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Effects of concentrate supplementation on fecal microbiota, serum metabolomics, and proteomics in grazing Yili horses

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Abstract

Background

Under grazing conditions, natural pasture alone may not fully meet the nutritional requirements of Yili horses. Seasonal variation and rainfall can also markedly affect pasture quality and nutrient composition, which may restrict the health management of Yili horses and the development of mare milk resources. Therefore, this study investigated the effects of concentrate supplementation on fecal fermentation parameters, serum metabolomic profiles, and serum proteomic profiles in grazing Yili horses.

Materials and methods

Sixteen healthy early-lactation Yili mares of similar age (3–4 years), foaling date (May 2023), and body weight (391.5 ± 13.74 kg) were randomly assigned to a grazing group (G) or a grazing plus concentrate supplementation group (GS), with eight mares in each group. Mares in the G group grazed naturally on pasture, whereas mares in the GS group received concentrate supplementation twice daily under grazing conditions, with 1 kg provided at each feeding (2 kg/day). The experiment lasted 100 days, including a 10-day adaptation period and a 90-day experimental period. On day 90 of the experimental period, fecal and blood samples were collected to determine fecal fermentation parameters, fecal microbial diversity, serum biochemical indices, serum metabolomic profiles, and serum proteomic profiles.

Results

Fecal fermentation and microbiota analyses showed that, compared with the G group, the GS group had significantly higher fecal propionate and butyrate concentrations, whereas fecal pH and the acetate-to-propionate ratio were significantly lower ($P < 0.05$). At the genus level, the relative abundances of *Lachnoclostridium*, *Roseburia*, *Anaerosacchariphilus*, and *Coprococcus* were significantly higher in the G group than in the GS group, whereas Treponema and Oliverpabstia were significantly less abundant in the G group than in the GS group ($P < 0.05$). Serum biochemical and metabolomic analyses showed that concentrate supplementation decreased total bilirubin (TBIL), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) concentrations, while increasing serum glucose (GLU) concentration ($P < 0.05$). Differential metabolites were mainly enriched in pathways related to the tricarboxylic acid cycle, nucleotide metabolism, bile secretion, vitamin B6 metabolism, and pyruvate metabolism. Serum proteomic analysis showed increased abundances of peroxiredoxin-5 (PRDX5), lactoylglutathione lyase (GLO1), transketolase, and several immune-related proteins, whereas phospholipid transfer protein, annexin, kallikrein-related peptidase 11, and several immunoglobulin light-chain fragments showed decreased abundance.

Conclusions

Concentrate supplementation under grazing conditions altered fecal fermentation patterns and fecal microbial composition in Yili horses, increased the production of fermentation products such as propionate and butyrate, and regulated pathways related to serum glucose and lipid metabolism, bile acid metabolism, antioxidant responses, and energy metabolism. These findings suggest that moderate concentrate supplementation may improve the nutritional and metabolic status of grazing Yili horses and provide a reference for feeding management during lactation.

Introduction

The Yili horse is a well-established dual-purpose breed developed in China. It is characterized by strong tolerance to roughage, cold resistance, and athletic performance [1]. Traditional grazing systems are constrained by season, climate, and pasture resources, and the supply of energy and protein may be insufficient to meet the nutritional requirements of horses used for different production purposes during certain periods. In addition, overgrazing can accelerate grassland degradation and desertification, which has become an important challenge for the sustainable development of grassland animal husbandry [2]. Therefore, improving the nutrient supply of grazing horses through appropriate concentrate supplementation, while maintaining grassland ecological stability, is important for optimizing feeding management and promoting the development of the horse industry.

Equids rely mainly on microbial fermentation in the cecum and colon for the digestion and utilization of dietary nutrients [3]. Hindgut microorganisms degrade structural carbohydrates, such as cellulose and hemicellulose, into volatile fatty acids (VFAs), including acetate, propionate, and butyrate, which provide energy for the host [4]. Appropriate concentrate supplementation can meet animals' nutritional requirements and improve energy utilization efficiency. However, an inappropriate concentrate proportion or imbalanced dietary structure may disturb the hindgut fermentation environment and thereby interfere with nutrient metabolism and health status in horses. Lu et al. [5] reported that concentrate and fat supplementation in grazing Yili mares effectively increased milk yield and altered fecal microbial structure. Harlow et al. [6] further found that different starch sources and levels significantly changed the structure of the fecal microbiota in horses. In addition, plasma proteomics and metabolomics have been used to monitor subtle physiological changes in horses under different feeding systems and training intensities [7–9]. Li et al. [10] found that increasing the proportion of dietary non-fibrous carbohydrates (NFC) affected intestinal microbial composition and altered blood metabolites in Yili horses, which may be associated with improved nutrient digestion and absorption. Nilsson et al. [11] compared the effects of starch-free and high-starch diets on the plasma metabolome of exercised horses and found that plasma glycine concentration was increased in horses fed the high-starch diet.

Few studies have integrated gut microbiota, fermentation parameters, serum metabolomics, and proteomics to evaluate the effects of feeding strategies in equids. Therefore, the present study investigated concentrate supplementation in grazing Yili horses. By combining fecal fermentation

parameters, serum biochemical indices, serum metabolomics, and serum proteomics, this study aimed to clarify the effects of concentrate supplementation on hindgut microbial community structure and host metabolism in grazing Yili horses and to provide a theoretical basis for precision nutritional management.

Materials and methods

Experimental Design

The experiment was conducted in Zhaosu Horse Farm, Zhaosu County, Ili Kazakh Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China. Sixteen Yili mares aged 3–4 years, with 2–3 parities and similar body weight (391.5 ± 13.74 kg), were selected for this study. The mares were randomly assigned to two groups: a grazing group (G) and a grazing plus concentrate supplementation group (GS), with eight mares in each group. Mares in the G group grazed naturally on pasture, whereas mares in the GS group received concentrate supplementation under grazing conditions at 11:00 and 19:00 each day, with 1 kg provided at each feeding (2 kg/day). The experiment lasted 100 days, including a 10-day adaptation period and a 90-day experimental period. All experimental mares were obtained from the same pasture and were monitored by professional veterinarians throughout the trial to ensure that they remained in good health. The only difference between the two groups was that mares in the GS group received an additional 2 kg/day of concentrate supplement.

The pasture was mainly composed of graminaceous plants. The concentrate supplement was formulated according to the nutrient requirements of lactating mares with a body weight of 400 kg, based on the NRC guidelines for horses [4]. The ingredient composition and nutrient levels of the concentrate supplement are shown in Table 1.

Table 1
Composition and nutrient levels of the concentrate supplement (dry matter-based, %)

Concentrate Ration		**Nutrient Levels** [2]		
Ingredients	**Contents**	**Item**	**Concentrate**	**Pasture**
Corn	52.00	CP	16.95	10.57
Bran	12.00	EE	7.52	3.31
Barley	10.00	NDF	25.86	60.44
Soybean meal	20.00	ADF	3.15	29.93
$CaHPO_4$	2.00	Ash	8.07	7.71
Salt	2.00	Ca	1.32	1.16
Limestone powder	1.00	P	0.74	0.23
Premix [1]	1.00	GE, MJ/kg	18.86	17.65
Total	100.00			

[1] The premix provided the following amounts per kilogram of concentrate supplement: Co, 0.5 mg; Cu, 100 mg; I, 3.5 mg; Fe, 500 mg; Mn, 400 mg; Zn, 400 mg; Se, 1 mg; vitamin A, 24,000 IU; vitamin D, 2,640 IU; vitamin E, 400 IU; vitamin B_1, 30 mg; vitamin B_2, 20 mg; vitamin B_3, 240 mg; choline chloride, 120 mg; and phytase, 50 mg. [2] Ingredient proportions of the concentrate supplement are formulated values. Nutrient levels of the concentrate supplement and pasture are measured values.

Sample collection

Five grazing exclusion cages were placed evenly along the mares' grazing route. Forage species consumed by the mares within the cages were selectively collected, cut at ground level, thoroughly mixed, and sampled to obtain 500 g of material. The forage samples were air-dried in a cool, ventilated place and then stored. Concentrate supplement samples were randomly collected from different bags and mixed to obtain a 1 kg composite sample, from which a 200 g subsample was retained. After transport to the laboratory, forage samples were dried, ground, passed through a 40-mesh sieve, and stored in sealed bags until analysis.

On the last day of the experiment, blood samples were collected 2 h after feeding. Approximately 5 mL of blood was collected from each mare using plain blood collection tubes. The samples were kept at 4°C for 4 h to allow clotting and then centrifuged at 3,000 r/min for 10 min. Serum was transferred into 1.5 mL cryotubes and stored at -80°C until analysis.

Fecal samples were collected on the last day of the experiment after natural defecation. Fresh feces from the middle portion of each fecal bolus were collected immediately using sterile shovels, placed into

5 mL sterile cryotubes, and stored at -80°C until analysis.

Sample analysis and calculation

Feed samples, including forage and concentrate supplement samples, were dried at 65°C for 48 h until a constant weight was achieved. The dried samples were ground and passed through a 1 mm sieve for subsequent analysis. The nutrient components analyzed included dry matter (DM; method 930.15, determined by drying at 105°C to constant weight), ether extract (EE; method 989.05, determined using Soxhlet extraction with ether), and crude protein (CP; method 976.05, determined by the Kjeldahl method using a Buchi B-324 distillation unit; Büchi Labortechnik AG, Switzerland). These analyses were performed according to the methods of the Association of Official Analytical Chemists (AOAC) [12]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the Van Soest method [13], with filter bags and an ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedonia, NY, USA).

For fecal pH measurement, 5 g of fecal sample was mixed with 15 mL of distilled water at a 1:3 (w/v) ratio and stirred at room temperature for 3–5 min [14]. The pH was then measured using a portable pH meter (FiveEasy22-Meter, Shanghai, China).

Fecal concentrations of acetate, propionate, butyrate, isobutyrate, and isovalerate were determined by gas chromatography. Sample pretreatment was performed as described by Huang et al. [15]. VFAs were quantified using a gas chromatograph (GC-2010, Shimadzu Corporation, Kyoto, Japan) equipped with a Stabilwax capillary column (30 m × 0.20 mm × 0.33 μm; Shimadzu Corporation, Kyoto, Japan). The chromatographic conditions were as follows: the initial column oven temperature was 55°C, which was increased to 200°C at 13°C/min, and then held for 0.5 min. The injector and detector temperatures were maintained at 230°C and 240°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 5.0 mL/min.

Extraction and sequencing of DNA

Fecal microbial DNA was extracted using a fecal genomic DNA extraction kit (TIANGEN, Shanghai, China). DNA quality was evaluated by 1% agarose gel electrophoresis. Full-length PCR amplification of the target 16S rRNA gene was performed using the universal primers 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-RGYTACCTTGTTACGACTT-3'), which were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

PCR amplification program was as follows: initial denaturation at 95°C for 5 min; followed by 27 cycles of denaturation at 98°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 60 s; and a final extension at 72°C for 5 min. The PCR products were purified using SMRTbell Cleanup Beads and quantified using Qubit. The second-round amplified 16S libraries were concatenated using the Kinnex Concatenation Kit, and sequencing was performed on the PacBio platform.

Serum concentrations of glucose (GLU), albumin (ALB), globulin (GLB), total protein (TP), urea (UREA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), alkaline phosphatase (ALP), triglycerides (TG), total cholesterol (CHO), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using an automatic biochemical analyzer (Beckman AU5800; Beckman Coulter Ltd., Brea, CA, USA).

Analysis of serum metabolomics

Samples were thawed at 4°C and vortexed thoroughly. A 200 µL aliquot of each sample was mixed with 800 µL of pre-chilled methanol and incubated at -20°C for 30 min. The mixture was then centrifuged at 16,000 × g for 20 min at 4°C, and the supernatant was collected for mass spectrometry analysis.

Chromatographic separation was performed using a Waters UPLC I-Class ultra-high-performance liquid chromatography system. A Waters ACQUITY UPLC HSS T3 column (1.7 µm, 2.1 × 100 mm) was used for separation. The injection volume was 4 µL, the column temperature was maintained at 40°C, and the flow rate was 300 µL/min. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The gradient elution program was as follows: 0-1.5 min, 0% B; 1.5-6 min, 0–48% B; 6–10 min, 48–100% B; 10–12 min, 100% B; 12-12.1 min, 100-0% B; and 12.1–15 min, 0% B.

Mass spectrometry was performed using an AB SCIEX TripleTOF® 5600 + mass spectrometer. An electrospray ionization (ESI) source was used, and data were acquired in both positive-ion (ESI+) and negative-ion (ESI-) modes under information-dependent acquisition (IDA).

Serum proteomic analysis

Serum samples were prepared for DIA-based proteomic analysis. Briefly, 5 µL of serum sample was mixed with 50 µL of denaturation buffer containing 8 M urea in 100 mM ammonium bicarbonate. Proteins were reduced with 10 mM dithiothreitol at 37°C for 30 min and alkylated with 50 mM iodoacetamide in the dark at room temperature. The samples were then diluted with 50 mM ammonium bicarbonate and digested with trypsin at an enzyme-to-protein ratio of 1:50 overnight at 37°C. The digested peptides were desalted using C18 StageTips for subsequent LC-MS/MS analysis. Peptide concentrations were determined at OD280 using a NanoDrop One spectrophotometer.

LC-MS/MS analysis was performed on an Orbitrap Astral mass spectrometer coupled to a Vanquish Neo UHPLC system (Thermo Fisher Scientific). Peptides from each sample were loaded onto a 50 cm Low-Load µPAC Neo HPLC column at a flow rate of 2.2 µL/min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in 80% acetonitrile. Peptides were eluted over 8 min using a linear gradient of mobile phase B at a flow rate of 1.25 µL/min. The gradient was set as follows: 0-0.1 min, 4–6% B; 0.1–1.1 min, 6–12% B; 1.1–4.3 min, 12–25% B; 4.3–6.1 min, 25–45% B; 6.1–6.5 min, 45–99% B; and 6.5-8 min, 99% B.

For DIA acquisition, the survey scan range was set from 380 to 980 m/z at a resolution of 240,000, with an AGC target of 500% and a maximum injection time of 5 ms. DIA MS/MS scans were acquired using Astral detection over a range of 150-2,000 m/z, with a 2 m/z isolation window, an AGC target of 500%, and a maximum injection time of 3 ms. The normalized collision energy was set to 25, and the cycle time was 0.6 s.

DIA data were analyzed using DIA-NN software version 1.8.1. The MS data were searched against the UniProtKB/Swiss-Prot database for Equus caballus. Trypsin was selected as the digestion enzyme, with a maximum of one missed cleavage allowed. The mass tolerance was set to 10 ppm for precursor ions and 10 ppm for fragment ions. Carbamidomethylation of cysteine was set as a fixed modification, whereas protein N-terminal acetylation and methionine oxidation were set as variable modifications. Peptide length was set from 7 to 30 amino acids, and peptide charge states ranged from 1 to 4, and the fragment ion m/z range was set from 150 to 2,000. The database search results were filtered using a false discovery rate (FDR) of < 1% at both the peptide-spectrum match and protein levels.

Statistical analysis

Preliminary data organization was performed using Microsoft Excel 2019. Normality and homogeneity of variance were assessed using the Shapiro-Wilk test and Levene's test in SPSS 26.0 (IBM Corp., Armonk, NY, USA), respectively. Fecal fermentation parameters and serum biochemical indices were analyzed using an independent-samples t-test. GraphPad Prism 10.0 was used to visualize fecal fermentation parameters, differential bacterial genera, and differential metabolites. All results are presented as the mean ± standard deviation. Statistical significance was defined as $P < 0.05$, and highly significant differences were defined as $P < 0.01$.

Raw sequencing data from fecal microbiota were denoised using DADA2 (v1.14.0). Taxonomic assignment was performed using BLAST (v2.10.0) against the NCBI NT database, with a threshold of 0.8. After quality control, each dereplicated sequence was defined as an amplicon sequence variant (ASV). The rarefaction depth was set to 95% of the minimum sequencing depth among all samples. Alpha diversity was analyzed using Mothur software (v3.8.31). Beta diversity was estimated in QIIME2 based on weighted UniFrac distances. LEfSe analysis was performed using LEfSe software, with the default LDA threshold set to 3.

Bioinformatic analyses of metabolomic and proteomic data were performed using Microsoft Excel and R software. Hierarchical clustering heatmaps and volcano plots were generated in R. Protein functional annotation was performed using the UniProtKB/Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO) databases. GO and KEGG enrichment analyses were performed using Fisher's exact test, and false discovery rate (FDR) correction was applied for multiple testing.

Results

Effects of concentrate supplementation on serum biochemical indices in grazing Yili horses

As shown in Table 2, compared with the G group, the GS group showed significantly lower serum levels of TBIL, TC, and HDL-C ($P < 0.01$ or $P < 0.05$), and a significantly higher GLU concentration ($P < 0.05$). No significant differences were observed in the other serum biochemical indices between the two groups ($P > 0.05$).

Table 2
Effects of concentrate supplementation on serum biochemical indices in grazing Yili horses

Items	G	GS	*P*-value
UREA	7.62 ± 0.29	8.00 ± 0.60	0.132
GLU	4.26 ± 0.47^{b}	4.97 ± 0.54^{a}	0.013
ALT	4.88 ± 0.99	4.50 ± 0.76	0.409
AST	263.25 ± 38.13	244.88 ± 23.38	0.269
TBIL	7.85 ± 0.98^{Aa}	5.93 ± 0.43^{Bb}	< 0.010
ALP	232.00 ± 34.20	241.50 ± 31.13	0.570
TP	74.91 ± 3.37	74.66 ± 2.81	0.875
ALB	20.74 ± 1.04	19.68 ± 1.10	0.068
GLB	54.18 ± 3.84	54.99 ± 3.35	0.659
TC	1.99 ± 0.17^{a}	1.66 ± 0.27^{b}	0.011
TG	0.18 ± 0.03	0.22 ± 0.05	0.075
HDL-C	1.76 ± 0.16^{a}	1.45 ± 0.26^{b}	0.012
LDL-C	0.31 ± 0.04	0.27 ± 0.08	0.340

Note: Within the same row, different uppercase superscript letters indicate a highly significant difference ($P < 0.01$), different lowercase superscript letters indicate a significant difference ($P < 0.05$), and the absence of superscript letters indicates no significant difference ($P > 0.05$). G indicates the grazing group ($n = 8$), and GS indicates the grazing with concentrate supplementation group ($n = 8$).

Table 2
Differentially expressed serum proteins identified by DIA-based proteomic analysis between the G and GS groups

Accession ID	Protein name	*P*-value	FC	Significant
F6RCA8	Peroxiredoxin-5	< 0.001	1454.91	Up
A0A9L0SSM0	Lactoylglutathione lyase	0.001	65.630	Up
A0A9L0TIU5	Sialic acid acetylesterase	0.001	295.590	Up
A0A0A1E513	Immunoglobulin lambda light chain variable region (Fragment)	0.002	2.780	Up
P18907	Sodium/potassium-transporting ATPase subunit alpha-1	0.002	557.75	Up
A0A0A1E9D0	Immunoglobulin lambda light chain variable region (Fragment)	0.003	1.620	Up
B5BV06	Alpha-1-antitrypsin	0.003	3.060	Up
F7CR03	Phospholipid transfer protein	0.003	0.510	Down
A0A3Q2GW64	Heterogeneous nuclear ribonucleoproteins A2/B1	0.003	0.001	Down
A0A5F5PPA5	Serotransferrin	0.005	2.110	Up
F7AMI1	Ig-like domain-containing protein	0.007	0.490	Down
A0A0A1E422	Immunoglobulin lambda light chain variable region (Fragment)	0.007	1.740	Up
A0A0A1E458	Immunoglobulin lambda light chain variable region (Fragment)	0.008	2.060	Up
A0A5F5PMP3	Cadherin-1	0.008	121.49	Up
A0A9L0RPM9	transketolase	0.009	80.120	Up
A0A9L0RJU8	Kallikrein related peptidase 11	0.009	0.090	Down
P82615	Latherin	0.011	0.650	Down
A0A3Q2L7X0	Proteasome subunit alpha type	0.016	6.960	Up
F6WZW6	Proteasome subunit alpha type	0.017	2.060	Up
A0A9L0TA06	G protein-coupled receptor 39	0.019	3.180	Up
Q8MJV1	Myosin-2	0.025	0.001	Down
A0A0A1E6J8	Immunoglobulin lambda light chain variable region (Fragment)	0.025	0.640	Down

Accession ID	Protein name	*P*-value	FC	Significant
A0A3Q2HLF7	Receptor-type tyrosine-protein phosphatase F	0.026	0.600	Down
A0A0A1E464	Immunoglobulin lambda light chain variable region (Fragment)	0.027	1.560	Up
A0A9L0T9L5	Proteasome subunit alpha type	0.027	1.560	Up
F7ATL5	Keratin, type I cytoskeletal 17	0.027	4.070	Up
A0A3Q2LPE6	Annexin	0.032	0.570	Down
A0A0A1E417	Immunoglobulin lambda light chain variable region (Fragment)	0.036	0.190	Down
A0A9L0T2K0	Proteasome subunit alpha type	0.036	2.020	Up
A0A3Q2GX82	Coiled-coil domain containing 66	0.041	0.500	Down
A0A3Q2H839	Ig-like domain-containing protein	0.047	2.010	Up

Effects of concentrate supplementation on fecal pH and volatile fatty acids in grazing Yili horses

As shown in Fig. 1, compared with the G group, the GS group had significantly higher fecal concentrations of propionate and butyrate ($P < 0.01$), whereas fecal pH and the acetate-to-propionate ratio were significantly lower ($P < 0.05$). No significant differences were observed in the other indices between the two groups ($P > 0.05$).

Effects of concentrate supplementation on fecal bacterial diversity in grazing Yili horses

A total of 1,549,804 raw reads were detected from 16 fecal samples in this study, and 1,547,079 reads were retained after quality control. On average, 96,692 reads were generated per sample, with an average sequence length of 1,450 bp. Alpha diversity analysis showed that the Simpson index was significantly higher in the GS group than in the G group ($P < 0.05$). No significant differences were observed in the Observed Species, Shannon, or Chao1 indices between the two groups ($P > 0.05$).

PCoA and NMDS analyses based on Bray-Curtis distances are shown in Fig. 2. The PCoA results showed a tendency toward separation between the G and GS groups, with PC1 and PC2 explaining 14.86% and 10.90% of the variation, respectively. The NMDS analysis showed a stress value of 0.14, indicating an acceptable ordination fit for evaluating differences in microbial community structure between the two groups. Taxonomic classification showed that, at the phylum level, the dominant phyla were Firmicutes, Verrucomicrobiota, Bacteroidota, and Spirochaetota (Fig. 2C). At the genus level, the dominant genera were *Eubacterium*, *Akkermansia*, *Oscillibacter*, and *Lachnospira* (Fig. 2D).

Figure 3 shows the LEfSe analysis results and the significantly different bacterial genera between the G and GS groups. The LDA score plot (Fig. 3A) showed that the discriminative taxa in the GS group included s__*Akkermansia_glycaniphila*, g__*Akkermansia*, c__Verrucomicrobiae, o__Verrucomicrobiales, f__Akkermansiaceae, and p__Verrucomicrobia. The discriminative taxa in the G group included g__*Lachnoclostridium*, s__*Ligilactobacillus_equi*, p__Firmicutes, and f__Lachnospiraceae. At the genus level (Fig. 3B), the relative abundances of *Lachnoclostridium*, *Roseburia*, *Anaerosacchariphilus*, and *Coprococcus* were significantly higher in the G group than in the GS group ($P < 0.05$), whereas the relative abundances of *Treponema* and *Oliverpabstia* were significantly lower in the G group than in the GS group ($P < 0.05$).

Effects of concentrate supplementation on serum metabolomic profiles in grazing Yili horses

Untargeted metabolomic analysis was performed on serum samples from the G and GS groups, and a total of 1,762 metabolites were identified (Table S1). Principal component analysis (PCA) was conducted to evaluate the overall differences between the two groups. As shown in Fig. 4A, the score plot based on the first two principal components showed a tendency toward separation between the G and GS groups. To further investigate changes in serum metabolites, OPLS-DA was performed. As shown in Fig. 4B and C, the model showed good explanatory and predictive ability, with $R^2Y = 0.956$ and $Q^2 = 0.758$. The permutation test indicated that the OPLS-DA model was not overfit. Samples from the two groups were distributed on opposite sides of the plot, indicating that the model effectively distinguished the G and GS groups and was suitable for screening differential metabolites. Differential metabolites were screened based on the variable importance in projection (VIP) values from the OPLS-DA model. The screening criteria were FC > 1.5 or FC < 0.67, $P < 0.05$, and VIP > 1. A total of 487 differential metabolites were identified (Table S2). The volcano plot and hierarchical clustering heatmap of these differential metabolites are shown in Fig. 4D and E. Compared with the G group, 78 metabolites showed increased abundance and 409 metabolites showed decreased abundance in the GS group.

To comprehensively interpret the biological significance of the differential metabolites between the G and GS groups, KEGG pathway enrichment analysis was performed. As shown in Fig. 5A, the differential metabolites were mainly enriched in pathways related to the tricarboxylic acid cycle, nucleotide metabolism, bile secretion, vitamin B6 metabolism, and pyruvate metabolism. To further verify the changing patterns of metabolites involved in these enriched pathways, key differential metabolites were selected based on $\log_2$FC values and visualized. As shown in Fig. 5B, the levels of estrone-3-sulfate, fumaric acid, uric acid, and citric acid levels were significantly higher in the GS group than in the G group ($P < 0.05$), whereas the levels of cis-aconitate, inosine, and guanine were significantly lower in the GS group than in the G group ($P < 0.05$).

Correlation analysis of fecal fermentation parameters, serum biochemical indices, differential bacterial genera,

and key metabolites

Spearman correlation analysis was performed to evaluate the relationships among fecal fermentation parameters, serum biochemical indices, differential bacterial genera, and key metabolites. The results showed clear associations between differential bacterial genera and fecal fermentation parameters (Fig. 6A). *Treponema* was significantly positively correlated with butyrate and valerate concentrations, but significantly negatively correlated with fecal pH. *Lachnoclostridium* was significantly negatively correlated with acetate, propionate, butyrate, isobutyrate, isovalerate, and total VFA concentrations. *Roseburia* was significantly negatively correlated with propionate, butyrate, and isobutyrate concentrations.

Correlation analysis between differential serum metabolites and serum biochemical indices showed that purine-related metabolites, including hypoxanthine, inosine, guanine, and guanosine, were significantly negatively correlated with GLU and TG, but significantly positively correlated with TBIL (Fig. 6B). cis-Aconitate was significantly positively correlated with TBIL, ALB, HDL-C, and TC, but significantly negatively correlated with GLB and UREA. Fumaric acid, glutamine, cortisol, and several bile acid-related metabolites were generally negatively correlated with TC, HDL-C, and TBIL.

Associations were also observed between differential bacterial genera and differential serum metabolites (Fig. 6C). Overall, *Oliverpabstia* and *Treponema* tended to be positively correlated with most organic acid-, bile acid-, and amino acid-related metabolites, but negatively correlated with purine-related metabolites. In contrast, *Lachnoclostridium*, *Roseburia*, *Anaerosaccharіphilus*, and *Coprococcus* were generally positively correlated with purine metabolites, including guanine, guanosine, hypoxanthine, and inosine. Among these genera, *Lachnoclostridium* and *Roseburia* were significantly positively correlated with these metabolites ($P < 0.05$). In addition, *Roseburia*, Anaerosaccharіphilus, and *Coprococcus* were generally negatively correlated with metabolites such as citric acid, uric acid, glutamine, and fumaric acid.

Effects of concentrate supplementation on serum proteomic profiles in grazing Yili horses

Serum proteins were separated and identified using DIA combined with UHPLC-QE-MS, and the serum proteomic profiles were subsequently evaluated. A total of 1,150 proteins and 16,164 peptides were identified, with an FDR of 1% (Table S3). Among the 1,150 identified proteins, differentially expressed proteins (DEPs) were screened using the following criteria: $P < 0.05$ and FC > 1.5 or FC < 0.67. A total of 31 DEPs were identified, including 20 upregulated and 11 downregulated proteins in the GS group compared with the G group (Table 2; Fig. 7A). The PCA score plot showed clear separation between the G and GS groups (Fig. 7B). In addition, a volcano plot of the DEPs (Fig. 7C) and a hierarchical clustering heatmap (Fig. 7D) were generated to visualize differences in protein abundance between the two groups. The heatmap showed distinct clustering between the G and GS groups, which was consistent with the PCA results.

Functional analysis of all DEPs was performed based on GO classification and the UniProt database (Fig. 8A). The 31 DEPs were assigned to three GO categories: biological process (BP), cellular component (CC), and molecular function (MF). GO classification showed that the DEPs were mainly associated with metabolism-related and cellular process-related functions. In the BP category, the DEPs were mainly involved in metabolic processes, cellular processes, responses to stimulus, and immune system processes. In the CC category, they were mainly assigned to cellular anatomical entity and protein-containing complex. In the MF category, they were mainly associated with binding and catalytic activity.

As shown in Fig. 8B, the DEPs were mainly enriched in pathways including mineral absorption, pentose phosphate pathway, pyruvate metabolism, and carbon metabolism. These results indicated that the DEPs may be primarily involved in substance metabolism, energy conversion, and stress-related biological processes.

Discussion

Grassland resources in Xinjiang show clear seasonal and regional characteristics. Natural pastures can provide a certain amount of crude fiber and some minerals during the growing season [16], but insufficient forage nutrients become more prominent when grasslands are degraded or enter the dormant season. In addition, the high fiber content of roughage often limits nutrient digestion, absorption, and energy utilization efficiency in animals [17]. However, few studies have investigated how different feeding systems affect hindgut fermentation in horses and subsequently influence host energy metabolism.

VFAs produced from carbohydrate fermentation in the equine hindgut mainly include acetate, propionate, and butyrate. After absorption, acetate can be converted into acetyl-CoA, which enters the tricarboxylic acid cycle for energy production or participates in lipid metabolism. Propionate is an important precursor for gluconeogenesis and plays an important role in maintaining glucose supply and energy metabolism in grazing horses during physical activity [18]. Butyrate is not only a major energy source for colonic epithelial cells, but also contributes to the maintenance of intestinal barrier integrity and has anti-inflammatory effects [19]. Previous studies have shown that increasing the proportion of grain or concentrate supplementation in equine diets can promote propionate production while decreasing acetate concentration [20]. Hintz et al. [21] found that a high-grain diet decreased acetate concentration and increased propionate, isovalerate, and valerate concentrations in the cecum of foals. Medina et al. [22] further reported that, compared with a high-fiber diet, a high-concentrate diet significantly decreased cecal pH and acetate concentration in stallions. In the present study, fecal propionate and butyrate concentrations increased in the supplemented group, whereas fecal pH and the acetate-to-propionate ratio decreased, which is consistent with previous findings. These results suggest that concentrate supplementation increased the intake of starch and other non-structural carbohydrates in Yili horses. Some fermentable carbohydrates may have escaped complete digestion and absorption in the small intestine and entered the hindgut, providing more readily fermentable substrates for hindgut

microorganisms and promoting the production of VFAs such as propionate and butyrate. In addition, the decreases in fecal pH and the acetate-to-propionate ratio further indicated that concentrate supplementation enhanced hindgut fermentation intensity and altered VFA composition.

Gut microbiota plays a key role in nutrient utilization, intestinal development, and immune regulation in animals. Changes in the hindgut microbial community may also contribute to shifts in fermentation patterns [23]. In the present study, fecal microbiota analysis showed a significant increase in the Simpson index in the supplemented group. In addition, PCoA and NMDS analyses revealed partial separation in microbial community structure between the two groups. These findings indicate that concentrate supplementation altered the fecal microbial community structure of Yili horses. Grazing horses consume natural pasture over long periods, and their hindgut microbiota may adapt to a substrate environment dominated by structural carbohydrates. Under natural grazing conditions, the diversity of roughage sources can provide a wider range of fiber substrates for hindgut microorganisms, potentially helping maintain higher microbial diversity. This is consistent with the findings of Zhu et al. [24]. In contrast, concentrate supplementation provides relatively simple, rapidly fermentable substrates that may promote the proliferation of starch-degrading bacteria and inhibit the growth of fiber-degrading bacteria, thereby reducing microbial richness and diversity. Raspa et al. [25] also reported that high-starch diets in horses may increase the flow of undigested starch into the hindgut and induce changes in microbial communities, hindgut acidification, and the risk of microbial dysbiosis.

At the phylum level, Firmicutes, Bacteroidota, and Verrucomicrobiota were the dominant bacterial phyla in the feces of Yili horses, which is consistent with previous studies on the equine gut microbiota [26, 27]. Firmicutes contains many fiber-degrading bacteria [28], whereas Bacteroidota is mainly involved in the degradation of pectin, polysaccharides, and proteins. The relative abundances and ratio of these two phyla are considered important indicators of host energy metabolism and nutrient absorption efficiency [29]. Verrucomicrobiota is widely distributed in the cecum, colon, and feces of horses, and its relative abundance has been positively associated with the expression of gastrointestinal immune-regulatory genes, suggesting an important role in host health [30]. At the genus level, changes in bacterial abundance can provide more detailed information on the effects of different feeding systems on the fecal microbial community of horses. In the present study, the main differential genera included *Lachnoclostridium*, *Oliverpabstia*, *Roseburia*, *Coprococcus*, and *Treponema*. *Lachnoclostridium*, a Gram-positive anaerobic genus, includes species capable of degrading complex carbohydrates in plant cell walls, such as cellulose and hemicellulose, and producing acetate and butyrate [31]. *Oliverpabstia*, which belongs to Bacteroidota, is involved in dietary carbohydrate degradation and intestinal carbon metabolism [32]. *Roseburia* is a fiber-degrading genus that contributes to intestinal health [33]. *Coprococcus* is an anaerobic coccus that produces acetate and butyrate through the fermentation of fibrous carbohydrates [34]. In the present study, concentrate supplementation increased the availability of fermentable carbohydrates in the hindgut, which may have increased VFA production and decreased fecal pH, thereby contributing to the reduced relative abundance of *Coprococcus*.

Correlation analysis between differential bacterial genera and fecal fermentation parameters further suggested that concentrate supplementation may affect VFA production patterns by altering hindgut microbial composition and substrate utilization. In addition, *Treponema* has often been considered a potentially unfavorable genus in the gut and has been associated with intestinal mucosal inflammation [35]. The significantly increased relative abundance of *Treponema* in the supplemented group may indicate a potential risk of hindgut microbial disturbance. However, because intestinal inflammatory markers and histological changes were not measured in this study, this interpretation should be considered with caution.

Blood biochemical indices are important indicators of nutrient digestion, absorption, and energy metabolism. GLU is a key indicator of host energy metabolism. Previous studies have shown that increasing dietary starch intake in horses can enhance postprandial glucose and insulin responses [36]. Stull et al. [37] reported that dietary structure, fiber content, and starch-protein interactions can alter GLU levels in horses. In the present study, serum GLU concentration was increased in the GS group, suggesting that concentrate supplementation enhanced the intake and utilization of digestible carbohydrates in Yili horses. Serum TBIL concentration was markedly decreased in grazing horses after concentrate supplementation. Previous studies have shown that when grazing horses experience insufficient energy intake due to fluctuations in pasture nutritional value, the risk of hyperbilirubinemia may increase. This phenomenon is primarily associated with reduced hepatic uptake and clearance of bilirubin and may also be influenced by elevated free fatty acid concentrations [38]. The higher serum TBIL level in the G group may indicate a relatively insufficient energy supply. In contrast, after concentrate supplementation, the increase in blood glucose and overall energy intake in the GS group may have improved bilirubin clearance [39]. Serum TC concentration is an important indicator of lipid metabolism. HDL-C participates in reverse cholesterol transport by carrying cholesterol from peripheral tissues to the liver for metabolism and excretion. Because HDL is the major lipoprotein component in equine plasma, the decrease in HDL-C may partly explain the reduction in TC [40]. Therefore, the decreased serum TC and HDL-C concentrations observed in the present study may suggest that concentrate supplementation reduced lactating mares' reliance on body fat mobilization and circulating cholesterol transport, and may have promoted the conversion of cholesterol toward bile acid synthesis or tissue utilization.

Metabolomic analysis identified 487 differential metabolites, mainly including lipids and organic acids. KEGG enrichment analysis showed that these metabolites were mainly enriched in pathways related to the tricarboxylic acid (TCA) cycle, pyruvate metabolism, vitamin B6 metabolism, and purine and nucleotide metabolism. In the TCA cycle, citric acid and fumaric acid were increased, whereas cis-aconitate was decreased. The increase in citric acid may reflect increased substrate supply from acetyl-CoA entering the TCA cycle, while the increase in fumaric acid may indicate enhanced activity in the later stages of the TCA cycle and closer links with amino acid metabolism and oxidative phosphorylation [41]. The decrease in cis-aconitate may be related to rapid conversion toward isocitrate or further downstream utilization. These changes in TCA cycle intermediates suggest that concentrate supplementation altered metabolite levels involved in the TCA cycle. In addition, glucose 1-phosphate, a

differential metabolite, was enriched in the galactose metabolism pathway. Glucose 1-phosphate is an important intermediate in carbohydrate metabolism and serves as a precursor for nucleotide sugar synthesis. Its increased level in the GS group suggests that concentrate supplementation may not only enhance energy metabolism efficiency but also promote glycogen turnover, glycosylation substrate production, and tissue anabolic processes [42].

Several metabolites, including glutamine, guanosine, inosine, guanine, and hypoxanthine, were enriched in purine metabolism and nucleotide metabolism pathways. Inosine consists of hypoxanthine and ribose and can contribute to energy metabolism by entering the tricarboxylic acid (TCA) cycle. It may also help maintain ATP production under glucose-limited or hypoxic conditions [43]. Glutamine is a conditionally essential amino acid in animals. It serves as an important nitrogen donor for nucleotide synthesis and can also participate in energy metabolism by replenishing TCA cycle intermediates. In addition, glutamine is involved in nitrogen transfer, energy supply, and immune regulation [44]. Therefore, concentrate supplementation may not only enhance glucose metabolism and TCA cycle-related energy supply, but also influence energy metabolism and nucleotide metabolism through glutamine-related pathways. In addition, in the bile secretion pathway, the differential metabolites bilirubin and deoxycholic acid showed a downward trend in the GS group. Bile acids emulsify dietary lipids and facilitate the digestion and absorption of triglycerides, cholesterol, and fat-soluble vitamins in the small intestine [45]. Combined with the significant decreases in serum TC and HDL-C observed in this study, these results suggest that concentrate supplementation may affect the bile secretion pathway by improving host energy supply and regulating bile acid-related metabolism.

Changes in the serum proteome further revealed the effects of concentrate supplementation on host metabolism in Yili horses. In the present study, 31 differentially expressed proteins (DEPs) were identified using DIA-based quantitative proteomics, including 20 upregulated and 11 downregulated proteins in the GS group. KEGG pathway enrichment analysis showed that these DEPs were mainly enriched in pathways related to pyruvate metabolism, the pentose phosphate pathway, and carbohydrate digestion and absorption. Pyruvate is a central metabolite linking glycolysis with the TCA cycle, whereas the pentose phosphate pathway is closely associated with NADPH production, reductive biosynthesis, and the maintenance of redox homeostasis [46, 47]. The enrichment of these pathways suggests that concentrate supplementation may not only improve energy metabolism efficiency in Yili horses but also affect redox-related metabolic processes.

At the protein level, peroxiredoxin-5 (PRDX5), lactoylglutathione lyase (GLO1), transketolase, ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1), and several proteasome subunits were upregulated in the GS group. These changes suggest that concentrate supplementation may enhance antioxidant defense, methylglyoxal detoxification, pentose phosphate pathway activity, and protein turnover capacity. PRDX5 is an important antioxidant enzyme, and its increased abundance may indicate an enhanced capacity to remove peroxides and maintain cellular redox balance [48]. The upregulation of GLO1 suggests an increased capacity to detoxify methylglyoxal (MGO), a highly reactive ketoaldehyde by-product of glycolysis [49, 50]. MGO has also been used as a monitoring biomarker for ketosis in dairy

cows [51]. Transketolase is a thiamine-dependent enzyme and a key enzyme in the pentose phosphate pathway, where it plays an important role in glucose metabolism [52]. Its increased abundance further supports the possibility that glucose metabolism was redirected toward this pathway to meet the requirements for NADPH production and antioxidant regulation. ATP1A1 is a transmembrane protein mainly involved in ion transport, metabolic regulation, cell migration, and signal transduction, and plays important roles in cell survival, growth, and differentiation [53, 54]. Taken together, these results suggest that improved glucose metabolism after concentrate supplementation was accompanied by the activation of metabolic buffering and defense mechanisms.

In addition, the upregulation of several immune-related proteins in the GS group suggests that concentrate supplementation may have affected immune-related serum protein profiles. Immunoglobulin light chains (IGL) are important components of passive immunity and contribute to mucosal defense and early immune protection in foals [55]. Their increased abundance may indicate an increase in immunoglobulin-related components. Transferrin is the major iron-binding protein in blood and is responsible for transporting iron absorbed from the digestive tract and iron released from erythrocyte degradation. It plays an important role in maintaining iron metabolism and has antibacterial effects by limiting bacterial iron availability [56, 57]. Alpha-1-antitrypsin (Spi2-7), as a protease inhibitor, is mainly expressed in epithelial cells, glandular tissues, and secretions, and is involved in inhibiting inflammatory responses and reducing cell apoptosis [58]. The upregulation of these proteins suggests that concentrate supplementation may also influence iron transport, inflammatory regulation, and protease inhibitor balance.

Conclusion

In the present study, supplementation with 2 kg/day of concentrate supplement increased fecal propionate and butyrate concentrations, decreased fecal pH and the acetate-to-propionate ratio, and altered hindgut fermentation patterns in grazing Yili horses. Concentrate supplementation also changed fecal microbiota structure, with higher relative abundances of *Treponema* and *Oliverpabstia* and lower relative abundances of *Lachnoclostridium*, *Roseburia*, *Anaerosaccharіphilus*, and *Coprococcus* in the GS group. These changes suggest that concentrate supplementation may affect hindgut fermentation by modulating microbial composition and substrate utilization. Serum metabolomic and biochemical analyses further indicated that concentrate supplementation increased glucose availability and altered purine metabolism and bile acid-related metabolic processes. Serum proteomic analysis showed that the differentially expressed proteins were primarily involved in glucose metabolism, antioxidant responses, detoxification, and metabolic homeostasis. Overall, these findings suggest that concentrate supplementation was associated with changes in the fecal microbiota, fermentation products, serum metabolites, and the host protein network, and may improve energy metabolism in grazing Yili horses. This study provides a theoretical basis for optimizing concentrate supplementation strategies for Yili horses under grazing conditions.

Declarations

Authors' contributions

M.L. and C.Z. conceived and designed the study. M.L., Z.X., and L.S. (Long Sun) carried out the animal experiment, sample collection, and laboratory analyses. Z.C., Y.Y., L.S. (Lingkai Sun), and M.A. assisted with the animal trial, sample processing, and manuscript preparation. M.L. performed data curation, formal analysis, visualization, and drafted the original manuscript. Z.X. and L.S. (Long Sun) contributed to data analysis, data interpretation, and manuscript revision. F.L. provided methodological guidance and supervision. Y.C. and C.Z. were responsible for supervision and project administration. C.Z. provided conceptualization, methodology, funding acquisition, project administration, and critical revision of the manuscript. All authors read and approved the final manuscript.

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Data availability

The data generated and analyzed during the current study have been deposited in Zenodo under DOI: 10.5281/zenodo.20635937. The 16S rRNA sequencing raw data are available in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1476471 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1476471].

Ethics approval and consent to participate

All protocols were approved by the Animal Care and Use Committee of Xinjiang Agricultural University (permission number 2023059). Informed consent - Owners gave informed consent for their animals’ inclusion in the study. All methods were carried out in accordance with relevant guidelines and regulations for the use of animal subjects.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Disclosure statement

The authors declare that they have no conflicts of interest.

Consent for publication

Not applicable.

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Figures

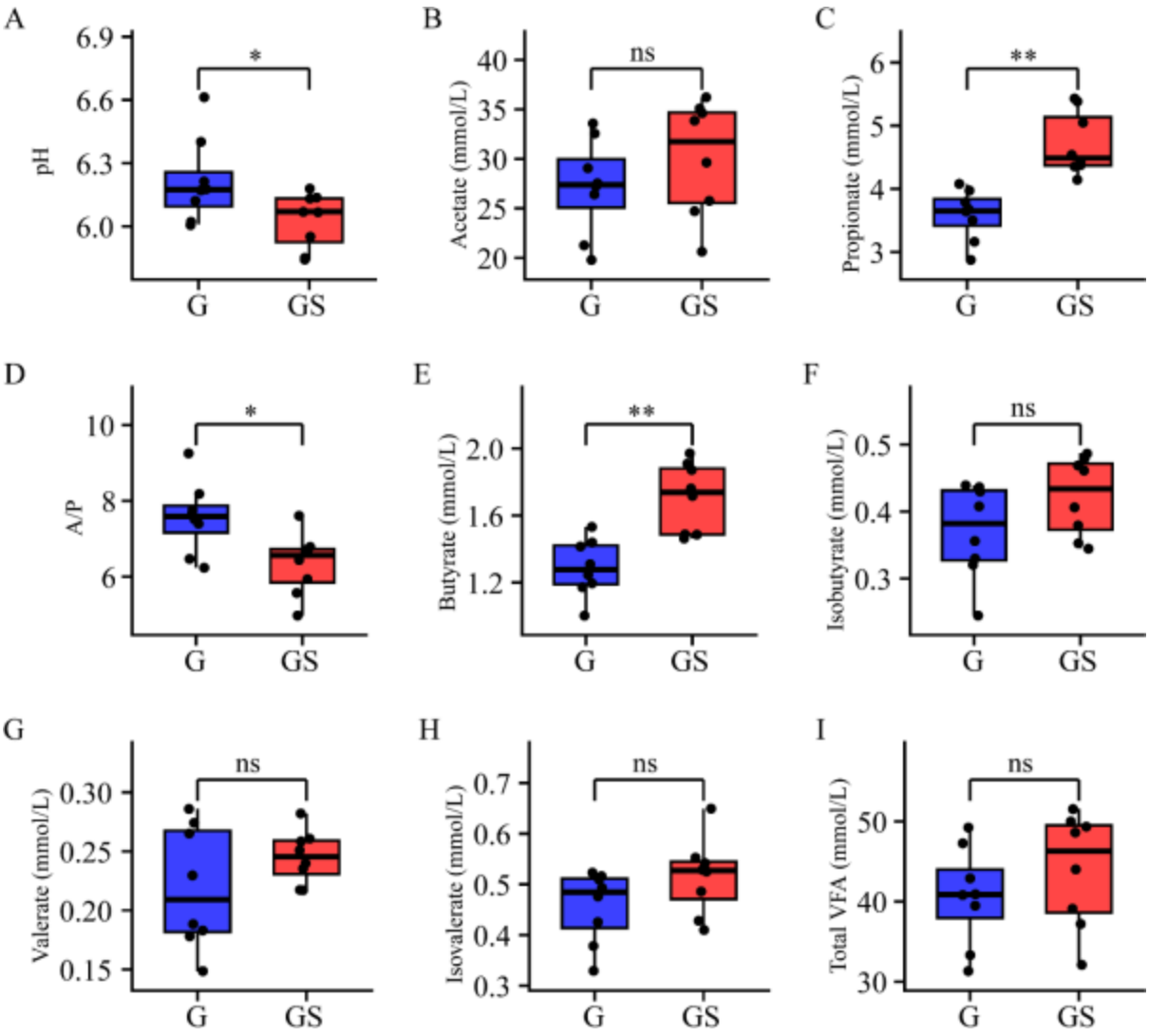


Figure 1

Effects of concentrate supplementation on fecal pH and volatile fatty acids in grazing Yili horses (n = 8 per group). A: Fecal pH. **B**: Acetate. **C**: Propionate. **D**: Acetate/Propionate. **E**: Butyrate. **F**: Isobutyrate. **G**: Valerate. **H**: Isovalerate. **I**: Total VFA. ** indicates $P < 0.01$, * indicates $P < 0.05$, and ns indicates $P > 0.05$.

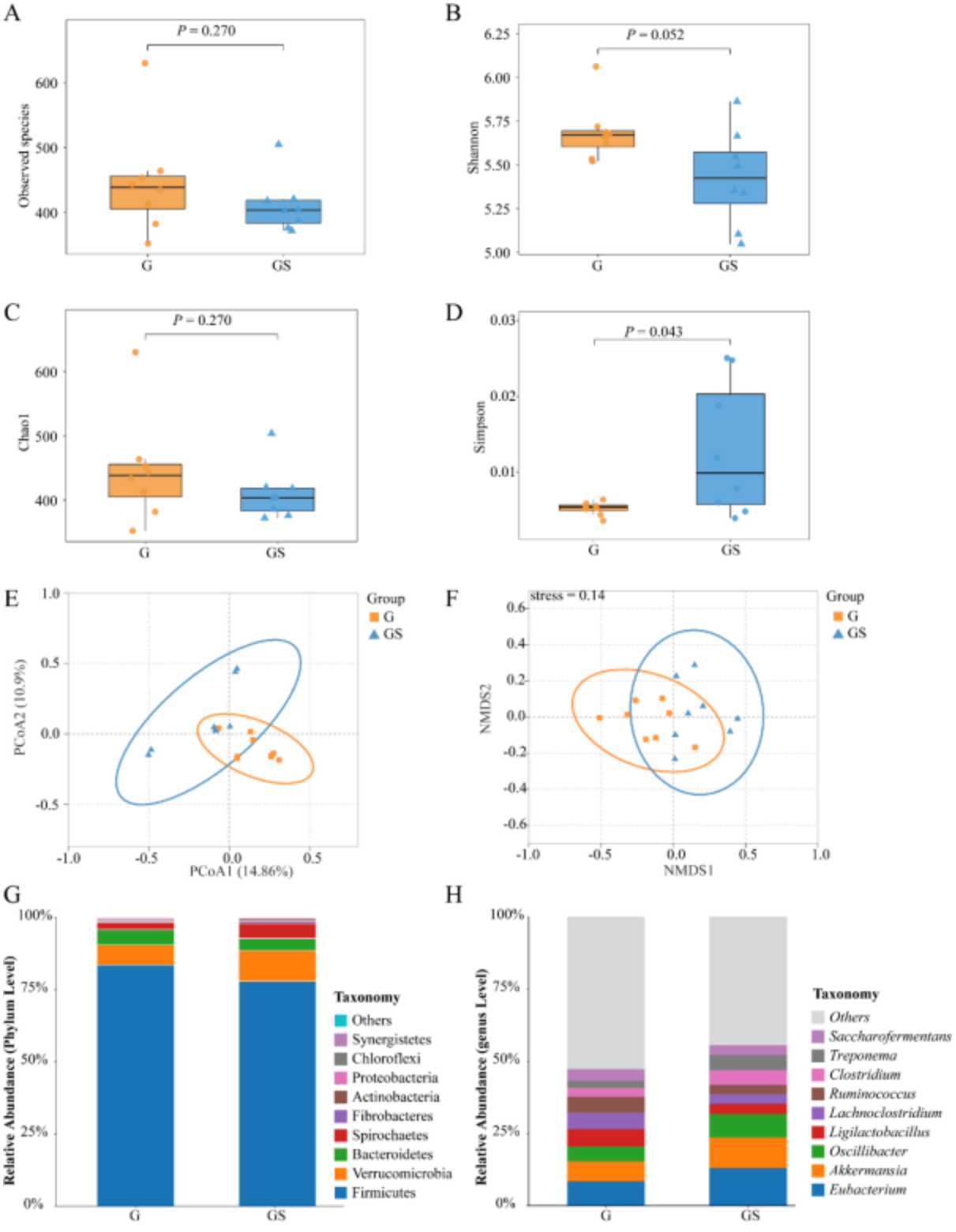


Figure 2

Effects of concentrate supplementation on fecal bacterial diversity and taxonomic composition in grazing Yili horses (n = 8 per group). A: Observed species. **B**: Shannon index. **C**: Chao1 index. **D**: Simpson index. E: Principal coordinate analysis (PCoA) based on Bray-Curtis distances. F: Non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis distances. G: Relative abundance of fecal bacteria at the phylum level. H: Relative abundance of fecal bacteria at the genus level.

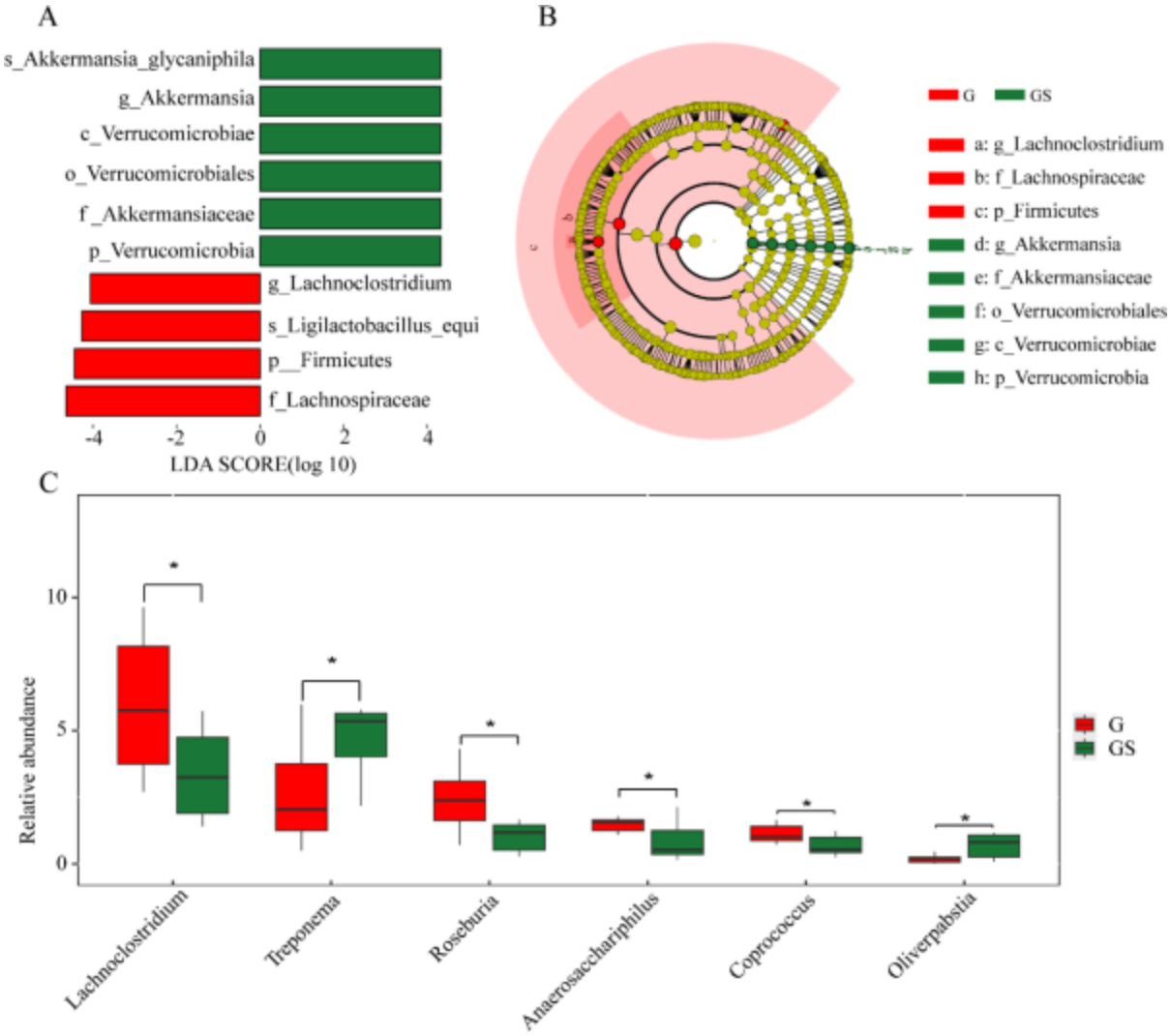


Figure 3

LEfSe analysis of fecal flora (n = 8 per group). A: LDA score plot showing taxa enriched in the G and GS groups. **B**: LEfSe taxonomic cladogram showing the phylogenetic distribution of differential taxa. **C**: Boxplots showing the relative abundances of significantly different genera between the two groups. * indicates $P < 0.05$.

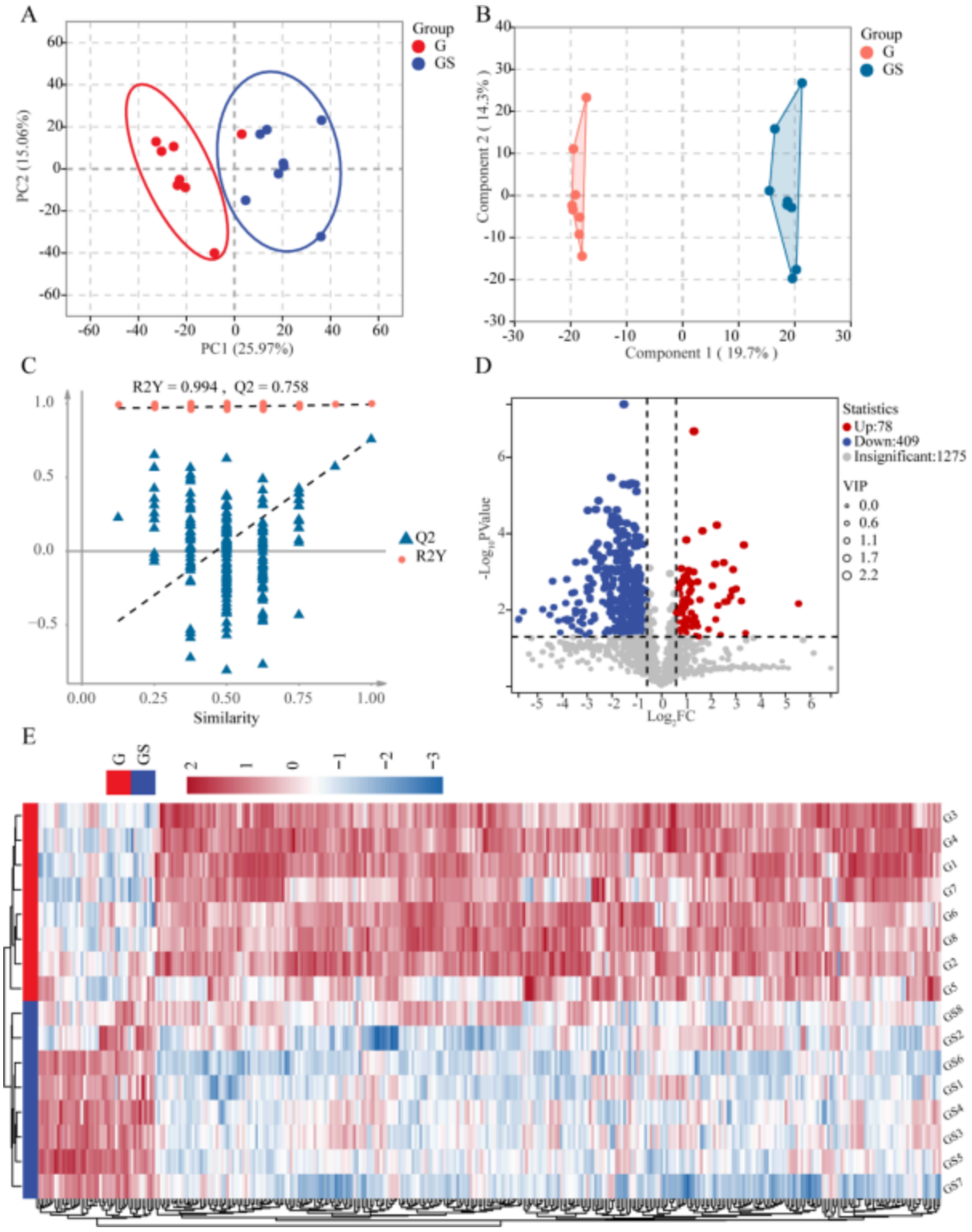


Figure 4

Effects of concentrate supplementation on serum metabolomic profiles in grazing Yili horses (n = 8 per group). A: PCA score plot of serum metabolites in the G and GS groups. **B**: OPLS-DA score plot showing the separation between the G and GS groups. **C**: Permutation test of the OPLS-DA model. **D**: Volcano plot of differential metabolites between the G and GS groups. **E**: Hierarchical clustering heatmap of differential metabolites between the G and GS groups.

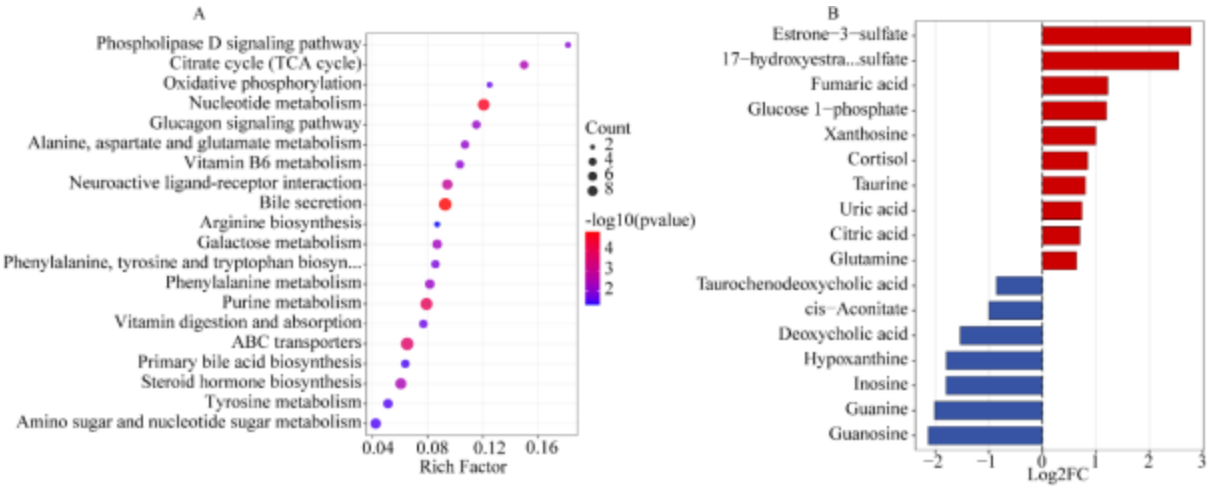


Figure 5

Effects of concentrate supplementation on KEGG pathway enrichment and key differential metabolites in serum of grazing Yili horses (n = 8 per group). **A**: KEGG pathway enrichment analysis of differential serum metabolites between the G and GS groups. The size of each bubble represents the number of differential metabolites enriched in the pathway, and the color represents -log10(*P*-value). **B**: Log_2FC values of key differential metabolites involved in the enriched pathways.

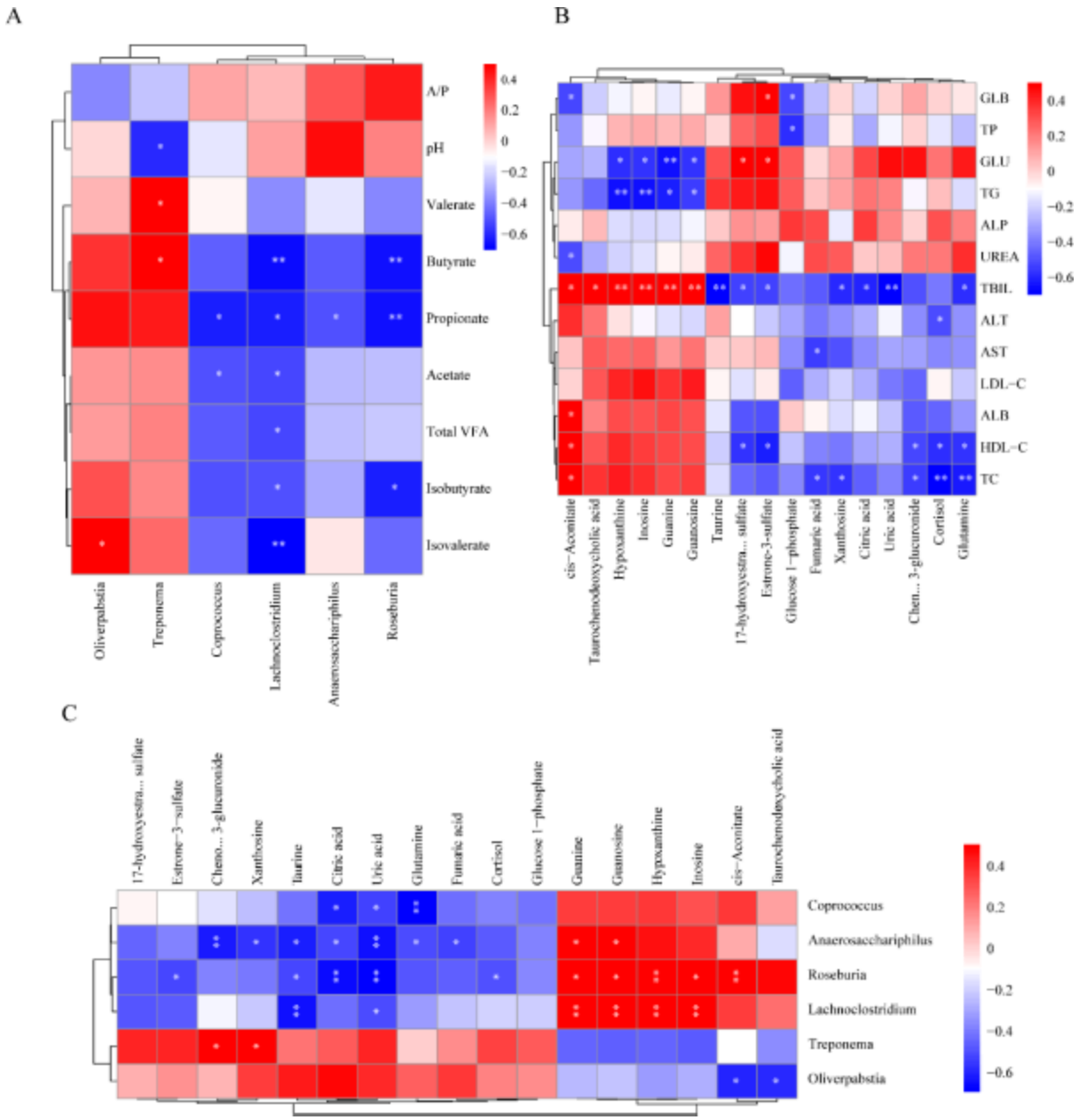


Figure 6

Correlation analysis among fecal fermentation parameters, serum biochemical indices, differential bacterial genera, and key serum metabolites. **A**: Spearman correlation heatmap between differential fecal bacterial genera and fecal fermentation parameters. **B**: Spearman correlation heatmap between key differential serum metabolites and serum biochemical indices. **C**: Spearman correlation heatmap between differential fecal bacterial genera and key differential serum metabolites. Red indicates positive correlations, and blue indicates negative correlations. * indicates $P < 0.05$, and ** indicates $P < 0.01$.

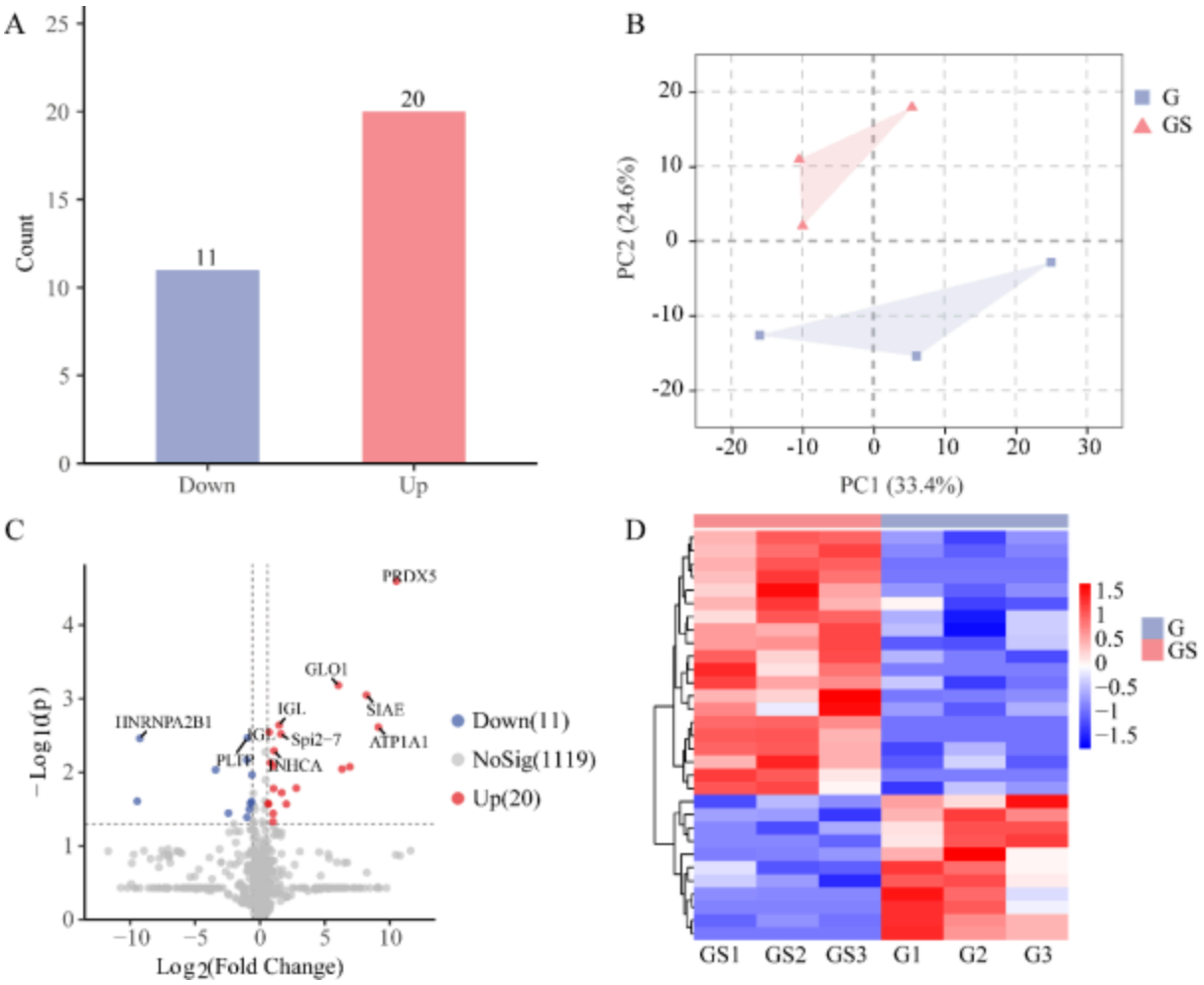


Figure 7

Effects of concentrate supplementation on serum proteomic profiles in grazing Yili horses (n = 3 per group). A: Number of upregulated and downregulated differentially expressed proteins between the G and GS groups. **B**: PCA score plot of serum proteins in the G and GS groups. **C**: Volcano plot of differentially expressed proteins between the G and GS groups. **D**: Hierarchical clustering heatmap of differentially expressed proteins between the G and GS groups.

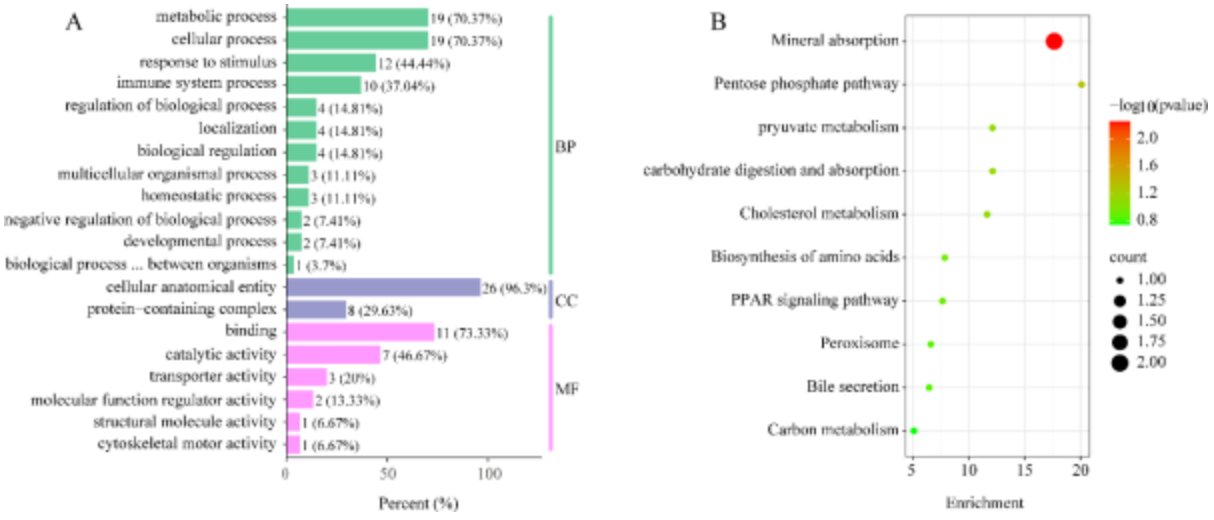


Figure 8

Functional annotation and KEGG pathway enrichment analysis of differentially expressed serum proteins. A: GO classification of differentially expressed proteins, including biological process, cellular component, and molecular function categories. **B**: KEGG pathway enrichment analysis of differentially expressed proteins between the G and GS groups. The size of each bubble represents the number of enriched proteins, and the color represents -log10(*P*-value). BP, biological process; CC, cellular component; MF, molecular function.