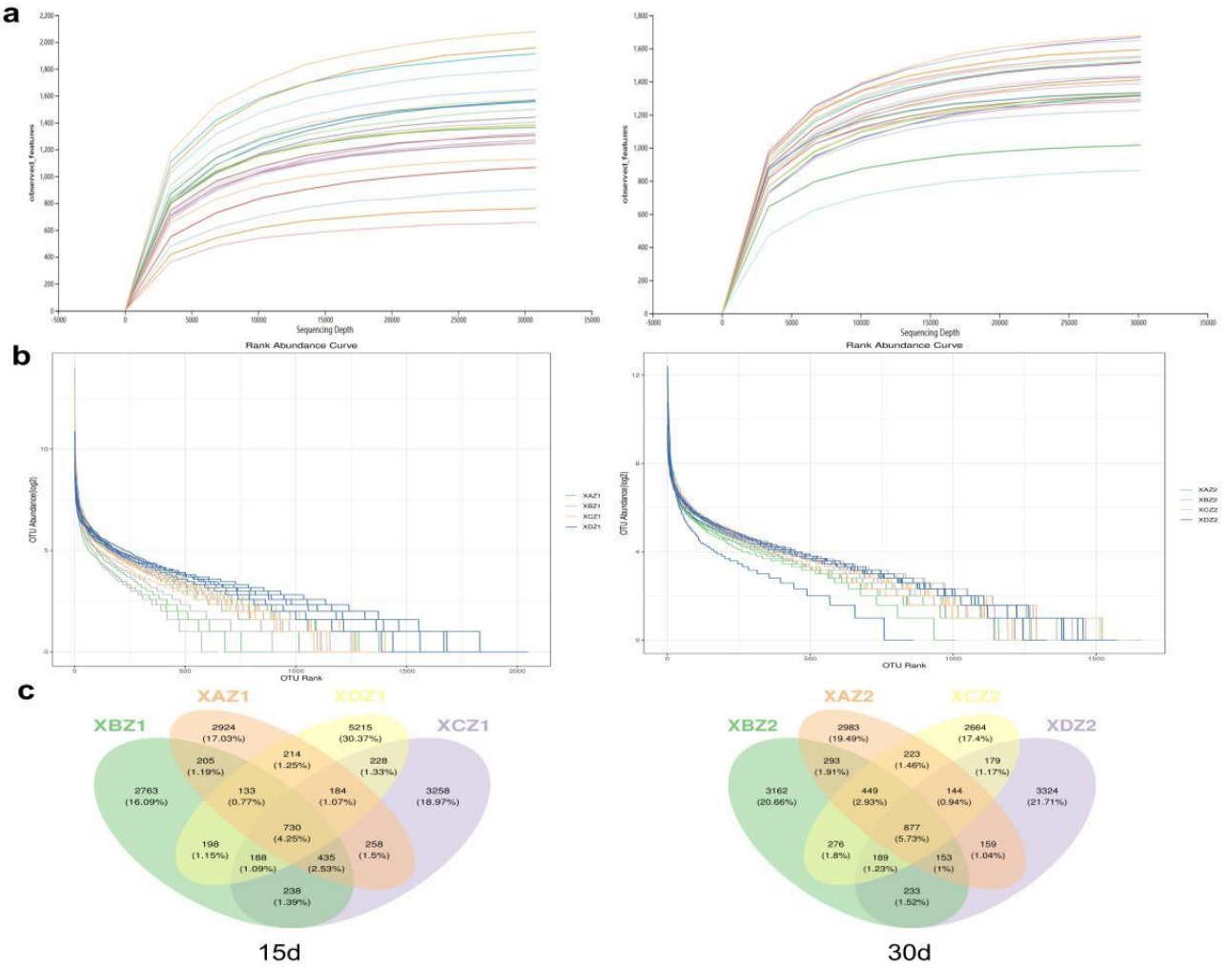

Fig. 2: Distribution of Operational Taxonomic Units (OTUs). (a) Rarefaction curves illustrating sequencing depth and species richness across samples. (b) Rank abundance curves showing species diversity and evenness within samples. (c) Venn diagram depicting the shared and unique OTUs among different groups or samples.

and *Clostridium* (*Firmicutes*) were more abundant in group XCZ2. Moreover, the phylum Proteobacteria exhibited lower abundance in group XCZ2 ($P<0.05$ or $P<0.01$) (Fig. 5b).

The Random Forest Analysis generated important scores for general prediction. On the 15th day, the top three genera were *Atopobium*, *Butyrivibrio*, and *BF311*, with the top two being less abundant and the third being richer in the control group (Fig. 5c). By the 30th day, the predominant genus was *Acinetobacter*, enriched in group XAZ2. The secondary genus, *Prevotella*, was enriched in group XDZ2, while the third, *Turicibacter*, was less prevalent in this group (Fig. 5d). Furthermore, T-test Analysis at phyla (Fig. 6a-6c) and genera level (Fig. 7a and 7b) reflected significant ($P<0.05$) differences between the control group and the three experimental groups.

Microbial associations Network analysis: Co-occurrence Network analysis of sample bacteria on the 15th day of the experiment revealed a total of 5036 co-occurrence relationships, including 4116 positive relationships and 920 negative relationships, with 118 connectors nodes and 185 peripheral nodes. On the 30th day, a total of 6041 co-occurrence relationships were found, including 4325 positive relationships and 1716 negative relationships, with one module hub (order *Clostridiales*) (Fig. 8a). *Firmicutes* are the node with the most relevant connections (69.31%;70.31%). On the 30th day, the *Arthrobacter* in *Actinobacteria* showed more positive connections compared to the 15th day (Fig. 8b).

Functional profiles of the intestinal microbiome: PCoA maps of PICRUSt2 analysis found that on the 15th day of the experiment, except for the control group, the points in each group were relatively scattered. On the 30th day of the experiment, it was discovered that the functional composition of the experimental group samples tends to be similar, especially in groups XBZ2 and XCZ2 (Fig. 9a).

The 16S rRNA gene sequences were aligned to 181 KEGG level 3, pathways that represent intestinal microbial metabolic pathways. At KEGG level 2, 33 categories were identified, including amino acid metabolism, carbohydrate metabolism, lipid metabolism, and metabolism all falling under the umbrella of metabolism, displaying high abundance (Fig. 9b). While no significant changes were observed, in comparison to the 15th day, pathways such as signaling molecules and interaction, signal transduction, and metabolism of terpenoids and polyketides increased on the 30th day, whereas infectious diseases decreased.

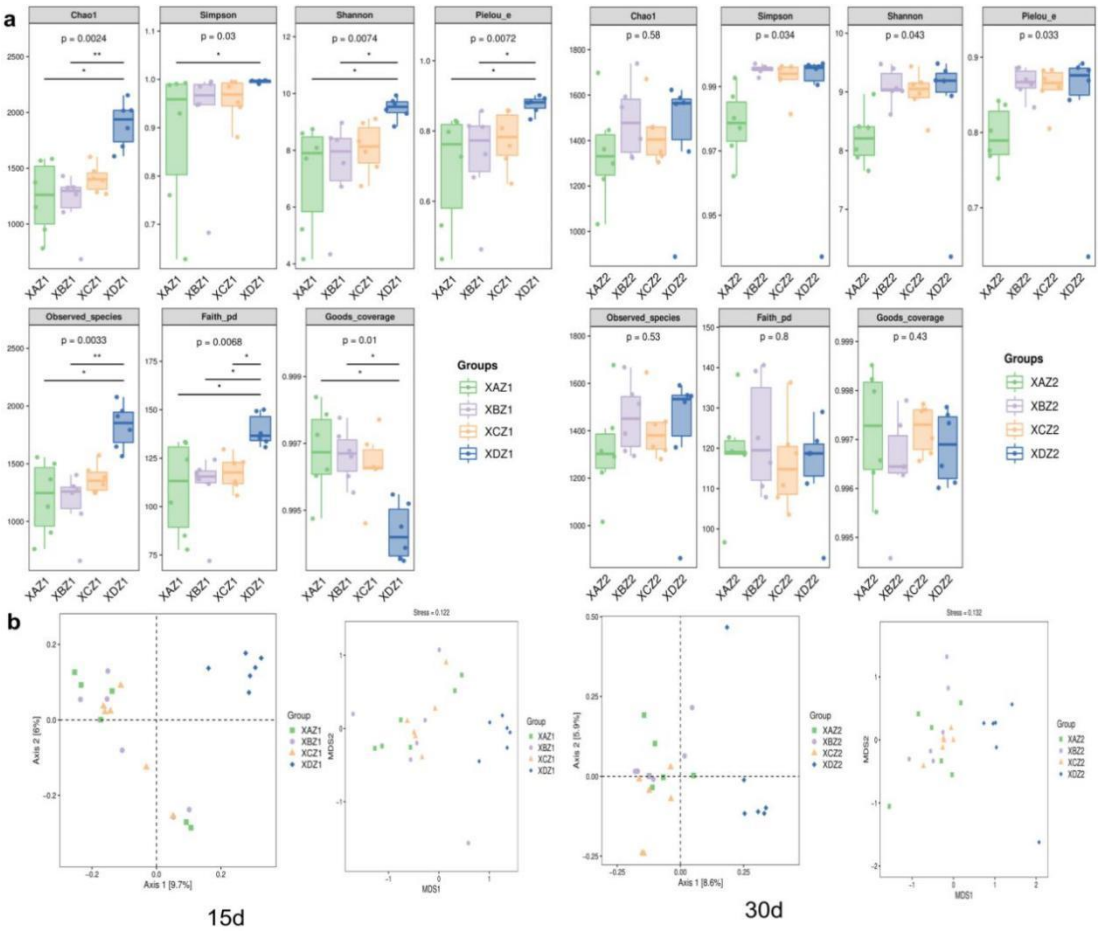

Fig. 3: Gut microbial diversity analysis. (a) Alpha diversity analysis, representing within-sample microbial diversity using metrics such as richness and evenness. (b) Beta diversity analysis, illustrating between-sample microbial variation through ordination or clustering methods.

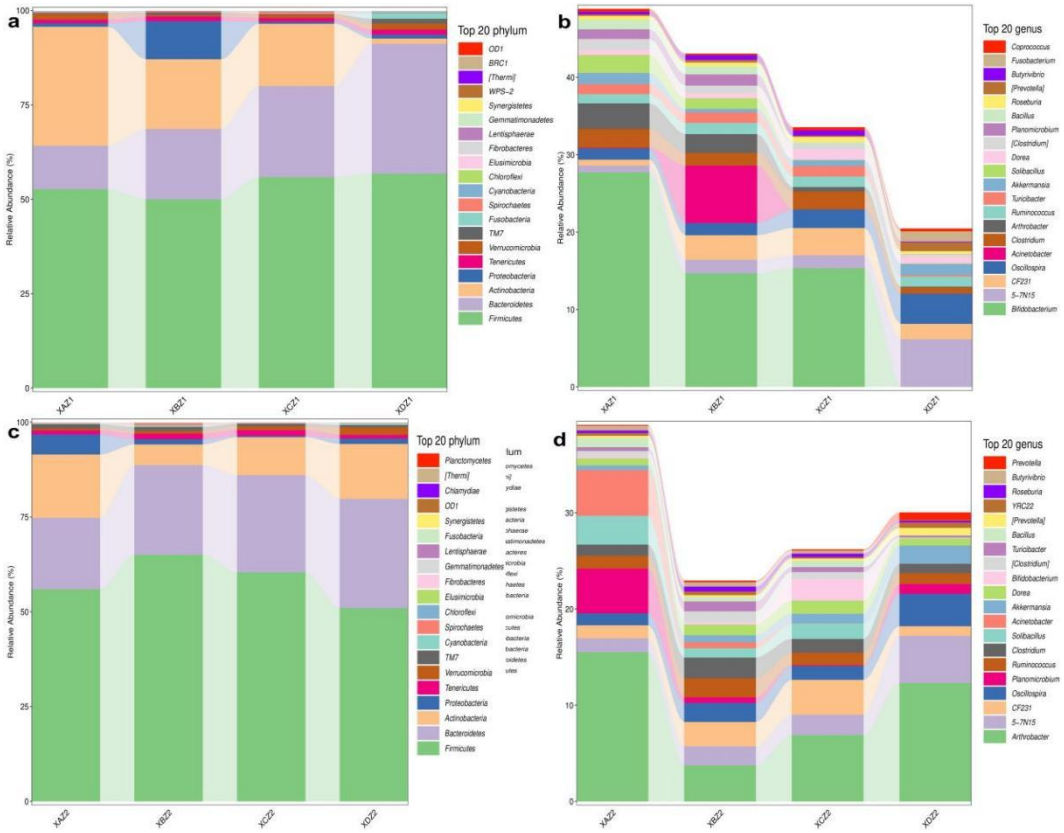

Fig. 4: Bar charts depicting species composition and relative abundance at the phylum and genus levels. (a, c) Relative abundance of microbial communities at the phylum level. (b, d) Relative abundance of microbial communities at the genus level.

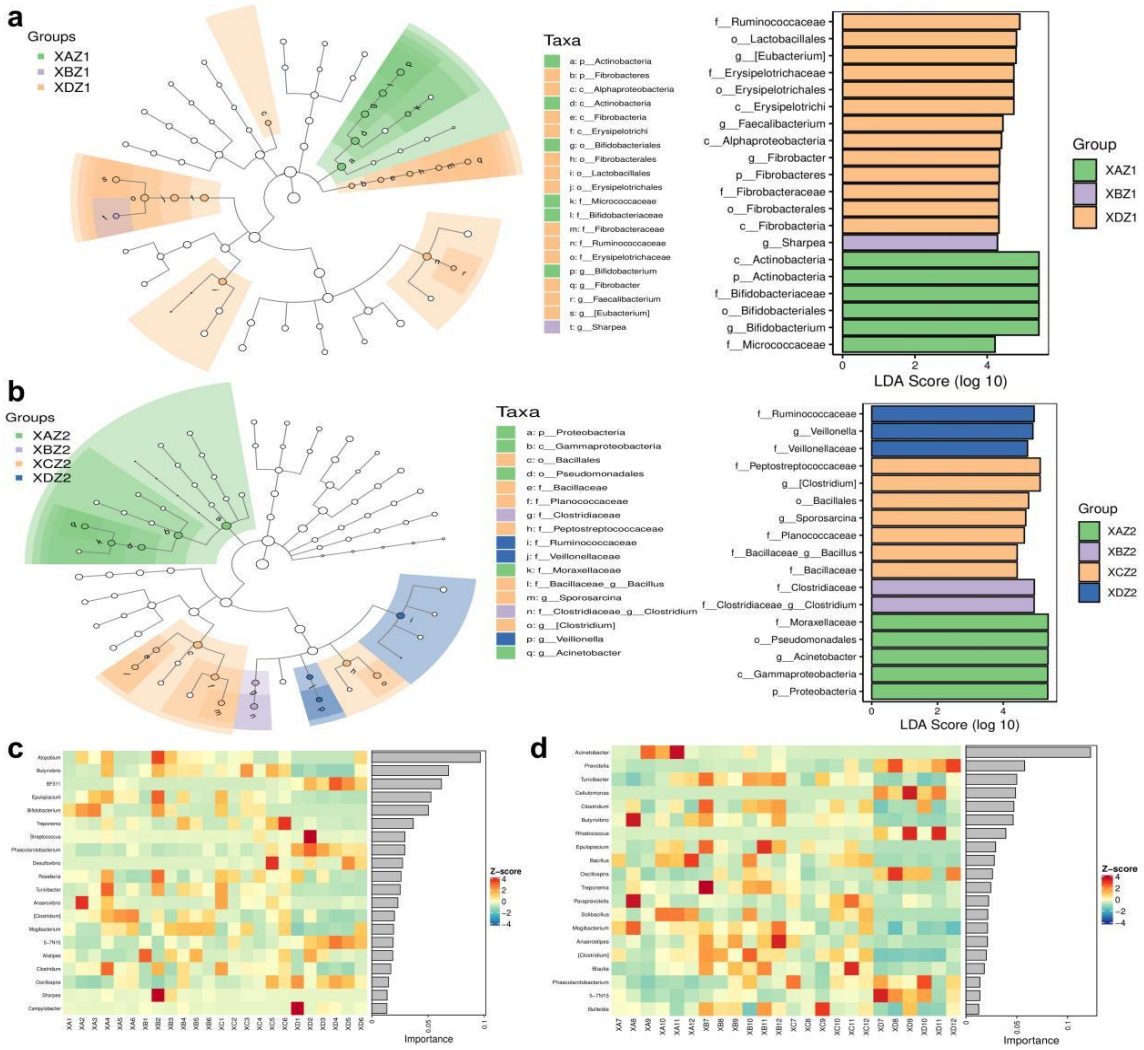

Fig. 5: Differential microbiota variation analysis. (a, b) Linear discriminant analysis effect size (LEfSe) analysis identifying key microbial taxa with significant differences between groups. (c, d) Random Forest analysis highlighting the most important microbial taxa contributing to group differentiation.

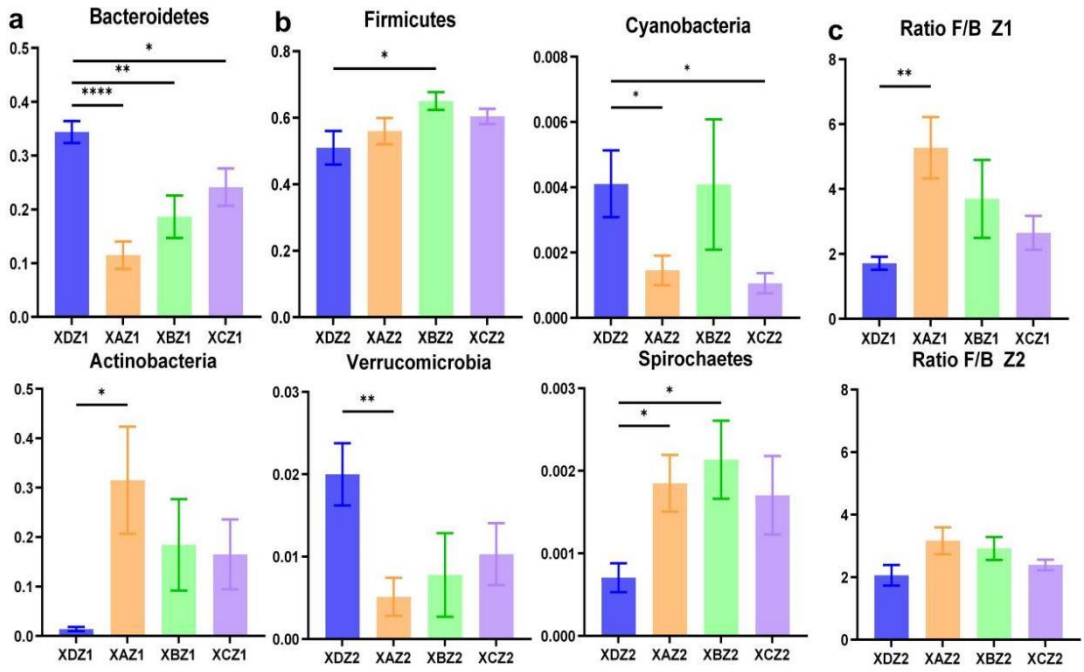

Fig. 6: Differential analysis at the phylum level via T-test. (a) Phyla with significant differences in group XZ1. (b) Phyla with significant differences in group XZ2. (c) Firmicutes/ Bacteroidetes ratio.

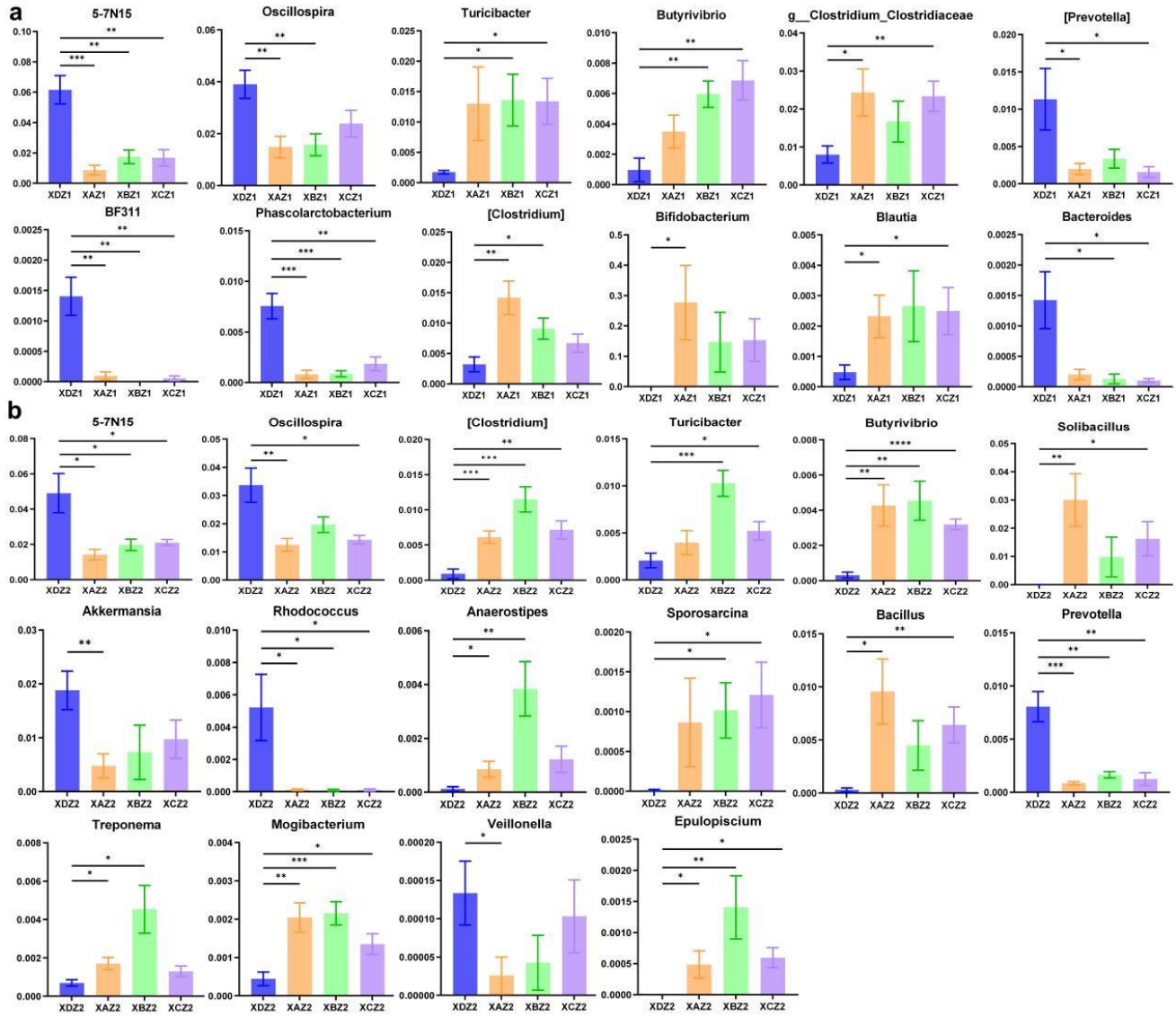

Fig. 7: Differential analysis at the genus level via T-test. (a) Genera showing significant differences in relative abundance in group XZ1. (b) Genera showing significant differences in relative abundance in group XZ2.

DISCUSSION

TCHMFs have been utilized in clinical practices for immuno-stimulation, as antioxidants (Matkowski *et al.*, 2013), regulators of energy metabolism, to reduce inflammation, and to beneficially modulate the gut microbiome (Ran *et al.*, 2022). Numerous studies have reported the positive impacts of TCHMFs on livestock production. For instance, TCHMFs as feed additives have shown their influence on growth paraments and intestinal morphology in weaned piglets (Wang *et al.*, 2020). Additionally, TCHMFs have enhanced the reproductive performance in perinatal cows through their antioxidant mode of action (Ran *et al.*, 2022).

In this study, it was observed that T-AOC was increased, while MDA content decreased in weaned yaks supplemented with TCHMFs in their diet. SOD activity also increased over the experimental period, indicating a reduction in oxidative stress in the experimental groups of yaks. Additionally, the key pro-inflammatory interleukin-6 decreased, while the anti-interleukin-10 content increased in TCHMFs diet groups. It can be inferred that TCHMF intake effectively enhanced the antioxidant capacity of weaned yaks and alleviated inflammatory reactions, consistent with the pharmacological effects of traditional Chinese medicine formulas.

Variations in the gut microbiota composition can reflect the host's intestinal health, and metabolic and immune changes, and are considered essential elements regulating the host's health (de Vos *et al.*, 2022). At the phylum level, *Bacteroidetes* were reduced, while *Firmicutes* and *Actinobacteria* increased significantly in the TCHMF diet groups. *Firmicutes* and *Bacteroidetes* are dominant bacteria in the intestine of ruminants, playing crucial roles in digestion, nutritional metabolism (Faniyi *et al.*, 2019), and intestinal metabolic homeostasis. The *Firmicutes/Bacteroidetes* ratio is related to inflammatory markers and gut homeostasis. The increase or decrease in the mentioned ratio indicates gut dysbiosis. The former often manifests metabolic diseases like obesity, while the latter is bound up with inflammatory bowel disease (Lozupone *et al.*, 2012; Stojanov *et al.*, 2020). Despite the potential presence of harmful species, *Firmicutes* still regulate immune response, inhibit pathogens' invasion,

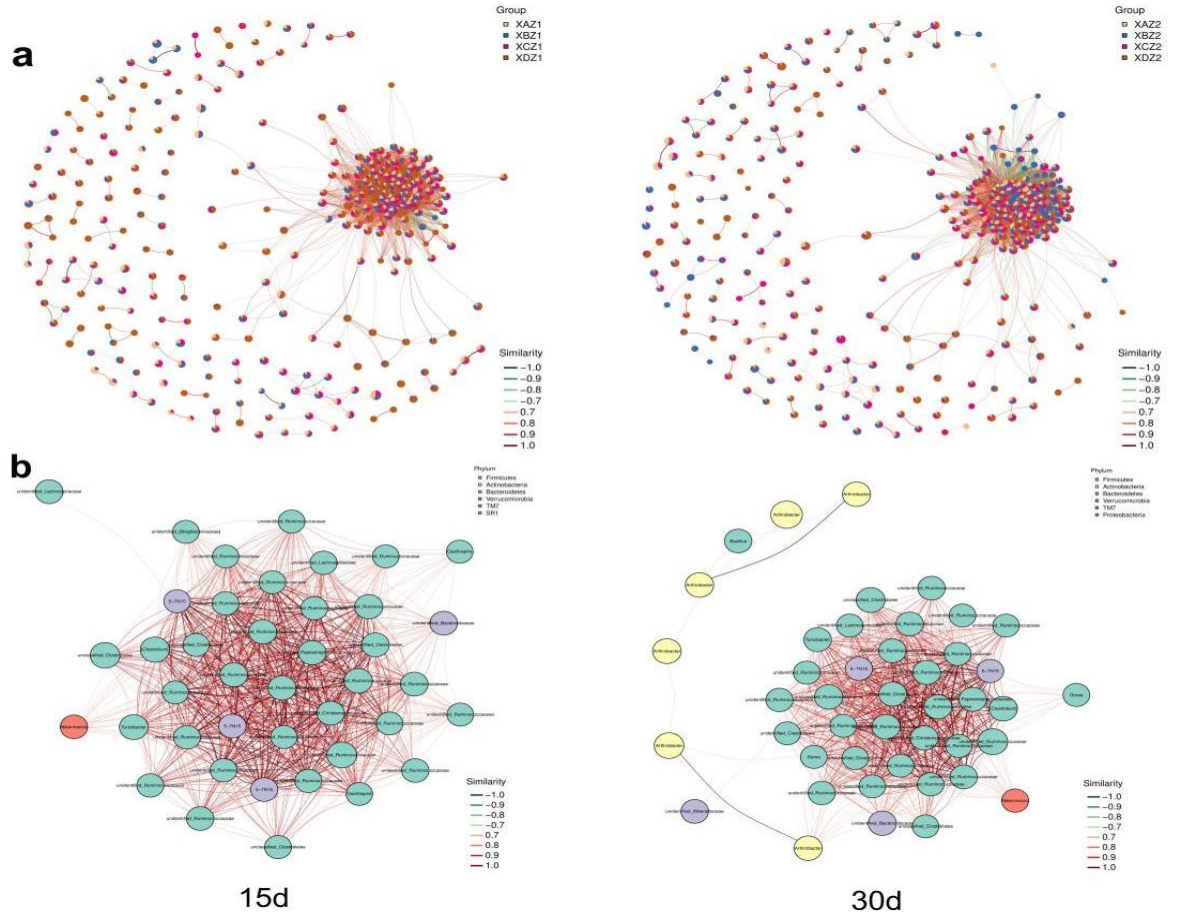

Fig. 8: Association network analysis of microbial communities. (a) Modular association network diagram illustrating co-occurrence patterns and modularity among microbial taxa. (b) Advantage seed network diagram based on phylum-level annotation, highlighting key taxa and their interactions within the network.

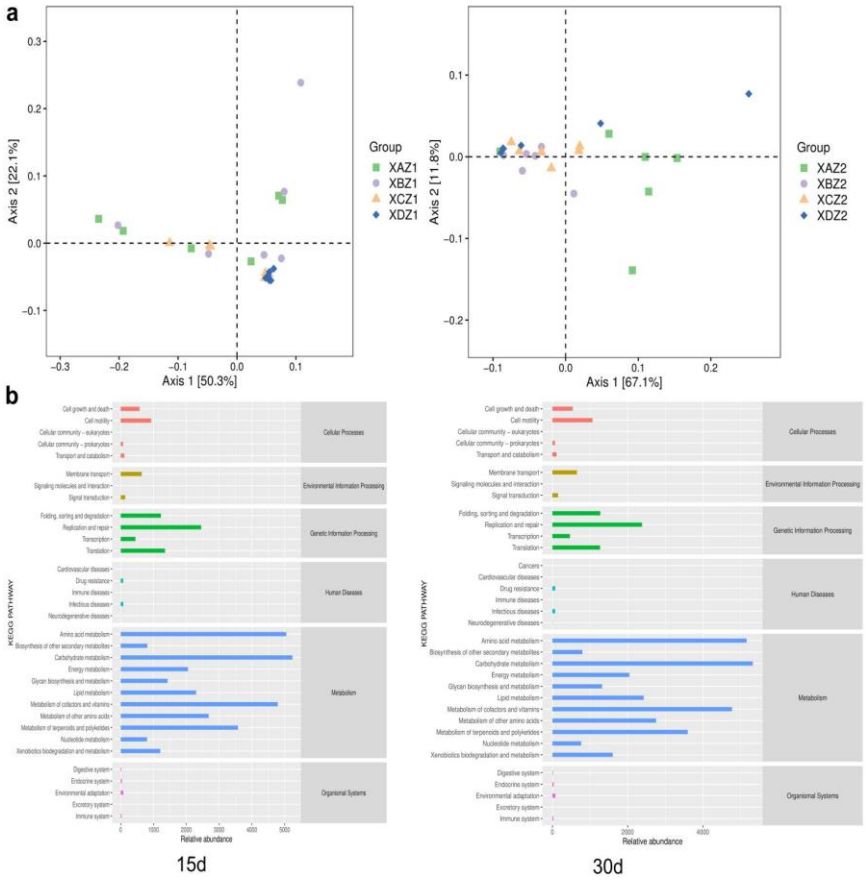

Fig. 9: Functional potential prediction. a. PCoA analysis of functional unit; b. Abundance of secondary functional pathways in the KEGG database.

and prevent intestinal inflammation (Li *et al.*, 2018; Faniyi *et al.*, 2019; Wang *et al.*, 2019). During the experiment, the captive experimental groups' diets contained more grain products. Therefore, the increase in *Firmicutes/ Bacteroidetes* ratio in the experimental groups may be due to short-term dietary changes. However, in the long run, Chinese herbal medicine intake reduced the increase in the ratio and stabilized it (Fig. 6c). The *Firmicutes/ Bacteroidetes* ratio slightly increased in the TCHMF diet groups. Considering the decrease in serum inflammatory factors, it can be speculated that TCHMF intake regulated the intestinal microbiota structure and reduced the likelihood of intestinal inflammation. *Actinobacteria* exhibited higher abundance in the experimental groups, especially in group XA. Although its proportion is relatively low, it holds a key position in maintaining intestinal homeostasis. Numerous species within *Actinobacteria*, particularly genus *Bifidobacterium*, serve as probiotics and demonstrate beneficial effects under many pathological conditions (Binda *et al.*, 2018). It was reported that *Bifidobacterium* plays a crucial role in reducing the occurrence of intestinal diseases during weaning in yaks, having beneficial homeostatic and anti-inflammatory immune regulatory properties (Gavzy *et al.*, 2023). Besides, some species in *Bifidobacterium*, as well as in *Anaerostipes* produce butyrate, which is an essential metabolite in the intestine that helps to maintain intestinal barrier function and has immunomodulatory and anti-inflammatory properties (Rivière *et al.*, 2016). Results found that *Bifidobacterium* and *Anaerostipes* were deficient in the control group but had a high abundance in the experiment groups, reflecting the anti-inflammatory and immune-promoting effects of Chinese herbal medicine.

Among other genera with significant variations, *5-7N15* was always enriched in the control group. According to Li *et al*.'s description, *5-7N15* is one of the genera with the highest abundance in the digestive tract of yaks before and after weaning, and there is a significant positive correlation between *5-7N15* and crude fiber digestion rate. In this study, the high abundance of *5-7N15* in the control group may be related to the free consumption of natural grass containing a large amount of crude fiber by yaks in the control group. However, over time, the abundance of *5-7N15* decreased in the control group, while remaining stable in the experimental group. Intestinal anaerobic bacteria *Butyrivibrio*, *Clostridium*, and *Blautia* are the core flora producing short-chain fatty acids (SCFAs) and play a vital role in maintaining intestinal health, adjusting the pH value of the intestinal environment and affecting mucosal immune function to maintain intestinal integrity (Palevich *et al.*, 2019; Liu *et al.*, 2021a; de Mooij *et al.*, 2023).

Genus that may have negative effects were enriched in the control group. Genus *Prevotella* consists of a variety of species, having rich ecological niches, some of the species were associated with chronic inflammation in the body (Larsen, 2017; Iljazovic *et al.*, 2021), and were believed to be significantly positively correlated with unhealthy fat accumulation in pigs through the occurrence of chronic inflammation (Chen *et al.*, 2021). In addition, the increase of *Prevotella* and decrease of *Bacteroides* was reported to be related to the loss of beneficial microorganisms in Chronic inflammatory diseases (Larsen, 2017). Sui Y *et al.* found that the abundance of *BF311* significantly increased in the gut of sheep treated with aflatoxin B1, which may be a marker genus related to liver injury (Sui *et al.*, 2022). In another study, it was observed that Chinese herbal medicine reduced the abundance of the above-mentioned genera, and reduced possible inflammatory reactions and liver damage (Sui *et al.*, 2022). Berberine present in these herbal formulas has strong antibacterial effects against *Hemolytic streptococcus*, *Pneumococci*, and *Staphylococcus aureus*, among others. Moreover, it enhances the sensitivity of intestinal bacteria to antibiotics such as streptomycin, chloramphenicol, and oxytetracycline. In husbandry production, Berberine supplementation has been shown to reduce Bovine endometritis (Fu *et al.*, 2021) and exerts positive effects on fatty liver in perinatal cows (Shi *et al.*, 2018).

Conclusions: In conclusion, this study affirms that supplementing Traditional Chinese Herbal Medicine Formulas (TCHMFs) can boost immunity and substantially enhance antioxidant stress capacity in weaning yaks. The supplementation of TCHMF in the diet promotes the stability of the gut microbiota in yaks, increases the ratio of *Firmicutes*/*Bacteroidetes*, and the abundance of beneficial bacteria genera like *5-7N15*, *Butyrivibrio*, *Clostridium*, *Blautia*, while suppressing the harmful genera like *BF311*. Consequently, incorporating healthy, antibiotic-free traditional Chinese medicine formulas into the diet of weaned yaks emerges as a practical and effective strategy for enhancing their physical well-being and encouraging the economic prospects of the yak breeding industry. The small sample size, short duration, and focus on young, weaned yaks limit the generalizability and long-term application of the study. Additionally, environmental influences, limited species-level resolution of 16S rRNA sequencing, and unassessed contributions of individual components in the Chinese medicine formulas need further investigation.

Ethical approval and consent to participate: All the experiment procedures were performed under the ethics committee of Nanjing Agricultural University (NJAU. No. 20240321054).

Data availability: The raw data in this study were deposited in the NCBI database under accession number (PRJNA1102654).

Competing interests: The authors declare no competing interests.

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Authors contribution: Conceptualization was from KL and WB, and methodology was designed by YZ, SL, XZ, FAK, and YC. The software was implemented by SL, validation was done by KL and WB, formal analysis was performed by YZ, SL, MA, and YC, the investigation was

conducted by YZ, SL, MA, and YC, resources were managed by KL and WB, data curation was conducted by SL and PD, writing-original draft preparation was performed by YZ, JT, MA, and KL, writing—review and editing was done by FAK, SMU, KL and WB, visualization by KL and WB, supervised by KL and WB, project administration was conducted by WB, funding acquisition was by KL and YBZ. All authors have read and agreed to the published version of the manuscript.

REFERENCES

Binda C, Lopetuso LR, Rizzatti G, *et al*., 2018. Actinobacteria: A relevant minority for the maintenance of gut homeostasis. Dig Liver Dis 50:421-428.

Chen C, Fang S, Wei H, *et al*., 2021. Prevotella copri increases fat accumulation in pigs fed with formula diets. Microbiome 9:175.

Chen X, Zhao X, Zhao C, *et al*., 2023. Cryptosporidium infection induced the dropping of SCFAS and dysbiosis in the intestinal microbiome of Tibetan pigs. Microb Pathog174:105922.

de Mooij CEM, van Groningen LFJ, Molendijk EBD, *et al*., 2023. Blautia Abundance and Mucosal Barrier Injury: A Complex Play of Cause and Effect. Clin Infect Dis 76:1152-1153.

de Vos WM, Tilg H, Van Hul M, *et al*., 2022. Gut microbiome and health: mechanistic insights. Gut 71:1020-1032.

Deng C, 2010. Thinking and practice on research of effective components of traditional Chinese medicine compound prescriptions. Zhong xi yi jie he xue bao= J Chin Integ Med 8:1107-1113.

Ding A and Wen X. 2018. Dandelion root extract protects NCM460 colonic cells and relieves experimental mouse colitis. J Nat Med 72:857-866.

Fan M, Zhang X, Song H, *et al*., 2023. Dandelion (Taraxacum Genus): A review of chemical constituents and pharmacological effects. Molecules 28(13):5022.

Faniyi TO, Adegbeye MJ, Elghandour M, *et al*., 2019. Role of diverse fermentative factors towards microbial community shift in ruminants. J Appl Microbiol 127:2-11.

Fox NJ, Marion G, Davidson RS, *et al*., 2013. Modeling parasite transmission in a grazing system: the importance of host behavior and immunity. PloS One 8:e77996.

Fu K, Wang Z and Cao R, 2021. Berberine attenuates the inflammatory response by activating the Keap1/Nrf2 signaling pathway in bovine endometrial epithelial cells. Int Immunopharmacol 96:107738.

Gavzy SJ, Kensiski A, Lee ZL, *et al*., 2023. Bifidobacterium mechanisms of immune modulation and tolerance. Gut Microbes15:2291164.

Iljazovic A, Amend L, Galvez EJC, *et al*., 2021. Modulation of inflammatory responses by gastrointestinal Prevotella spp. - From associations to functional studies. Int J Med Microbiol 311:151472.

Johnson JS, Spakowicz DJ, Hong BY, *et al*., 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun 10:5029.

Larsen JM, 2017. The immune response to Prevotella bacteria in chronic inflammatory disease. Immunology 151:363-374.

Lee HS, Beon MS, Kim MK, 2001. Selective growth inhibitor toward human intestinal bacteria derived from *Pulsatilla cernua* root. J Agric Food Chem 49:4656-61.

Leite CDS, Bonafé GA, Carvalho Santos J, *et al*., 2022. The anti-inflammatory properties of Licorice (*Glycyrrhiza glabra*)-derived compounds in intestinal disorders. Int J Mol Sci 23(8):4121.

Li A, Liu C, Han X, *et al*., 2023a. Tibetan Plateau yak milk: A comprehensive review of nutritional values, health benefits, and processing technology. Food Chem 20:100919.

Li L, Xiao S, Dai X, *et al*., 2024. Multi-omics analysis and the remedial effects of Swertiamarin on hepatic injuries caused by CCl4. Ecotox Environ Saf 282:116734.

Li Y, Chen Y, Sun-Waterhouse D, 2022a. The potential of dandelion in the fight against gastrointestinal diseases: A review. J Ethnopharmacol 293:115272.

Li Y, Hu X, Yang S, *et al*., 2018. Comparison between the fecal bacterial microbiota of healthy and diarrheic Captive Musk Deer. Front Microbiol 9:300.

Li Y, Li X, Liu Y, *et al*., 2022b. Comparison of bacterial and fungal community structure and potential function analysis of yak feces before and after weaning. Biomed Res Int 2022:6297231.

Li Z, Song Y, Xu W, *et al*., 2023b. Pulsatilla chinensis saponins improve SCFAs regulating GPR43-NLRP3 signaling pathway in the treatment of ulcerative colitis. J Ethnopharmacol 308:116215.

Liu X, Mao B, Gu J, *et al*., 2021a. Blautia-a new functional genus with potential probiotic properties? Gut Microbes 13:1-21.

Liu Y, Zhou M, Yang M, *et al*., 2021b. Pulsatilla chinensis Saponins ameliorate inflammation and dss-induced ulcerative colitis in rats by regulating the composition and diversity of intestinal flora. Front Cell Infect Microbiol 11:728929.

Lozupone CA, Stombaugh JI, Gordon JI, *et al*., 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220-30.

Lu S, Zou W, Chen X, *et al*., 2023. Effects of *Cryptosporidium parvum* infection on intestinal fungal microbiota in yaks (*Bos grunniens*). Microb Pathog 183:106322.

Matkowski A, Jamiołkowska-Kozlowska W, Nawrot I, 2013. Chinese medicinal herbs as source of antioxidant compounds--where tradition meets the future. Curr Med Chem 20:984-1004.

Meng A, Zhang X, Pubu P, *et al*., 2024. Protective effect of Lentinan against LPS-Induced injury in mice via influencing antioxidant enzyme activity, inflammatory pathways and gut microbiota. Pak Vet J 44(3):647-656.

Palevich N, Kelly WJ, Leahy SC, *et al*., 2019. Comparative genomics of rumen butyrivibrio spp. uncovers a continuum of polysaccharide-degrading capabilities. Appl Environ Microbiol 13:86:e01993-19

Ran M, Cha C, Xu Y, *et al*., 2022. Traditional Chinese herbal medicine complex supplementation improves reproductive performance, serum biochemical parameters, and anti-oxidative capacity in periparturient dairy cows. Anim Biotechnol 33:647-656.

Rivière A, Selak M, Lantin D, *et al*., 2016. Bifidobacteria and Butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. Front Microbiol 7:979.

Rognes T, Flouri T, Nichols B, *et al*., 2016. VSEARCH: a versatile open-source tool for metagenomics. Peer J 4:e2584.

Rowland I, Gibson G, Heinken A, *et al*., 2018. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr 57: 1-24.

Sham T-T, Chan C-O, Wang Y-H, *et al*., 2014. A review on the traditional Chinese medicinal herbs and formulae with hypolipidemic effect. BioMed Res Int 2014:925302.

Shi Z, Li XB, Peng ZC, *et al*., 2018. Berberine protects against NEFA-induced impairment of mitochondrial respiratory chain function and insulin signaling in bovine hepatocytes. Int J Mol Sci 19(6):1691.

Stojanov S, Berlec A, Štrukelj B, 2020. The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. Microorg 8(11):1715.

Sui Y, Lu Y, Zuo S, *et al*., 2022. Aflatoxin B (1) exposure in sheep: insights into hepatotoxicity based on oxidative stress, inflammatory injury, apoptosis, and gut microbiota analysis. Toxins (Basel):14(12):840.

Wang L, Zhang G, Xu H, *et al*., 2019. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of Holstein cows fed different forage-to-concentrate ratios. Front Microbiol10:649.

Wang M, Huang H, Hu Y, *et al*., 2020. Effects of dietary supplementation with herbal extract mixture on growth performance, organ weight and intestinal morphology in weaning piglets. J Anim Physiol Anim Nutr (Berl)104:1462-1470.

Xi L, Song Y, Han J, *et al*., 2021. Microbiome analysis reveals the significant changes in gut microbiota of diarrheic Baer's Pochards (Aythya baeri). Microb Pathog 157:105015.