Animal Microbiome

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Omics integration reveals how the gut microbiota of Warmblood horses responds to equestrian show jumping—a short-duration, high-intensity technical exercise stress

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Abstract

Background Intestinal microbial homeostasis and metabolic balance play a crucial role in maintaining normal physiological function in horses. Exogenous stress involving abrupt turns and jumps during show jumping significantly impacts intestinal microbial homeostasis and metabolic balance in these animals.

Results By comparing rectal (faecal) samples from 10 Warmblood horses collected before and immediately after a show jumping competition on the same day, we observed substantial alterations in intestinal microbial homeostasis and metabolic balance post-exercise. Microbial evenness significantly increased following the competition, accompanied by enrichment of specific taxa such as Bacteroides, Ruminococcus, Prevotella, and Fibrobacter. Metabolite analysis revealed a marked decrease in antioxidant-related compounds, including orsellinic acid, 2,3-dimethyl-2-cyclohexen-1-one, and (1 R,6 R)-1,4,5,5a,6,9-hexahydrophenazine-1,6-dicarboxylate. Conversely, glucosan and thiamine pyrophosphate levels increased. Post-competition, membrane lipid metabolism pathways were significantly downregulated, while antioxidant responses and energy metabolism pathways were upregulated. Spearman correlation analysis indicated positive associations between Fibrobacter, Ruminococcus, and Prevotella with energy metabolism-related metabolites, whereas Lysinibacillus correlated positively with metabolites involved in antioxidant activity and intestinal mucosal protection.

Conclusion Collectively, our findings demonstrate that show jumping induces shifts in intestinal microbial homeostasis and metabolic balance in Warmblood horses. These adaptations appear conducive to preserving epithelial integrity and enhancing energy provision to meet the demands of high-intensity exercise. This study provides novel insights into the impact of acute high-intensity exercise on equine gut microbial dynamics and metabolism, offering a theoretical basis for probiotic-based interventions to support intestinal health in sport horses.

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Keywords Changes, Gut microbial homeostasis, Metabolic balance, Equestrian show jumping competition, Warmblood horses

Background

The gastrointestinal tract of animals hosts diverse microbial communities that are intricately linked to host health, immune function, and disease regulation. In horses, the gut microbiota encompasses various microorganisms, including fungi, parasites, protozoa, archaea, viruses, and bacteria, with bacteria being the dominant group, estimated at approximately $10^{1\ 5}$ cells [1, 2], primarily located in the cecum and colon [3]. These microbial communities execute critical functions, including nutrient digestion, immune modulation, and both beneficial and pathogenic interactions with the host [4–6], with functional variations among different microbial taxa. As obligate herbivores, horses rely extensively on hindgut microbial fermentation of plant materials to generate short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which are essential for meeting their energy demands [7]. However, gut microbiota composition and abundance are highly sensitive to external influences, including diseases, dietary modifications, antibiotic usage, and aging, often resulting in dysbiosis, impaired fermentation, and metabolic disturbances [1, 8]. Therefore, maintaining microbial homeostasis and metabolic balance is paramount for equine health and performance.

Exercise, recognized as a significant physiological stressor, has been shown to modulate gut microbiota. Studies across various species have demonstrated that exercise influences gut microbial composition and metabolite profiles. In humans, athletes generally exhibit higher microbial α-diversity, elevated SCFA levels, and increased populations of SCFA-producing bacteria compared to sedentary individuals, although such changes typically require more than eight weeks of sustained training [9]. In mice, seven weeks of anaerobic exercise altered cecal microbiota, influencing pathways related to carbohydrate metabolism, signal transduction, and transcription [10]. Similarly, in rats, exercise reduced body fat, elevated microbial α-diversity, and enriched anaerobic bacteria such as Christensenellaceae and Spirochaetaceae [11]. Anaerobic exercise also modified gut β-diversity in male rats, increasing Tremblaya abundance and reducing inflammatory markers [12]. In sheep, exercise elevated Bacteroidetes while reducing Firmicutes and Proteobacteria, with higher abundances of Bacteroides and Prevotella in exercised individuals compared to controls [13].

Despite considerable progress, most studies have concentrated on long-term exercise effects, while the influence of acute exercise on gut microbiota remains less understood. In humans, acute exercise-induced shifts in gut microbial composition and fecal metabolites have been reported after endurance events. Half-marathon completion led to changes in specific bacterial taxa and 40 fecal metabolites, characterized by increases in organic acids and decreases in nucleic acid components [14]. Ultramarathon participation increased potential pathogenic bacteria while reducing beneficial taxa [15]. Post-Boston Marathon samples showed elevated Veillonella abundance [16]. Conversely, no significant changes were detected following ultra-endurance triathlons or elite women's soccer tournaments [17, 18]. These conflicting findings suggest that the effects of acute exercise on the gut microbiota may vary depending on the type of exercise or the duration of exercise. Additionally, evidence indicates a dose–response relationship between exercise duration and metabolic shifts, with longer events inducing more pronounced metabolite changes than shorter efforts [14, 19].

Equestrian competitions encompass diverse disciplines such as show jumping, flat racing, and dressage, involving distinct exercise types [20]. Similar to human studies, the impact of exercise on equine gut microbial homeostasis and metabolic balance remains inconclusive. In Standardbred horses, strenuous acute exercise does not alter the faecal microbiota [21], whereas 12 weeks of exercise training does [22]. Interestingly, 12 weeks of exercise training did not alter the pattern of gut microbial response to acute exercise. [21]. However, metabolic studies in Standardbred report positive correlations between maximal speed and both fiber degradation efficiency and post-exercise blood acetate concentrations [23], highlighting the potential role of fiber-fermenting microbiota during intense exercise. Additionally, a group comparison between Yili racehorses and age-matched Yili nonracehorses revealed that the former exhibited higher relative abundances of fiber-degrading taxa (e.g., Lachnospiraceae, Oscillospiraceae, and Ruminococcus), enabling enhanced engagement in dietary fiber fermentation pathways [24]. Collectively, these findings imply that exercise may remodel equine gut microbial composition and metabolic activity, with the specific outcomes possibly dependent on the duration of exercise exposure and the experimental paradigm used to assess microbial changes.

Notably, current research predominantly focuses on speed-racing breeds (e.g., Standardbred, Thoroughbreds), with scarce data on sport horses used in equestrian disciplines. Building on this evidence, we investigated the effects of show jumping—a high-intensity, skill-based exercise—on gut microbial homeostasis and metabolic

balance in Warmblood horses, a primary breed in equestrian sports.

Methods

Study design and sample collection

Ten warmblood horses were randomly selected from participants competing on the same competition day during the 2022 Equuleus Premium Open CSI. Center faecal samples were collected from these horses both before the event (BR group: Before recording group) and after the event (AR group: After recording group) on 25 September 2022, following strict aseptic procedures. The interval between the two sampling time points did not exceed 10 hours. All samples were immediately frozen in liquid nitrogen and subsequently transported to the laboratory for storage at −80 °C. Detailed information on the experimental horses and sample collection is provided in Supplementary Table S1. All horses are kept under the same feeding and management conditions from two days before competition day until the end of the competition. All animals included in this study were healthy and had not received any antimicrobial treatments (Including antibiotics, anthelmintics, or non-steroidal anti-inflammatory drugs) within two months prior to sampling. All of them received uniform training at the fixed training ground of Equuleus Corp Club before the competition. An overview of the experimental design is illustrated in Fig. 1.

Metagenomic sequencing procedures

Microbial DNA was extracted from horse faecal samples using the cetyltrimethylammonium bromide (CTAB) method described by Griffith et al. [25] supplemented with a modified phenol/chloroform method [26]. Briefly, 100 mg of each sample was mixed with 1000 µL of CTAB lysis buffer containing 2% β-mercaptoethanol and 20 µL of lysozyme, and incubated at 65 °C for 2–3 hours. After centrifugation, the supernatant was sequentially extracted with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. DNA was precipitated with isopropanol, washed with ethanol, and dissolved in double-distilled water (ddH_2O). RNase A was added to remove residual RNA. DNA concentration was quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and DNA integrity was assessed by agarose gel electrophoresis.

For library preparation, 10 ng of genomic DNA was used to construct Illumina sequencing libraries using the VAHTS® Universal Plus DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. DNA was fragmented, purified, end-repaired, ligated to sequencing adapters, and amplified by PCR. Library quality was evaluated using the Qsep-400 system (Bioptic, Taiwan), ensuring a concentration ≥1 ng/µL and an average fragment size between 430 and 530 bp. Metagenomic sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using the NovaSeq 6000 S4 Reagent Kit.

Metagenomic data analysis

The metagenomic sequencing data were processed and analyzed following a standardized pipeline. Quality control was conducted using fastp to remove adapter sequences, reads with an N content greater than 10%, and low-quality reads (defined as reads with >50% of bases having a Q-score ≤20). To minimize host contamination, reads aligning to the horse genome (EquCab3.0) were removed using Bowtie2 (version 2.2.4) [27].

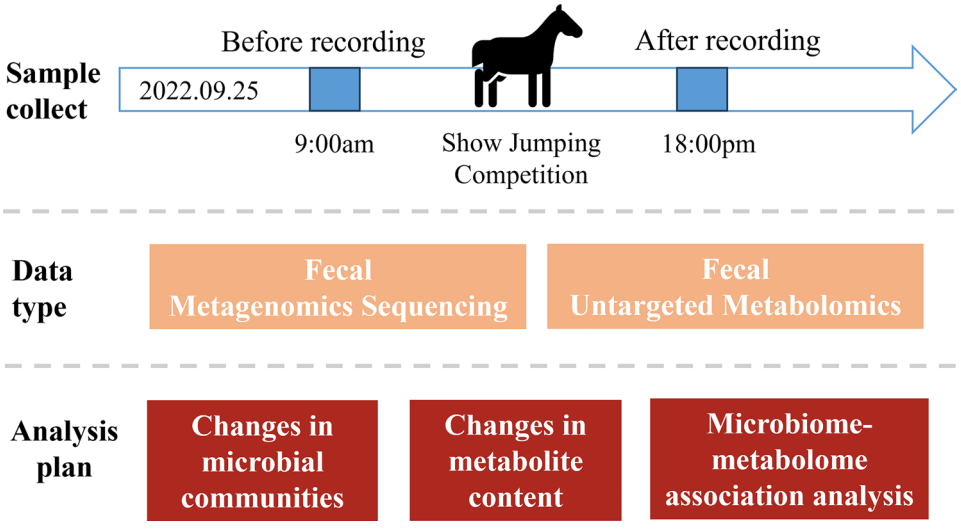

Fig. 1 Design and framework of the experiment

Metagenomic assembly was performed using SOAPdenovo2 (version 2.04) with optimized parameters [28]. Scaffolds were processed to generate high-quality Scaftigs (scaffold contigs) longer than 500 bp, and sequences containing ambiguous bases (N) were removed. The assembled Scaftigs were then mapped back to the clean reads to recover paired-end relationships [29].

Gene prediction was carried out using MetaGeneMark [30]. A non-redundant gene catalog was constructed with CD-HIT by clustering similar sequences and removing genes supported by ≤ 2 mapped reads [31]. Gene abundance was calculated based on the number of reads mapped to each gene normalized by gene length.

Taxonomic annotation was performed by aligning unigenes against the NCBI non-redundant (NR) database using DIAMOND [32], and taxonomic classifications were assigned with MEGAN [33]. Functional annotation was conducted by aligning predicted genes to the KEGG database using DIAMOND. Additionally, metagenome-assembled genome (MAG) classification was performed using GTDB-Tk [34].

Untargeted metabolomics experimental procedures

Metabolites were extracted by adding 50 mg of each sample to 1000 µL of extraction solvent (methanol:acetonitrile = 2:2:1, v/v) containing an internal standard (20 mg/L). The mixture was vortexed, ground, and subjected to ultrasonication. Subsequently, samples were cooled at −20 °C for 1 hour, centrifuged at 4 °C, and the supernatant was collected. The collected extracts were dried in a vacuum concentrator, re-dissolved in 160 µL of extraction solvent (acetonitrile: water = 1:1, v/v), vortexed, sonicated, and centrifuged again. The resulting supernatant was transferred to autosampler vials. Additionally, 10 µL aliquots from each sample were pooled to generate quality control (QC) samples for system monitoring [35, 36]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a Waters Acquity I-Class PLUS UPLC system coupled with a Waters Xevo G2-XS QTOF high-resolution mass spectrometer (Waters Corporation, Milford, MA, USA). Analytes were separated on a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm particle size) with an injection volume of 1 µL and a flow rate of 300 µL/min. The UPLC system was operated in both positive and negative electrospray ionization (ESI) modes, with the following parameters: capillary voltage of 2500 V (positive mode) and −2000 V (negative mode), cone voltage of 30 V, ion source temperature of 100 °C, desolvation gas temperature of 500 °C, and backflush and desolvation gas flow rates of 50 L/h and 800 L/h, respectively. Mass spectra were acquired over an m/z range of 50–1200. Quantitative data were collected using the multiple reaction monitoring (MRM) method. QC samples were analyzed throughout the run to evaluate system stability and reproducibility [37].

Untargeted metabolomics data preprocessing and analysis

Raw data generated by MassLynx V4.2 (Waters Corporation) were processed using Progenesis QI software for peak detection, extraction, and alignment. Metabolite identification was performed based on the METLIN database, public metabolite databases, and BaiMaiKe's proprietary database. Theoretical fragment matching was applied, with mass deviations controlled within 100 ppm for parent ions and 50 ppm for fragment ions, to ensure reliable qualitative and quantitative analysis [37].

Metabolic pathway annotation was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Integrated analysis of metagenome and metabolome

The top 10 metabolites with the highest fold change and p-values < 0.05 identified from the metabolomic analysis, along with five microbial biomarkers identified by metagenomic analysis before and after racing, were selected for correlation analysis. Redundancy analysis (RDA) was performed to conduct multiple direct gradient analysis and to explore the relationships between the gut microbiota and metabolites. A weighted correlation network was constructed based on Spearman correlation analysis.

Statistical analysis

Statistical analyses were performed to assess differences in microbial diversity, community composition, and metabolite profiles before and after the competition. Alpha diversity indices, including Shannon, Simpson, Chao1, and ACE, were calculated using the vegan package in R (version 3.6.1), and differences were assessed using the Wilcoxon signed-rank tests. Beta diversity was assessed through Principal Coordinates Analysis (PCoA) to visualize changes in microbial community structures, with significance determined using permutational multivariate analysis of variance (PERMANOVA). Linear discriminant analysis of effect size (LEfSe) was employed to identify taxa with significantly different relative abundances between groups. For metabolomic data, Principal component analysis (PCA) was conducted using the prcomp function in R (version 3.6.1), a combination of univariate and multivariate statistical methods was applied to comprehensively analyse the dataset, enabling the accurate identification of differential metabolites. Differential metabolites were identified by univariate statistical analysis, using a p-value threshold of <0.05 and a fold change (FC) greater than 2. Pathway enrichment analysis was performed with the R packages clusterProfiler and enrichplot [38]. Correlations between microbial taxa and

metabolites were assessed using Spearman's correlation analysis, with statistical significance set at $p < 0.05$.

Results

Changes in the composition of gut microbiota

Bacteria were the most abundant microorganisms in the hindgut microbiota of horses both before and after the competition, followed by fungi, protozoa, and viruses (Fig. 2a). It is noteworthy that although there were no significant differences in the relative abundance of bacteria, fungi, protists and viruses, they all tended to be up-regulated and the within-group differences were all reduced. Across all samples, a wide range of microbial taxa was detected, including 44 phyla, 94 classes, 203 orders, 513 families, and 1895 genera. After filtering based on relative abundance, the top 10 phylum, top 24 class, and top 25 genus in terms of relative abundance were selected for further analysis (Fig. 2b, c, d). The most prevalent bacterial genera included Streptococcus (5.49%), Clostridium (3.30%), Prevotella (2.44%), Bacteroides (2.25%), Butyrivibrio (2.20%), Streptomyces (1.91%), and Fibrobacter (1.60%). At the species level, a total of 13,965 microbial species were identified, among which 2331 were unnamed novel bacterial species (Detailed species information is provided in Supplementary Table S2, S3).

Changes in the hindgut microbiota and functional pathways before and after the competition

Analyses showed significant changes in the gut microbiota of horses before and after the competition. Principal Coordinates Analysis (PCoA) showed a significant reorganisation of the overall microbial community structure, with more homogeneous clustering of the post-competition samples (Fig. 3a). Similarly, the alpha-diversity indices were more consistent after the competition, although Chao 1, ACE, Shannon's index and Simpson's index were not significantly different (Fig. 3b). Further biomarker analyses identified marker microorganisms before and after the competition. Lysinibacillus was enriched before the competition, whereas Prevotella, Ruminococcus, Bacteroides, and Fibrobacter were more abundant after the competition (Specific changes in relative abundance are provided in Supplementary Figure S1, S2, S3, S4, S5). A phylogenetic tree constructed based on these biomarkers revealed that post-competition taxa predominantly clustered within the Fibrobacter and Bacteroidia lineages (Fig. 3c, d). STAMP analysis based on KEGG pathway annotation showed a significant upregulation of pathways such as Carbon fixation pathways in prokaryotes, One carbon pool by folate, Protein phosphatases and associated proteins, Phenylpropanoid biosynthesis and Arginine biosynthesis. Conversely, pathways including Glycerophospholipid metabolism, Ether lipid metabolism, Glycerolipid metabolism, and "D-Alanine metabolism" were significantly downregulated. Additionally, the abundance of pathways related to beta-lactam resistance and the Biosynthesis of siderophore group nonribosomal peptides markedly decreased following the competition (Fig. 3e).

Untargeted metabolomics data evaluation

Among the identified metabolites, lipid-related compounds, particularly fatty acyls and polyketides, were the most abundant (Fig. 4a). Principal component analysis (PCA) indicated that the overall metabolite profiles of the horse gut exhibited minimal changes between the before and after competition conditions, with a high degree of similarity observed in the major components of the metabolome (Fig. 4b). However, subtle differences between specific metabolites were detectable after the competition.

Differential metabolite screening and pathway enrichment

Untargeted metabolomics analysis identified a total of 3832 metabolites and revealed significant differences between the BR and AR groups. Comparative analysis showed that 30 metabolites were downregulated and 14 metabolites were upregulated following the competition. Among the upregulated metabolites were Glucosan, Thiamin pyrophosphate, (2S,3R,4R,5S,6S)-2-(hydroxymethyl)-6-phenylmethoxyoxane-3,4,5-triol, Delphinidin 3-O-(acetylglucoside) and Novapikromycin. Significantly downregulated metabolites included Orsellinic acid, 2,3-dimethyl-2-cyclohexen-1-one, (1R,6R)-1,4,5,5a,6,9-hexahydrophenazine-1,6-dicarboxylate, β-alanyl-L-lysine and 3-phenyl-4-pentenal (Fig. 5a). Correlation analysis showed that these altered metabolites may participate in multiple interconnected biological processes (Fig. 5b). KEGG pathway enrichment analysis highlighted significant downregulation of pathways associated with carotenoid biosynthesis, longevity regulation, β-alanine metabolism, and biofilm formation, whereas propanoate metabolism was notably upregulated. In addition, a slight upward trend was observed in the pathway related to carbohydrate digestion and absorption (Fig. 5c).

Integrated analysis of Metagenomics and Metabolomics

We conducted a correlation analysis between the microbial biomarkers at the genus level identified before and after the competition and the top ten differential metabolites (Fig. 6a). Notably, the pre-competition biomarker Lysinibacillus exhibited a significant negative correlation with metabolites that were upregulated post-competition, and a significant positive correlation with those that were downregulated. Furthermore, clustering analysis revealed that Bacteroides and Prevotella, Fibrobacter

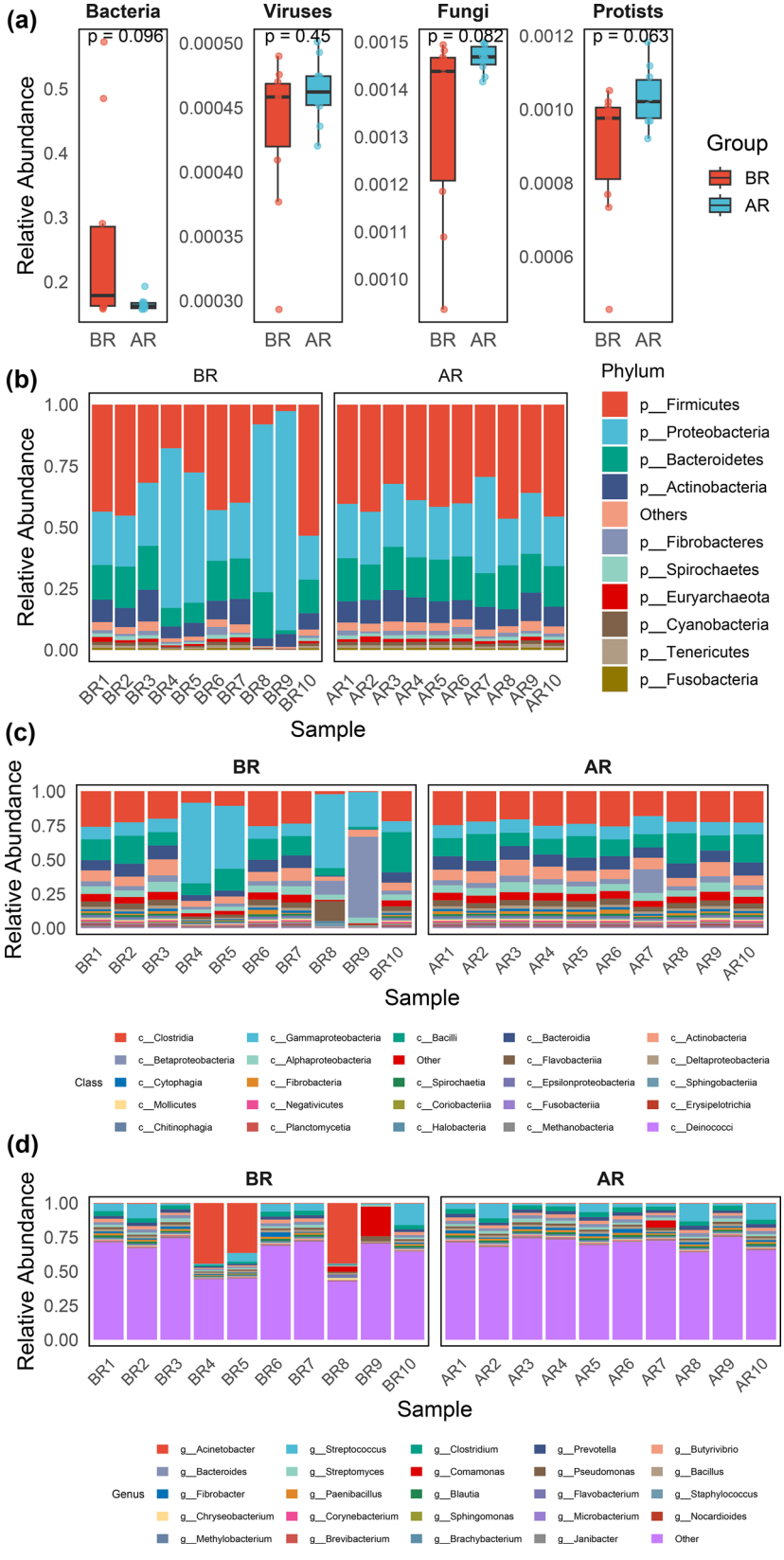

Fig. 2 The relative abundance of gut microbiota. (**a**) the relative abundance and changes of microorganisms at the domain level. (**b**) top 10 bacteria with the highest relative abundance in both groups at the phylum level. (**c**) top 24 bacteria with the highest relative abundance in both groups at the class level. (**d**) top 25 bacteria with the highest relative abundance in both groups at the genus level

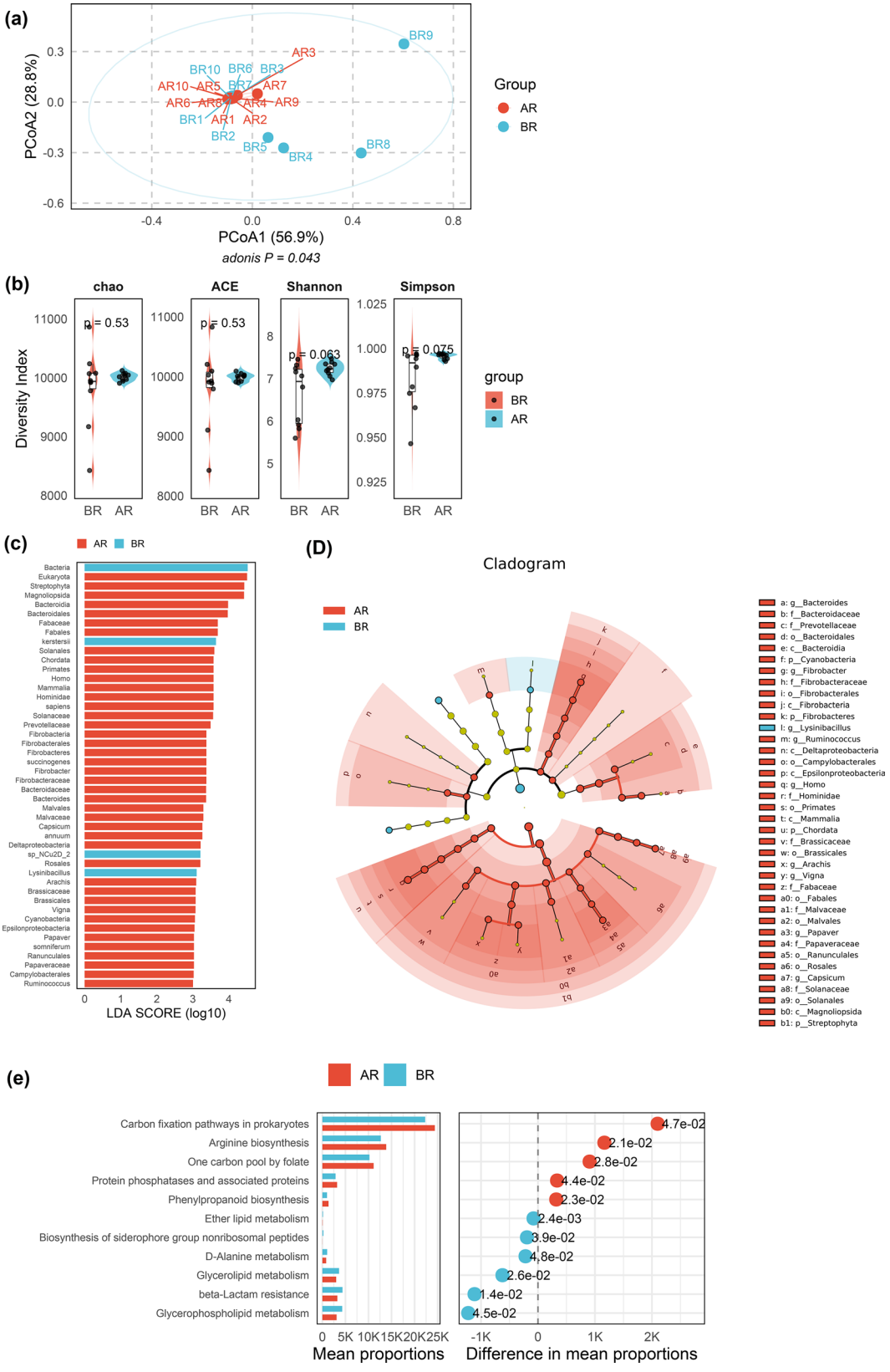

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Fig. 3 Diversity and differential analysis of equine gut microbiota before and after competition. (**a**) Principal Coordinates analysis (PCoA) showing the microbial community structure, with blue circles indicating samples collected before competition and red circles for samples after competition. Circle size reflects data dispersion within each group. (**b**) α-diversity indices (Shannon, Simpson, Chao1, ACE) comparing samples before and after competition. (**c**) LEfSe analysis highlighting species with LDA scores > 3 indicating significant differences. (**d**) phylogenetic tree of differential species, with concentric circles representing taxonomic levels from phylum to genus, color-coded by group, and yellow representing species without significant differences. (**e**) stamp analysis reveals significantly up- and down-regulated metabolic pathways in microorganisms

and Ruminococcus displayed similar correlation patterns with the differential metabolites (Fig. 6b).

Discussion

This study aimed to investigate the differences in the gut microbiota and metabolites of warmblood horses before and after show jumping competition. We employed metagenomic and metabolomic approaches to analyze genes, microbial communities, functional pathways, and untargeted metabolites from pre- and post-competition fecal samples collected from 10 healthy warmblood horses. To further explore the potential impact of gut microbiota changes on fecal metabolite profiles, we performed correlation analyses to integrate metagenomic and metabolomic datasets, facilitating the identification of potential exercise-related biomarkers. Our results revealed notable alterations in specific gut microbial taxa and key metabolites post-competition, suggesting that both the microbiota and metabolome were affected by exercise-induced stress. These findings provide valuable insights into the adaptive responses of the equine hindgut ecosystem to intense exercise. To our knowledge, this is the first study to use metagenomics and metabolomics to comparatively analyse faecal samples before and after a professional horse competition.

Focusing on warmblood horses participating in show jumping events, we observed a not significant increase in hindgut microbial alpha diversity and a more homogeneous community structure after the competition. These findings suggest that competition-related stress may promote a more homogeneous gut microbial composition. Notably, the relative abundances of microbial taxa such as Prevotella, Ruminococcus, and Bacteroides increased post-competition compared to pre-competition levels, suggesting their important roles in metabolism and energy supply. These observations are consistent with the findings of Zhang Yanni [13] in Sunit sheep, where exercise promoted an increase in Prevotella and Bacteroides. Moreover, our results showed a significant rise in the relative abundance of Fibrobacter after the competition, in agreement with previous studies on Standardbred horses [23]. As a cellulose-degrading bacterium, Fibrobacter facilitates the breakdown of cellulose into short-chain fatty acids such as acetate [39]. The enrichment of cellulose-degrading bacteria during exercise likely enhances cellulose degradation and elevates blood acetate levels, thus providing substantial energy to meet the heightened demands of strenuous exercise. These findings offer theoretical support for developing dietary strategies aimed at modulating the abundance of cellulolytic bacteria in the gut microbiota prior to competition to ensure adequate energy supply during competions.

Functional analysis of microbial metabolism revealed significant enrichment of pathways related to carbon fixation in prokaryotes, the folate-mediated one-carbon pool, and phenylpropanoid biosynthesis post-competition. Carbon fixation pathways provide precursors for energy production [40], while folate-dependent one-carbon metabolism contributes to nucleotide and amino acid synthesis and plays critical roles in maintaining redox homeostasis [41]. Additionally, 3-phenylpropionic acid produced through phenylpropanoid metabolism helps maintain intestinal epithelial barrier function [42]. The upregulation of these pathways suggests that the gut microbiota may adjust its metabolism to meet the increased energy and antioxidant demands imposed by strenuous exercise. Conversely, pathways involved in lipid metabolism, such as glycerophospholipid metabolism, ether lipid metabolism, and glycerolipid metabolism, were significantly downregulated after the competition. Given that these pathways are essential for maintaining biological membrane integrity [43–45], their downregulation may reflect adjustments in microbial membrane lipid metabolism or structural remodeling. Changes in arginine biosynthesis and D-alanine metabolism pathways were also observed, likely indicating microbial modulation of amino acid synthesis and cell wall construction [46]. As arginine has been shown to enhance exercise performance through various metabolic mechanisms [47], its increased biosynthesis post-competition may contribute to supporting the horses' athletic performance. Additionally, pathways related to β-lactam resistance and siderophore group nonribosomal peptide biosynthesis were significantly reduced post-competition ($p < 0.05$), suggesting that strenuous exercise may suppress microbial functions associated with antibiotic resistance [48] and iron metabolism [49]. Moreover, the enrichment of protein phosphatases and associated proteins after the competition implies potential stress responses or signal transduction adjustments between the microbiota and host [50]. Collectively, these findings suggest that the equine hindgut microbiota undergoes metabolic reprogramming after intense exercise,

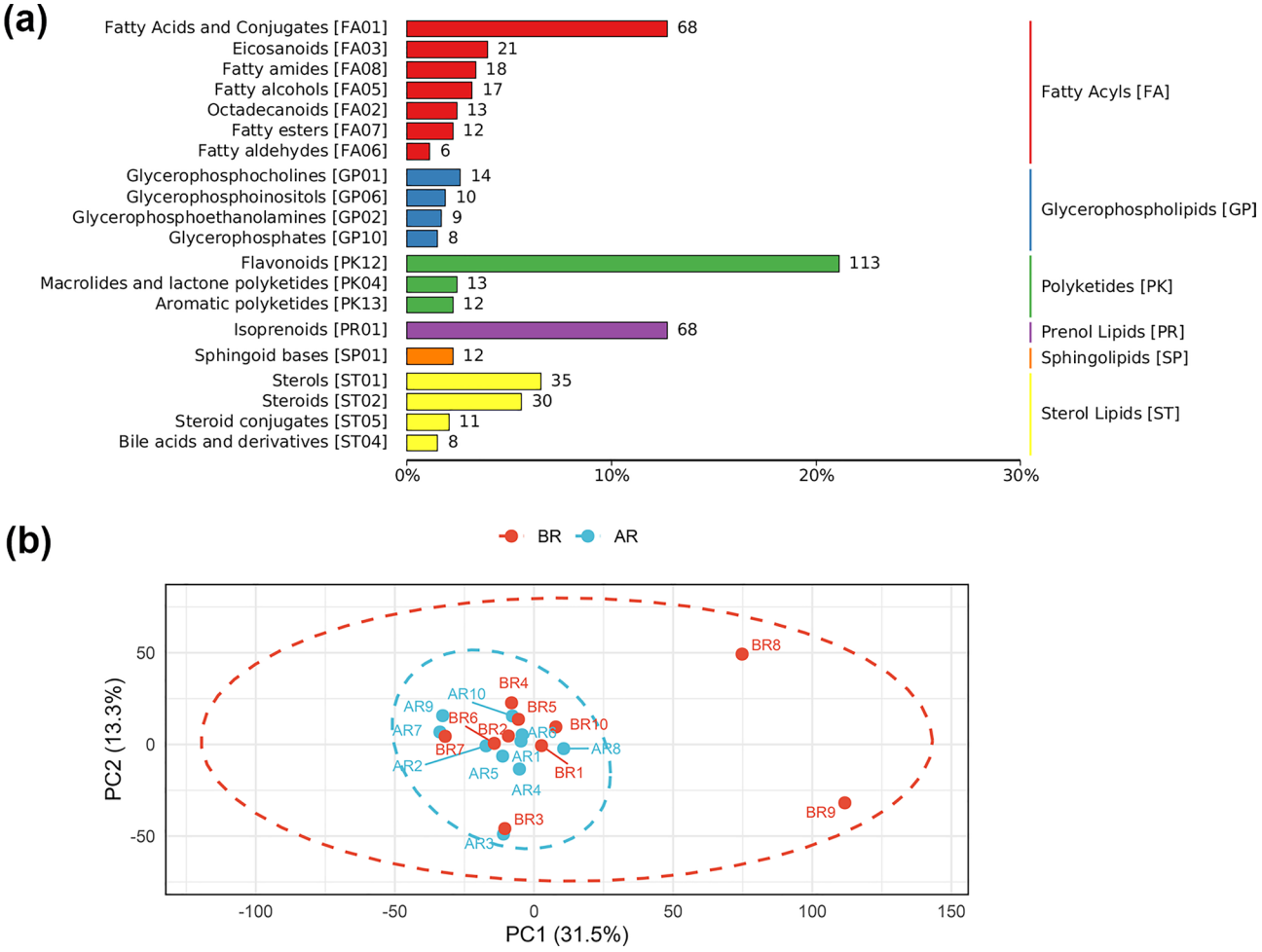

Fig. 4 Evaluation of Untargeted metabolomics data. (**a**) summary of LIPID MAPS database classifications. (**b**) PCA analysis based on the types of metabolites before and after competition

enhancing energy conversion efficiency and stress adaptation capacity.

Metabolomic analysis revealed that metabolites such as orsellinic acid, ergosteryl-3-β-D-glucoside, and 3-phenyl-4-pentenal were significantly downregulated after the competition, whereas glucosan and thiamin pyrophosphate were significantly upregulated. The upregulation of glucosan (a pyrolysis product of cellulose and glycogen) [51] and thiamin pyrophosphate (a key coenzyme in glycolysis and the tricarboxylic acid cycle) [52] indicates that horses enhanced glucose metabolism to meet the high-intensity energy demands during exercise. These metabolic shifts reflect a reprioritization of energy utilization during strenuous activity, shifting from energy storage to rapid energy supply, particularly relying on glycolysis and glycogenolysis. Beyond energy metabolism, glucosan and thiamin pyrophosphate also exhibit antitumor and immune-modulatory activities [52, 53]. We also observed specific metabolic adaptations related to oxidative stress. Levels of (1 R,6 R)-1,4,5,5a,6,9-hexahydrophenazine-1,6-dicarboxylate and β-alanyl-L-lysine decreased significantly post-competition, while novapikromycin and delphinidin 3-O-(acetylglucoside) levels increased. Previous studies have shown that ergosteryl-3-β-D-glucoside possesses free radical scavenging activity [54], while orsellinic acid derivatives and phenazine compounds exhibit antimicrobial, antioxidant, and anti-inflammatory properties [55, 56]. β-alanyl-L-lysine was found to increase following wheat straw feeding and strongly correlated with ruminal Streptococcus levels in Tibetan sheep [57], consistent with our observations. Delphinidin 3-O-(acetylglucoside) is an exogenous antioxidant compound [58], and novapikromycin has demonstrated potential in combating multidrug-resistant respiratory pathogens [59]. Together, these findings indicate that horses maintain redox balance during exercise by consuming endogenous antioxidants and upregulating exogenous antioxidant production, while simultaneously activating anti-inflammatory and antimicrobial mechanisms to protect tissues.

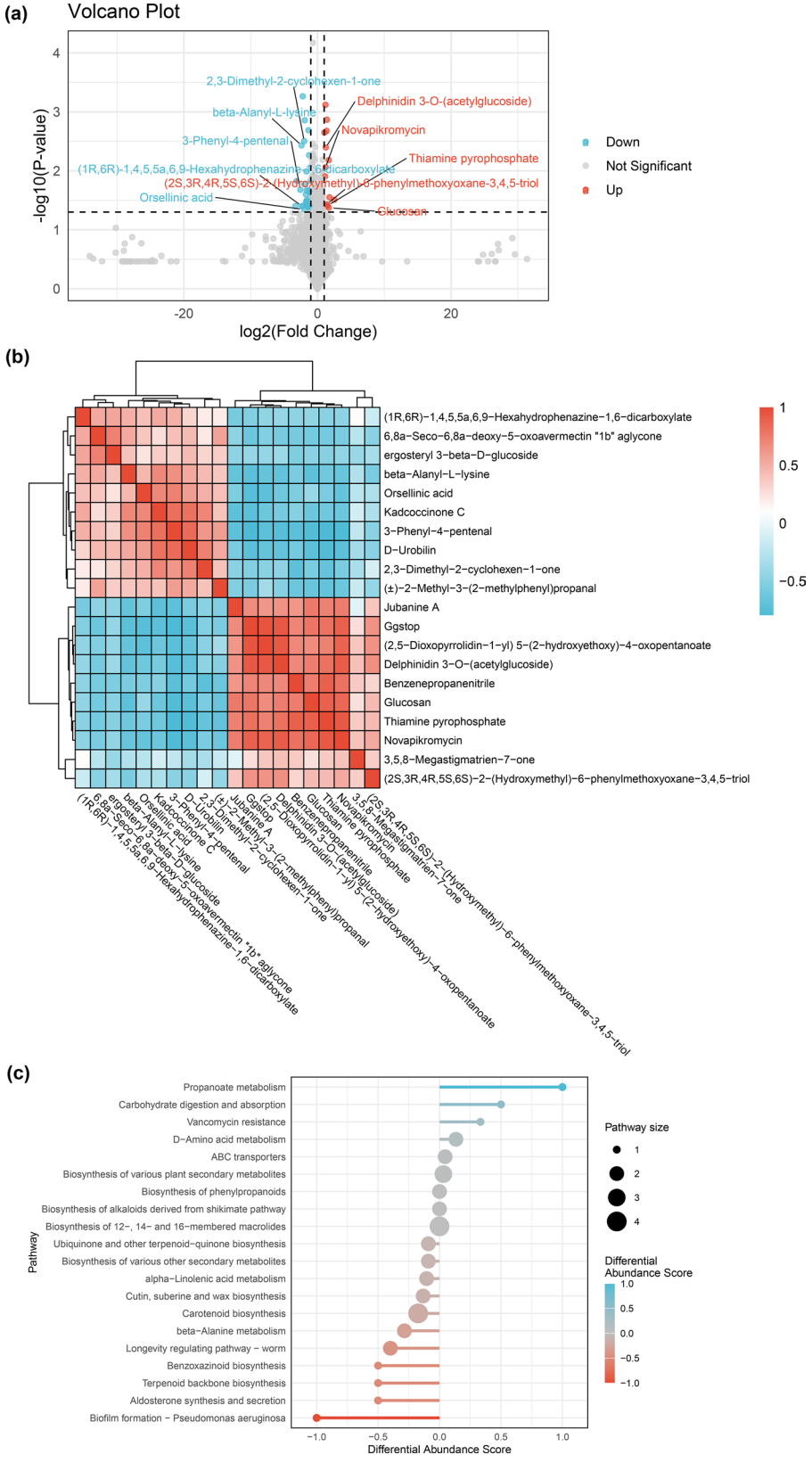

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Fig. 5 Changes in metabolites related to the gut microbiota of horses before and after competition. (**a**) volcanic map of differential metabolites: metabolites with FC > 2 and $p < 0.05$ are shown in red; metabolites with FC < 0.05 and $p < 0.05$ are shown in blue; metabolites with non-significant changes are shown in gray. The top five significantly changed metabolites with the largest and smallest Fold changes are labelled in the figure. (**b**) Heatmap of correlation analysis for these significantly dysregulated metabolites: red indicates positive correlation, blue indicates negative correlation, and white indicates no significant correlation. (**c**) differential metabolite differential abundance score plot: the DA score reflects the overall change of all metabolites in the metabolic pathway, the length of the line segment indicates the absolute value of the DA score, and the size of the dots at the endpoints of the line segment indicates the number of differential metabolites in the pathway

Pathway enrichment analysis of differential metabolites revealed significant downregulation of pathways related to biofilm structure and significant upregulation of pathways associated with immune response and nutrient metabolism, aligning with the microbial analysis results. These findings suggest that the gut microbiota plays a critical role in alleviating stress on the intestinal epithelium and maintaining gut health during exercise.

Correlation analysis between microbial biomarkers and differential metabolites revealed distinct, taxon-specific association patterns, with many reaching statistical significance. Lysinibacillus exhibited strong negative correlations with metabolites such as glucosan, thiamin pyrophosphate, delphinidin 3-O-(acetylglucoside), and novapikromycin, indicating that higher Lysinibacillus abundance corresponded to lower levels of these metabolites. Given that these metabolites are primarily involved in energy metabolism [51], vitamin transformation [60], and antioxidant activity [58], it is plausible that Lysinibacillus enrichment before exercise may inhibit metabolic activity to regulate energy output and antioxidant capacity under resting conditions. In contrast, genera such as Prevotella, Fibrobacter, and Ruminococcus showed positive correlations with these metabolites, suggesting that their post-competition enrichment may be closely associated with energy replenishment and antioxidant synthesis. As typical cellulolytic bacteria [61, 62], Fibrobactor and Ruminococcus showed positive correlations with carbohydrate-derived and anthocyanin-derived metabolites, implying their potential role in energy recovery and gut barrier restoration after exercise. Interestingly, certain metabolites displayed "directional differentiation" in their correlations; for example, 3-phenyl-4-pentenal was strongly positively correlated with Bacteroides but weakly negatively correlated with Fibrobacter, indicating that it may accumulate under pre-competition microbial dominance and be degraded or utilized post-competition. These aromatic compounds may be involved in stress regulation or energy metabolism [63] and warrant further investigation. In summary, the correlation network revealed that exercise-induced shifts in gut microbiota not only affect metabolic flux but also exhibit targeted regulatory effects. Lysinibacillus may inhibit specific metabolic pathways during resting states, whereas Prevotella, Ruminococcus, Bacteroides, and Fibrobacter may promote the synthesis of beneficial metabolites post-exercise to support host energy demands and antioxidant defenses. Future studies should focus on functional validation and pathway analysis of these key genera to elucidate their roles in exercise adaptation, energy transformation, and gut homeostasis.

This study has certain limitations. A control group that was present during the competition but did not participate should have been established to account for the effects of dynamic environmental factors such as weather conditions, time of day, and competition venue. Given the presence of multiple confounding factors on the competition day, it cannot be concluded that the observed changes in the experimental results were exclusively induced by the competition. In addition, this study lacks blood metabolite data, as the competition organizers prohibited any unauthorized blood collection during the event.

Conclusion

This study systematically investigated changes in the gut microbiota of warmblood horses before and after show jumping competition using metagenomic sequencing combined with untargeted metabolomics. The results revealed that after competition, the microbial composition among individuals became more homogeneous. Specific microbial genera, including Bacteroides, Prevotella, Fibrobacter, and Ruminococcus, were significantly enriched after show jumping competitions. Meanwhile, metabolites highly associated with energy supply, such as Glucosan and Thiamin pyrophosphate, were significantly upregulated, whereas antioxidant-related metabolites, such as Orsellinic acid, 2,3-dimethyl-2-cyclohexen-1-one, and (1R,6R)-1,4,5,5a,6,9-hexahydrophenazine-1,6-dicarboxylate, were significantly downregulated. These changes suggest a transition of the gut microbial ecosystem from a maintenance-oriented state to an enhanced energy conversion and stress adaptation mode following the competition. Integrated analysis revealed Fibrobacter and Ruminococcus were positively correlated with metabolites related to energy metabolism, while Lysinibacillus showed significant positive correlations with metabolites involved in antioxidative activity and intestinal mucosal protection. In conclusion, this study provides new insights into the effects of equestrian show jumping on equine intestinal microbial homeostasis and metabolic balance, and provides a theoretical basis for developing intervention strategies aimed at maintaining intestinal microecological homeostasis during intense

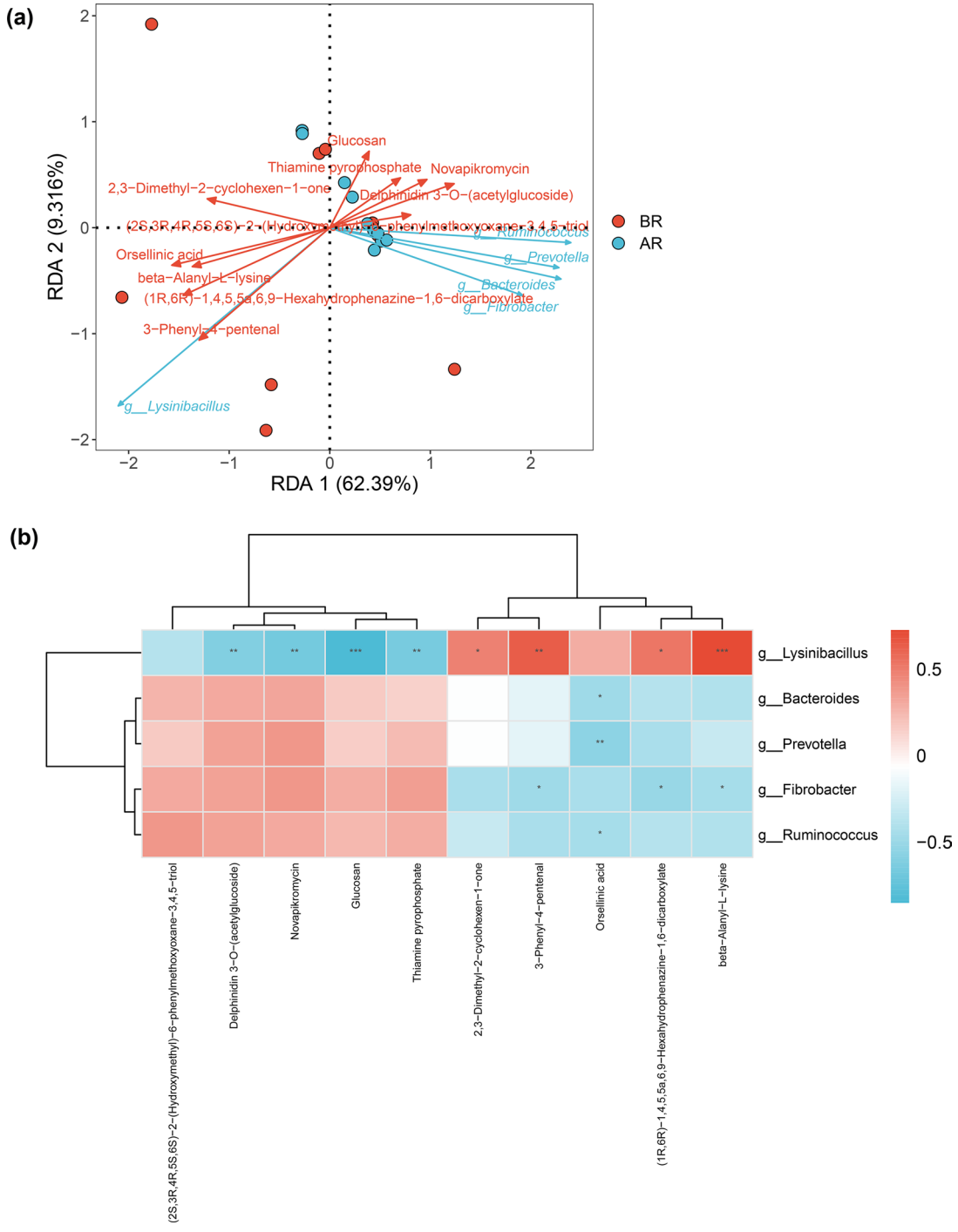

Fig. 6 Integrated analysis of Metagenomics and Metabolomics. (**a**) redundancy analysis (RDA) at the species level: different colored dots represent different samples. Arrows indicate metabolic information. The distance from metabolite projection points to arrows represents the relative impact of metabolites on the sample community distribution. (**b**) Heatmap of the association between the top 10 differential metabolites and the top 5 dominant genera: each row represents a microorganism at the genus level. Each column represents a differential metabolite. The correlation coefficient (R) is indicated by color, where $R > 0$ indicates positive correlation (red), $R < 0$ indicates negative correlation (blue). * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$

exercise. Furthermore, the results lay a foundation for the development of probiotic products specifically tailored to the physiological needs of equestrian horses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-026-00535-y.

Supplementary material 1

Supplementary material 2

Supplementary material 3

Supplementary material 4

Supplementary material 5

Supplementary material 6

Supplementary material 7

Supplementary material 8

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Author contribution
Research conception and Design, resources, supervision, funding acquisition, Q. W.; Writing (preparation of the original manuscript), S. Y.; Methodology and formal analysis, Q. W., S. Y.; Investigation and data management, W. T., X. Y.; Writing-review and editing, Q. W., S. Y.; Visualisation, S. Y., P. X., H. Y.; Project management, Q. W.，Q. Y., Y. L. All authors have read and agreed to the published version of the manuscript.

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Data availability
Metagenomic data available were deposited in the NCBI Sequence Read Archive (SRA) under the accession number: PRJNA1125130.

Declarations

Ethics approval and consent to participate
The samples used for metagenomic and metabolomic analysis in this study were collected with the informed consent of the animal owners for commercial omics testing and have been authorised for use in research. Consequently, institutional animal research ethics was not required. The Animal Research Ethics Committee of China Agricultural University (AW72203202-1-1) has approved the collection and transfer of genetic material for metagenomic sequencing analysis. We have complied with all ethical regulations pertaining to the use of animals.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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