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The rumen microbiome and its metabolome together with the host metabolome regulate the growth performance of crossbred cattle

Wei Zhao[1†], Lina Ma[1†], Lin Xue[1], Qiufei Jiang[2], Yuan Feng[2], Suwan Wang[1], Jinli Tian[1], Xiaohua Tian[1], Yaling Gu[1*] and Juan Zhang[1*]

Abstract

Background Although it has been demonstrated that gastrointestinal microorganisms greatly influence livestock performance, the effect of gastrointestinal microorganisms on the growth performance of crossbred cattle remains unclear. Due to their superior production characteristics, understanding the impact of gastrointestinal microorganisms on the growth performance of crossbred beef cattle is of significant importance for improving farming efficiency.

Result In this study, healthy Simmental with similar birth date and weight were selected as dams, Simmental (Combination I), Belgian Blue (Combination II) and Red Angus (Combination III) were used as parents for crossbreeding. The progeny of the three combination crosses were measured for growth performance under identical conditions from birth rearing to 18 months of age ($n=30$). Rumen fluid and plasma were collected for macro-genomic and non-targeted metabolomic analysis ($n=8$). The results showed that Combination II was superior to Combination I and Combination III in body weight (BW) and body height (BH) ($P<0.05$). Mycoplasma, Succinivibrio, Anaerostipes, Methanosphaera, Aspergillus, and Acidomyces were significantly increased in the rumen of Combination II ($P<0.05$), whereas differentially expressed metabolites (DEMs) 9,10,13-Trihome (11), 9,12,13-Trihome and 9(10)-Epome, and 9(S)-Hpode were reduced in abundance. In addition, plasma DEM PC (14:0/P-18:1(11Z)), PC (16:0/0:0), and PC (17:0/0:0) were down-regulated in combination II. Correlation analysis revealed that Anaerostipes, Methanosphaera, and Succinivibrio were associated with PC (14:0/P-18:1(11Z)), 9(10)-Epome, 9,10,13-Trihome (11), 9(S)-Hpode, 9,10,13-Trihome, PC (17:0/0:0), and PC (16:0/0:0). Growth traits were significantly positively correlated with the three dominant genera, Anaerostipes, Methanosphaera, and Succinivibrio, while significantly negatively correlated with key rumen metabolites and plasma metabolites ($P<0.05$).

†Wei Zhao and Lina Ma contributed equally to this work.

*Correspondence:
Yaling Gu
guyaling@sina.com
Juan Zhang
zhangjuannxy@nxu.edu.cn

Full list of author information is available at the end of the article

Conclusions Our study reveals the role of rumen microorganisms and its metabolites with host metabolism in the regulation of growth performance of crossbred cattle, which will contribute to the development of modern cattle breeding.

Keywords Simmental, Hybrid offspring, Growth performance, Rumen microorganisms, Metabolite

Introduction

According to data released by the Food and Agriculture Organization of the United Nations (FAO), global beef production has shown regular and steady growth. In 2022, global beef production reached 69 million tonnes, and China's beef production was 7,182,600 t, with a compound annual growth rate (CAGR) of 1.77%, ranking 3rd globally [1]. With the continuous improvement of people's living standards, consumers' consumption of beef has gradually increased, which makes the increase of beef production become a pressing issue [2].

Studies show that the dominant variance for growth, carcass, and reproduction traits in cattle ranges from 0 to 42%, with growth and carcass traits specifically varying between 0% and 9% [3, 4]. Crossbreeding significantly enhances production traits, which have led to global interest in strategies for more consistent and efficient profitability [5]. The Simmental, a dual-purpose breed from the Swiss Alps, is known for both dairy and meat production and is a major breed in China due to its high meat performance [6, 7]. Calves from Pinzgauer-Simmental crosses were heavier at birth compared to those from Tarentaise crosses [8], and Simmental crosses outperformed yak hybrids in growth and carcass traits [9]. Angus, an old British breed, is globally recognized for its excellent meat quality [10]. Crossbreeding Lowline Angus with Bos indicus significantly increased weight in both bulls and cows compared to native Thai cattle [11, 12]. Similarly, Qinchuan-Angus crosses exhibited superior live weight, carcass weight, fat content, and dorsal thickness compared to pure Qinchuan cattle [13]. Belgian Blue, renowned for its double-muscle genetics and exceptional meat production, also shows enhanced traits when crossed with Piemontese, outperforming purebred Piemontese in growth, feed efficiency, and carcass yield [14, 15]. Crossbreeding effectively combines the dominant traits of different strains, making it a reliable method for improving cattle production, aligning with market demands [16–18].

The rumen hosts a diverse community of microorganisms (e.g., protozoa, bacteria, fungi) that ferment cellulose and complex polysaccharides, producing short-chain fatty acids (acetic, propionic, butyric acids), gases (methane), and other metabolites [19]. Some of these metabolites are absorbed, influencing energy metabolism and nutrient uptake in cattle [20]. Recent studies highlight the gut microbiota's role in regulating cattle growth and development [21]. For example, adding 45% peanut vines and 55% whole maize silage to Simmental roughage increased *Firmicutes* abundance, improving growth performance and meat quality [22]. de Freitas identified rumen microbes linked to weight gain in Braford heifers, such as *RFN20*, *Prevotella*, and *Anaeroplasma* [23]. Other studies have shown that feed type can alter the Simmental rumen microbiota, enhancing growth performance and fermentation [24]. Additionally, heating water for cattle during cold seasons increased daily weight gain, with *Prevotella*, *Succinivibrionaceae*, and *Lachnospiraceae* potentially regulating propionic acid production [25]. Understanding rumen microbiota composition is crucial for improving cattle growth performance.

In order to explore the growth performance of crossbred progeny of different cattle breeds, Simmental was selected as the dam, and Simmental (Combination I), Belgian Blue (Combination II), and Red Angus (Combination III) were selected as the parents in this experiment. The comparison of body weight and size of hybrid offspring from different combinations under the same rearing conditions was used to screen for the best crossbreeding combinations in Simmental. We hypothesised that the host genetics of crossbred offspring influenced rumen microbial composition and metabolite activity, resulting in differences in the growth performance of crossbred offspring between breeds. Meanwhile, based on the above hypotheses, we characterised the rumen microbiota, its metabolites, and plasma metabolites in the three combined hybrid offspring using macrogenomic sequencing and non-targeted metabolomics, respectively. We aimed to investigate the possibility that rumen microbiota, its metabolites, and host metabolites in hybrid offspring regulate the growth performance of crossbred cattle, thus deepening the understanding of the rumen internal environment and crossbreeding selection.

Materials and methods

Animals and experimental design

All the animals required for the test in this study were provided by Ningxia Yitai Herding Co Ltd (Yinchuan, China). We selected Simmental with a close date of birth and good health condition as the dam, and Simmental (Combination I), Belgian Blue (Combination II), and Red Angus (Combination III) as the parents for artificial insemination (Table 1). The hybrid offspring of the pregnant females after calving relied mainly on breast milk to provide nutrients needed for growth and development. W Weaning was carried out until 6 months of age. For

Table 1 Combination programme between the three varieties

Combination type	Male	Female
Combination I (XX-18)	Simmental ♂	Simmental ♀
Combination II (LX-18)	Belgian Blue ♂	
Combination III (HX-18)	Red Angus ♂	

each combination, 30 hybrid offspring each of similar health and weight were selected. Forage was provided to the animals at 8:30 a.m. and 6:30 p.m. daily under the same feeding environment and conditions. The feed is mainly silage, as well as wheat straw, alfalfa, and other roughage, supplemented with concentrates at the right time: mainly cornmeal 47.5%, wheat bran 5%, cottonseed cake (meal) 10%, 1% additives, salt 0.5%, bone meal 1%, wheat straw powder or grass meal 35%. The entire feeding process was housed, with free access to feed and water.

Sample collection and processing

The hybrid offspring of the three combinations were reared until 18 months of age to determine growth performance ($n = 30$). Meanwhile, samples were taken 9 h after the end of the last feeding. At the same time, 8 cows with similar body weight were selected from each of the three combinations. Samples of rumen fluid (Combination I: XX-18-LW, Combination II: LX-18-LW, Combination III: HX-18-LW) and plasma (Combination I: XX-18-XJ, Combination II: LX-18-XJ, Combination III: HX-18-XJ) were collected 9 h after the last feeding. Sample collection was completed between September and November 2023. The rumen fluid sampler was fully rinsed with warm water and thoroughly sterilised and disinfected before use, then one end with a metal filter was fed into the rumen by bovine swallowing, the other end was connected to a 50 mL syringe to aspirate the first tube of rumen fluid and discarded, followed by the second tube of rumen fluid (30–40 mL) (solids and liquids) as a representative sample. At the time of collection, the collected rumen fluid samples were hand-filtered using 4 layers of sterile coarse cotton cloth, and the samples were snap-frozen in liquid nitrogen for macrogenomics and metabolomics analyses. At the same time, we collected blood through a blood collection tube using EDTA as an anticoagulant in the tail vein in the morning before the last feeding. After standing for 1 h at room temperature, plasma was obtained by centrifugation at 2000 rpm for 10 min and subsequently stored at -80 °C for metabolomic analysis.

Rumen fluid macrogenome sequencing

DNA extraction and sequencing

The rumen fluid samples were thawed at room temperature and centrifuged at 15,000 ×g, then the supernatant was removed. The genomic DNA of the samples was extracted using a DNA extraction kit according to the manufacturer's instructions, after which the concentration of DNA was detected using agarose gel electrophoresis and NanoDrop2000. 130 μL (260 ng) of Covaris S220 interrupted DNA transfer fragmented DNA was added to a Covaris tube into a new 1.5 mL centrifuge tube. 100 ng of the interrupted DNA was taken for end repair and splice ligation. Subsequent to library amplification, 5 uL of PCR Primer Mix 3 for Illumina and 25 uL of VAHTS HiFi Amplification Mix were mixed into the purified elution product, a limited cycle (7–8) polymerase chain reaction (PCR) was performed, and the PCR amplification product was purified. The final concentration of the library was quantified using Qubit 3.0 and the average library size was assessed using an Agilent 2100.

Data processing and statistical analysis

After sequencing, the raw sequence data files were demultiplexed and stored in FASTQ format. The reads containing N bases (fuzzy bases) were first removed by excising the junctions and filtering out the low quality bases using fastp (v0.20.1) 1. Subsequently, the bipartite reads were aligned to the host genome using bbmap (v38.93-0) 2, and this portion of the sequence was removed. After obtaining the validreads, the sequences were spliced to the macrogenome using MEGAHIT (v1.2.9) 3. ORF prediction of spliced contigs (≥ 500 bp) was performed using prodigal (v2.6.3) 4 and translated into amino acid sequences. Non-redundant gene sets were constructed using MMSeqs2 (v13.45111) 5 for the predicted genes in all the samples, with clustering parameters of 95% identify between sequences and 90% coverage. The longest gene in each cluster was selected as the representative sequence for that gene set. The cleanreads of each sample were compared with the non-redundant gene sets separately (95% identity) using salmon (v1.8.0) to count the abundance information of the genes in the corresponding samples.

Bioinformatics analysis

The representative sequences (amino acid sequences) of the gene set were annotated with Non and Gene KEGG using DIAMOND (v0.9.10.111) 6 software, and the BLAST comparison parameter was set with an expected value of 1e-5. Species annotations were obtained from the taxonomic information database corresponding to the NR database, and the abundance of the species was calculated by using the combined abundance of the corresponding genes of the species. And the abundance of the species in the sample was counted at the Domain, Kingdom, Phylum, Class, Order, Family, Genus, and Species taxonomic levels to construct the abundance profile at the corresponding taxonomic levels. The gene set was compared to the Carbohydrate-Active enzymes database

(CAZy database, http://www.cazy.org/) using the CAZy database counterpart tool hmmscan (v3.1) to obtain information about the functional classification of carbohydrate-active enzymes. The R package (v 3.2.0) was used to calculate Principal Components Analysis (PCA), Principal Co-ordinates Analysis (PCoA) and Non-metric multi-dimensional scaling (NMDS) equidistance matrices for species abundance spectra or functional abundance spectra and to visualise and analyse the results. Based on R package, Kruskal Wallis was used for analysis of variance, and Linear discriminant analysis Effect Size (LEfSe) was used to analyse the species abundanceprofile. Sequencing analysis was performed by Shanghai OE Biomedical Technology Co.

Rumen fluid and plasma untargeted metabolome sequencing

Metabolite extraction and analysis

Frozen rumen fluid was thawed gradually on ice at room temperature. 1 mL of the sample was taken over the SPE solid-phase mini-column, and 3 mL of methanol eluate was collected; it was nitrogen-blown with a nitrogen blowing apparatus, and after blow-drying, 300 μL of pre-cooled methanol-water (V: V = 4:1, containing mixed internal standard, 4 μg/mL) was added. Plasma samples stored at -80 °C were thawed in an ice-water mixture. 100 μL of plasma sample was pipetted into a 1.5 mL EP tube, and 400 μL of protein precipitant methanol-acetonitrile (V: V = 2:1, containing mixed internal standard, 4 μg/mL) was added. Both metabolites were vortexed and shaken for 1 min, ice-water bath for 10 min, and left at -40 ℃ overnight. On the next day, the samples were centrifuged at 12,000 rpm and 4 ℃ for 10 min, and 150 μL of the supernatant was aspirated, filtered using a 0.22 μm organic-phase pinhole filter and stored at -80 ℃ until Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed. Quality control (QC) samples were prepared by mixing equal volumes of extracts from all rumen fluid samples to verify the stability and reproducibility of the system. All extraction reagents were pre-cooled at -20 °C before use. Analyses were performed using a liquid-mass spectrometry (LMS) system consisting of a Waters ACQUITY UPLC I-Class plus/Thermo QE plus ultra-high performance liquid tandem high-resolution mass spectrometer (UPLC HSS). An ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm) was used with a column temperature of 45 ℃. The mobile phases consisted of 0.1% formic acid aqueous solution (phase A) and acetonitrile (phase B), and the injection volume was 5 μL with a flow rate of the metabolites of 0.35 mL/min. The mass spectrometry was performed in both positive and negative modes. The Spray Voltage was 3,800 V for the positive ion mode and 3000 V for the negative ion mode; the capillary temperature was set at 320 ℃; the flow rate of the sheath gas was controlled at about 35 Arb, and the flow rate of the auxiliary gas was controlled at 8 Arb.

Data processing and statistical analyses

Raw data were baseline filtered, peaks identified, integrated, retention time corrected, peaks aligned and normalised using Progenesis QI v3.0 software (Nonlinear Dynamics, UK). Compound identification was based on multiple dimensions such as retention time (RT), exact mass number, secondary fragmentation, and isotopic distribution, and was analysed using the Human Metabolome Database (HMDB), Lipidmaps (v 2.3) and METLIN databases, as well as the LuMet-Animal 3.0 database. Data matrices were imported into the R package for PCA analysis to observe the overall distribution between samples and the stability of the whole analysis process. The Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to distinguish metabolite differences between groups. To prevent overfitting, 7-fold cross-validation and 200 Response Permutation Test (RPT) were used to assess the quality of the models. The Variable Importance Projection (VIP) obtained from the OPLS-DA model was used to rank the overall contribution of each variable to the population. A two tailed Student's t-test was further used to verify whether the differences in metabolites between groups were significant. Selected differentially expressed metabolites (DEMs) with VIP value > 1.0 and P value < 0.05. Using KEGG database and Gene Set Enrichment Analysis (GSEA), metabolic pathway enrichment analysis was performed on DEMs and the results were visualised.

Result

Analysis of growth performance

In this study, we analysed the differences in growth performance among the hybrid offspring of the three combinations. The body weight (BW) and body height (BH) of Combination II were significantly higher than those of Combination I and Combination III ($P < 0.05$, Fig. 1A, B), while the cross section height (CSH) and body slant length (BSL) were significantly higher than those of Combination III ($P < 0.01$, Fig. 1C, D). The breast measurement (BM), abdominal circumference (AC) and tube circumference (TC) were not significantly different between the three groups ($P > 0.05$, Fig. 1E, F, G). The above study showed that the BW and BH of Combination II were superior to those of Combination I and Combination III.

Rumen fluid macrogenomic analysis

Analysis of rumen microbial diversity

We used the Illumina platform to perform macrogenomic sequencing of collected rumen fluid,

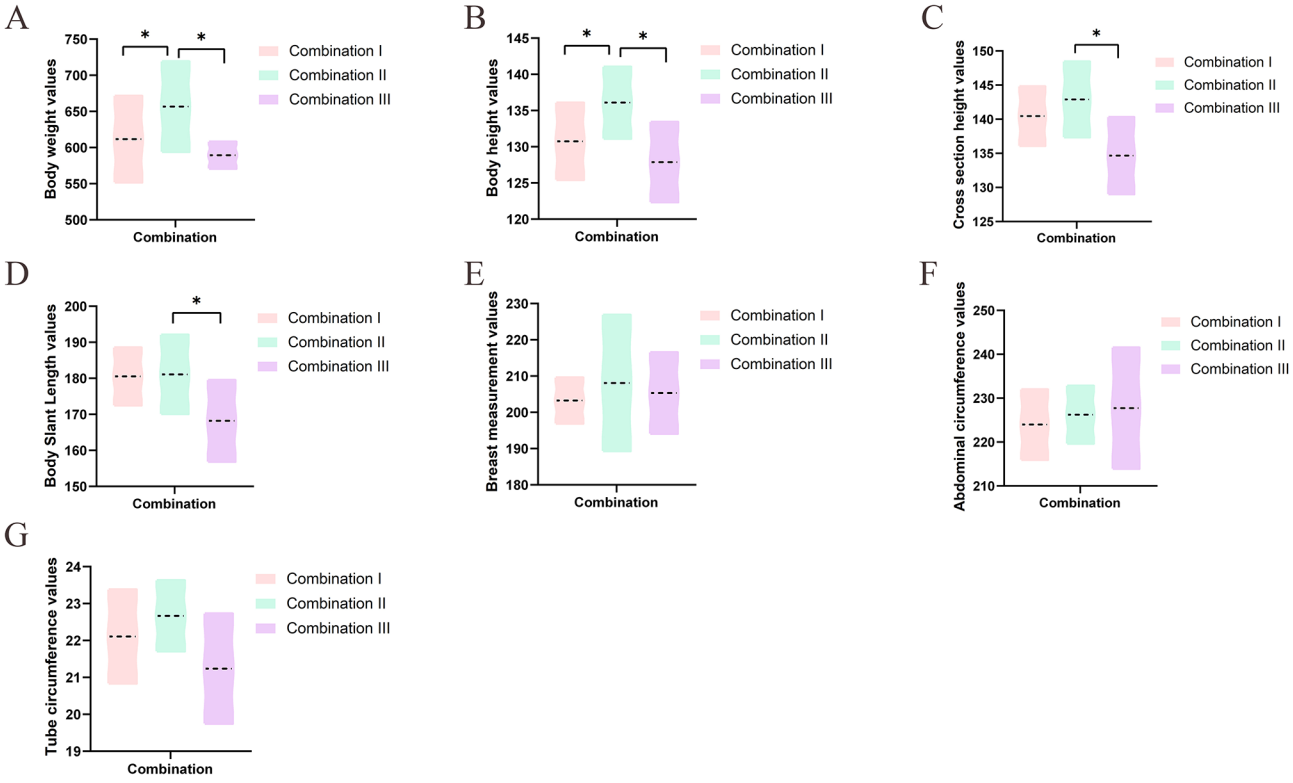

Fig. 1 Determination of growth performance of progeny from crossbreeding of three combinations. **(A-G)** Represents body weight (BW), body height (BH), cross section height (CSH), body slant length (BSL), breast measurement (BM), abdominal circumference (AC), and tube circumference (TC) of the progeny of the crossbreeding of three combinations, respectively

yielding a total of 1,911,841,956 reads, with 79,660,082 reads ± 2,050,756 reads per sample (mean ± standard deviation [SD]). After quality control and removal of host genes, a total of 1,910,652,482 reads were obtained, with 79,610,520 reads ± 2,050,449 reads per sample. After de novo assembly, a total of 32,371,666 contigs were generated (N50 length of 779 ± 84 bp), with each sample being 1,348,819 ± 300,843 contigs (Table 2). Firstly, we performed a comparative analysis of the diversity within and outside the rumen habitat of the three combinations. Alpha diversity reflects the degree of species diversity within the rumen environment. At the genus level, the Chao1, Simpson, Shannon, Ace, and Obs indices were not statistically significant among the three combinations (Fig. 2A - E). Beta diversity analyses illustrated the composition of the microbial communities among the three combinations. No significant separation among the three combinations was found by PCA, PCoA, NMDS and Anosim analyses (Fig. 2F - I).

Analysis of rumen microbial composition and differences

Species annotation was performed through NR Database, and the abundance of the species was calculated by summing the abundance of the corresponding genes of the species, and in total we annotated to 4 Kingdom, 210 Phylum, 227 Class, 471 Order, 1,168 Family, 4,846 Genus, and 29,133 Species (Table S1). The annotated species were differentiated into Bacteria (k__Bacteria), Fungi (k__Fungi), Archaea (k__Archaea), and Viruses (k__Viruses) by Kingdom. At the Phylum level, k__Bacteria had the highest abundance of Bacteroidota and Bacillota (Fig. 1A); k__Fungi had the highest abundance of Chytridiomycota, Mucoromycota, Ascomycota, Zoopagomycota, and Basidiomycota were the most abundant (Fig. 1C); k__Archaea had the highest abundance of Euryarchaeota, Candidatus_Thermoplasmatota, and Candidatus_Bathyarchaeota (Fig. 1E); k__ Uroviricota and Nucleocytoviricota had the highest abundance among Viruses (Fig. 1G). At the Genus level, the species identified in highest abundance in k__Bacteria were *Prevotella*, *Ruminococcus*, and *Hallella* (Fig. 1B); in k__Fungi *Neocallimastix*, *Piromyces*, *Anaeromyces*, and *Rhizopus* had the highest abundance (Fig. 1D); in k__Archaea had the highest abundance of *Methanobrevibacter*, *Methanocorpusculum* and *Methanosphaera* (Fig. 1F); in k__ Viruses had the highest abundance of *Buhlduvirus* and *Hacihdavirus* had the highest abundance (Fig. 2H).

Based on the Kruskal-Wallis test with P value < 0.05 as the threshold, a total of 210 differential species were screened among the three combinations at the Phylum level, and the top 10 Phylums in abundance were taken and analysed, and the relative abundance of

Table 2 Macrogenomic sequence data generated from rumen samples of progeny from three combined crosses ($n=8$)

Sample	RawReads	CleanReads	Contigs	N50
XX-18-1-LW	79,760,690	79,708,050	1,451,129	743
XX-18-2-LW	79,614,600	79,569,712	1,260,601	789
XX-18-3-LW	81,445,092	81,398,484	1,627,755	720
XX-18-4-LW	78,541,316	78,493,332	1,440,323	758
XX-18-5-LW	77,507,486	77,462,572	1,252,898	799
XX-18-6-LW	79,316,158	79,279,468	1,416,498	741
XX-18-7-LW	80,118,114	80,073,966	1,392,264	788
XX-18-8-LW	79,085,520	79,030,702	1,331,723	777
LX-18-1-LW	82,846,962	82,792,990	1,113,861	778
LX-18-2-LW	81,358,220	81,306,606	1,376,071	761
LX-18-3-LW	77,471,786	77,419,208	1,595,330	732
LX-18-4-LW	81,910,040	81,850,946	1,529,382	776
LX-18-5-LW	77,526,216	77,468,700	1,586,683	725
LX-18-6-LW	77,157,216	77,100,640	1,221,942	819
LX-18-7-LW	80,885,422	80,822,350	1,344,823	751
LX-18-8-LW	77,656,592	77,593,498	1,178,836	732
HX-18-1-LW	78,336,040	78,288,160	1,477,662	712
HX-18-2-LW	83,186,484	83,138,152	1,766,964	722
HX-18-3-LW	76,977,990	76,934,198	1,626,469	739
HX-18-4-LW	76,936,896	76,899,050	1,533,985	737
HX-18-5-LW	81,890,630	81,846,584	1,232,208	813
HX-18-6-LW	80,262,640	80,216,816	440,886	939
HX-18-7-LW	83,249,886	83,203,790	621,837	1,102
HX-18-8-LW	78,799,960	78,754,508	1,551,536	744
Total	1,911,841,956	191,065,24,82	32,371,666	18,697
mean	79,660,082	79,610,520	1,348,819	779
SD	2,050,756	2,050,449	300,843	84

Thermodesuifobacteriota in Combination I was significantly higher than that of Combination II and Combination III. Combination II had significantly higher abundance of Elusimicrobiota and Candidatus_Riflebacteria than Combination I and Combination III. Acidobacteriota was significantly less abundant in Combination III than in Combination I and Combination II. (Fig. 3I, Table S2). At the Genus level, a total of 658 differential Genus were screened, and the top 10 Genus in terms of abundance were taken and analysed, among which the abundance of *Methanocorpusculum* in Combination I was significantly higher than that of Combination II and Combination III. The abundance of *unclassified* microorganisms was higher in Combination II than in Combination I and Combination III. The abundance of *Prevotella* was higher in Combination III than Combination I and Combination II, (Fig. 3J, Table S3). We used LEfSe to analyse the composition of species that differed between the three combinations, and found that *Eubacterium*, *Methanocorpusculum*, *Aeriscardovia*, *Klebsiella* and *Desulfovibrio* were more abundant in Combination I than in the other two combinations; in Combination II key microorganisms included *Mycoplasma*, *Succinivibrio*, *Anaerostipes*, *Methanosphaera*, *Aspergillus* and *Acidomyces*; *Prevotella*, *Hallella*, *Sodaliphilus* and *Bacteroides* were the most abundant in Combination III (Fig. 3K).

Functions of the rumen microbiome

Functionally annotated by non-redundant genes, 32.13% of the unique genes from the rumen microbiota were classified in the KEGG pathway, and 2.39% of the genes were annotated to the CAZy database. Functional annotation of the sequencing data based on the KEGG database resulted in a total of 271 pathways for the three combinations. According to Kruskal-Wallis test ($P<0.05$), 34 biological pathways differed significantly among the three combinations, and the top 10 most significant functions are shown in the figure. (Fig. 4A). The unique pathways in the three combinations were analysed by LEfSe analysis, and the main functions in Combination I were porphyrin and chlorophyll metabolism, amino sugar and nucleotide sugar metabolism, sulfur metabolism, and oxidative phosphorylation; unique metabolic pathways in Combination II included insulin signaling pathway and steroid hormone biosynthesis; arginineand proline metabolism and histidine metabolism were considered as notably enriched pathways in Combination III (Fig. 4B, Table S4). As well, the CAZy database was used to analyse the microbial contribution to carbohydrate metabolism, and a total of 56 carbohydrases were obtained from the three combinations, and we visualised the top 10 carbohydrases with the most marked differences between groups (Fig. 4C). We found that glycoside hydrolases (GHs) were most abundant in Combination I and Combination III when LDA scores were higher than 2, and Combination II included the highest enrichment of glycosyltransferases (GTs) and polysaccharide hydrolases (PLs) (Fig. 4D, Table S5).

Non-targeted metabolomic analysis of rumen fluid

In the rumen metabolome, PCA was used to analyse the degree of separation of samples between groups. The positive and negative ion modes of PCA analysis revealed that the samples between the three groups were not clearly distinguished (Fig. S1A, D, G). In addition, OPLS-DA analysis yielded good predictability of the three intergroup samples without overfitting (Fig. S1B, C, E, F, H, I). These results suggest that OPLS-DA analysis can be used to identify differences among Combinations I, II, and III. We screened DEMs at VIP > 1 and $P<0.05$, and identified a total of 317 DEMs (65 up-regulated and 252 down-regulated) for Combination II vs. Combination I in both positive and negative ion modes; 190 DEMs for Combination II vs. Combination III (49 up-regulated and 141 down-regulated); Combination I vs. Combination III, a total of 213 DEMs were identified (163 up-regulated and 50 down-regulated) (Fig. 5A, B,

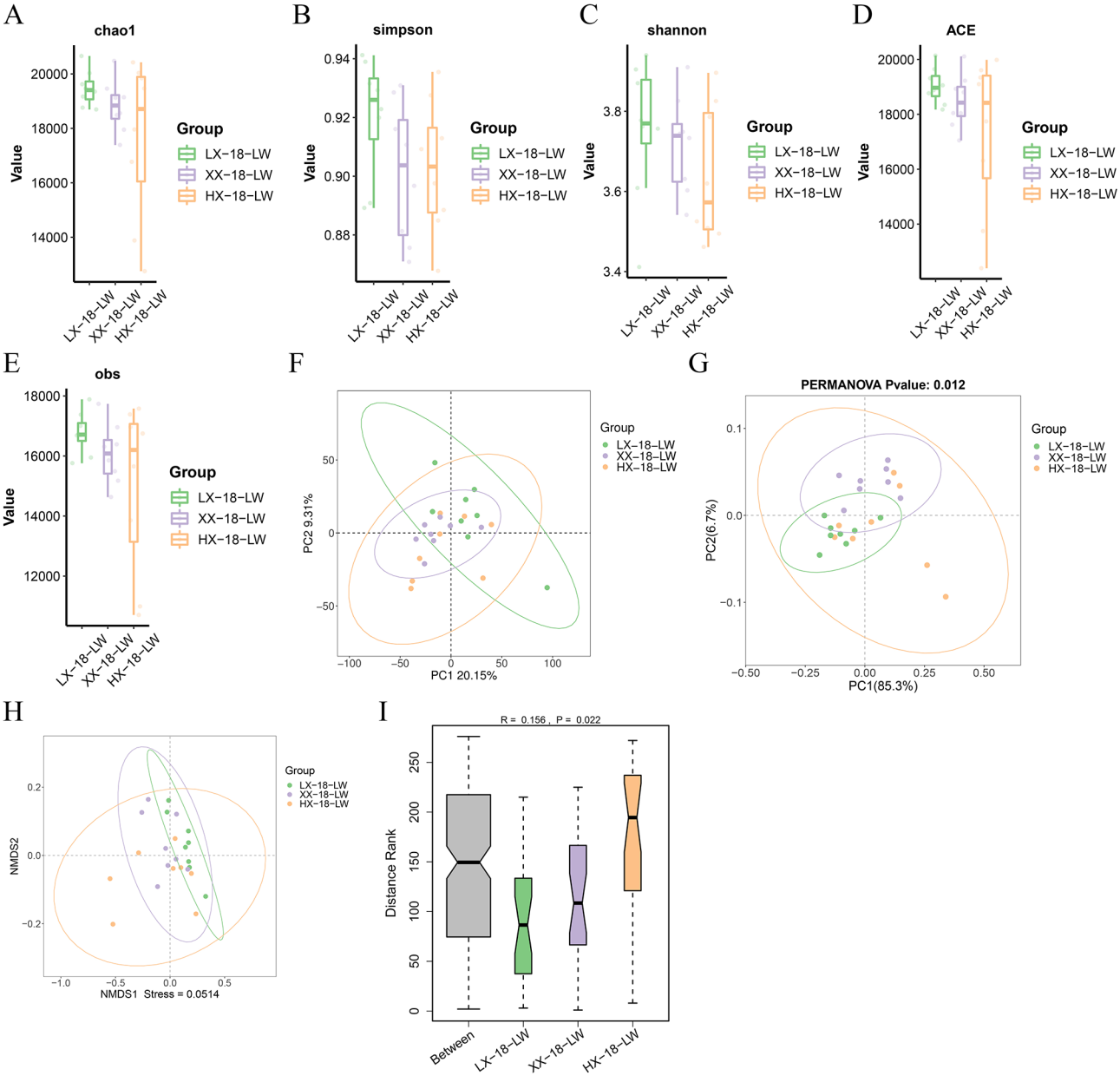

Fig. 2 Diversity of three combinations of rumen microbial communities ($n=8$). **(A - E)** Alpha diversity of different combinations of rumen microbes (Chao1, Simpson, Shannon, Ace and Obs indices). **(F - I)** Bata diversity of different combinations of rumen microorganisms (PCA, PCoA, NMDS and Anosim analysis)

C, Table S6). The relative expression of the top 50 identified prominently different DEMs was used to generate a hierarchical clustering heatmap (Fig. S2). Our selected DEMs were analysed for KEGG pathway enrichment. Pathways significantly enriched in Combination II vs. Combination I were linoleic acid metabolism, purine metabolism, pyrimidine metabolism, pentose phosphate pathway, glutathione metabolism, and alpha-Linolenic acid metabolism (Fig. 5D, Table S7). We found that Combination II vs. Combination III, DEMs were remarkably enriched in four pathways including primary bile acid biosynthesis, valine, leucine and isoleucine biosynthesis, valine, leucine and isoleucine biosynthesis and linoleic acid metabolism (Fig. 5E, Table S8). Combination I vs. Combination III includes alpha-linolenic acid metabolism, PPAR signalling pathway, arachidonic acid metabolism, oxidative phosphorylation, mineral absorption, linoleic acid metabolism, pentose phosphate pathway, primary bile acid biosynthesis, and ABC transporters, purine metabolism were greatly enriched (Fig. 5F, Table S9). From these analyses we found that Combination II was significantly enriched in linoleic acid metabolism for

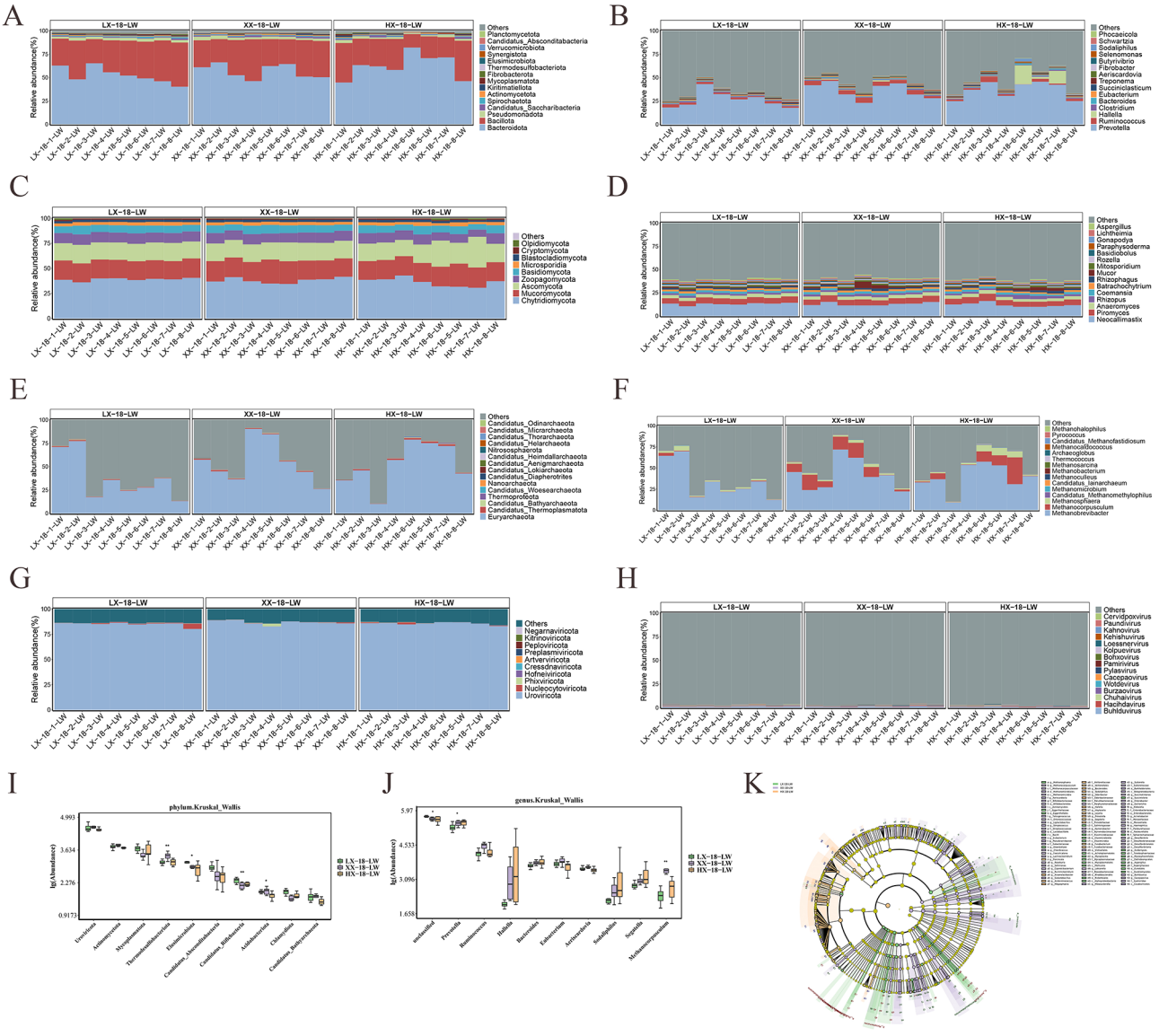

Fig. 3 Rumen microbial composition and analysis of variance for three combinations ($n = 8$). **(A, C, E, G, I)** Phylum-level abundance of bacteria (k__Bacteria), fungi (k__Fungi), archaea (k__Archaea), viruses (k__Viruses). **(B, D, F, H)** Genus-level abundance of bacteria (k__Bacteria), fungi (k__Fungi), archaea (k__Archaea), viruses (k__Viruses) **(I, J)** Differential species among the three combinations at the Phylum and Genus levels based on the Kruskal-Wallis test with a threshold of P-value < 0.05. **(K)** LEfSe analyses of the relative abundance of differential species across the three assemblages

both DEMs compared to Combination I and Combination III, respectively. We performed GSEA analysis of this pathway and found that it was significantly enriched for 11 metabolites, with 9,10,13-Trihome (11), 9,12,13-Trihome, and 9(10)-Epome expression down-regulated in Combination II vs. Combination I; 9(10)-Epome, 9(S)-Hpode expression down-regulated in Combination II vs. Combination III (Fig. 5G, H).

Non-targeted metabolomic analysis of plasma

A total of 2,722 metabolites were detected in plasma by LC-MS/MS. PCA analysis revealed no differences between combinations (Fig. S3A, D, G). Identification of metabolites by OPLS-DA showed significant separation between combinations (Fig. S3B, C, E, F, H, I). We screened 282 DEMs with VIP > 1 and $P < 0.05$, of which Combination II vs. Combination I had 81 DEMs (up-regulated 49, down-regulated 32), Combination II vs. Combination III identified 94 DEMs (up-regulated 33, down-regulated 61), Combination I vs. Combination III had 107 DEMs identified (up-regulated 49, down-regulated 45) (Fig. 6A, B, C, Table S10). The relative expression of the top 50 identified markedly different DEMs was used to generate a hierarchical clustering heatmap (Fig. S4). We further performed KEGG functional enrichment analysis of the identified DEMs and found that the

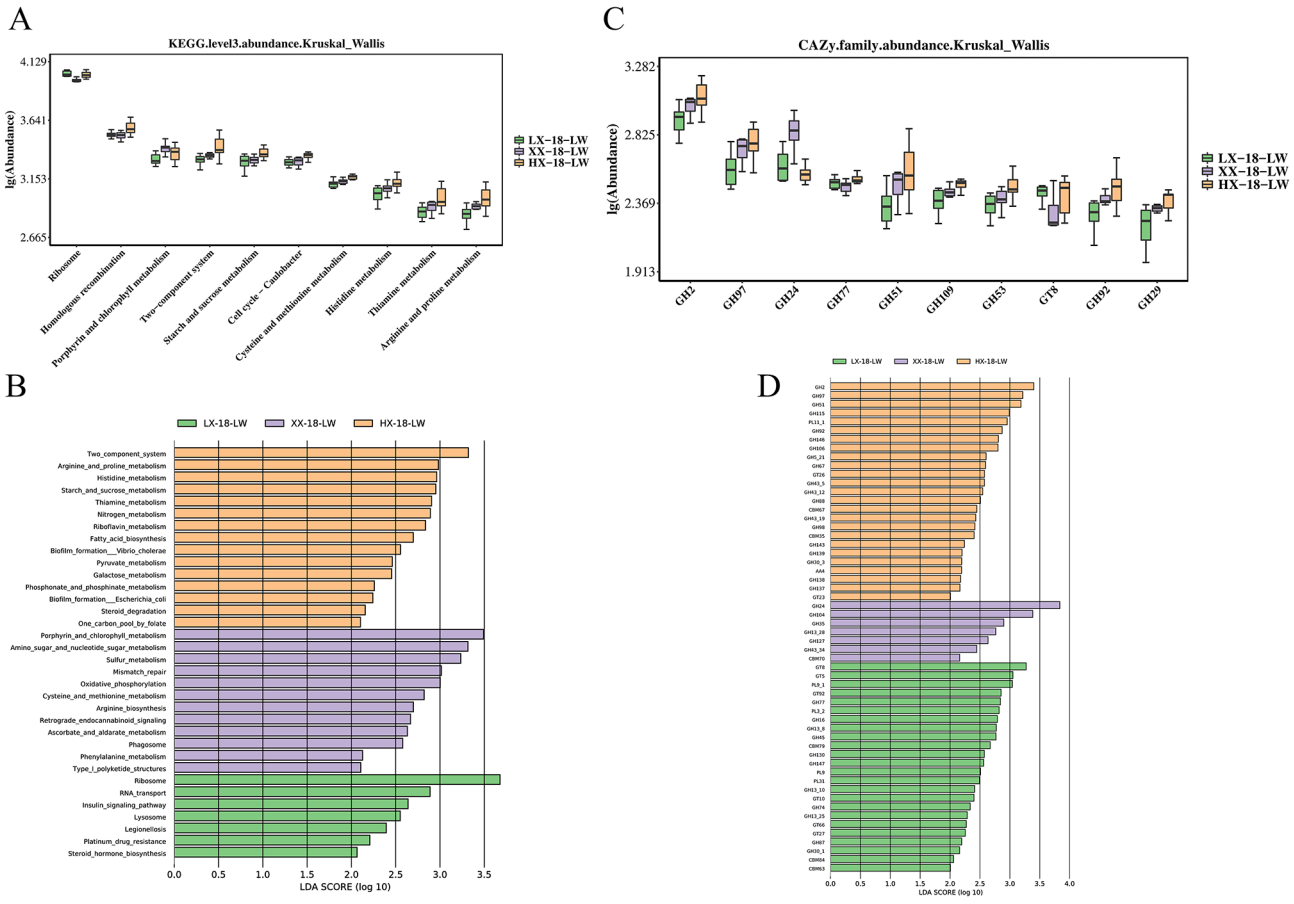

Fig. 4 Functional analysis of the rumen microbiome between the three combinations ($n=8$). **(A)** KEGG analysis yielded the top 10 functional pathways with the most significant non-redundant gene enrichment across the three combinations. **(B)** LEfSe analysis to screen for unique pathways in the three combinations. **(C)** CAZy analysis of the top 10 carbohydrate enzymes with the most significant differences among the three combinations. **(D)** LEfSe analysis screening for functional carbohydrases with significant differences among the three combinations

pathways significantly enriched in Combination II vs. Combination I included histidine metabolism, glycerophospholipid metabolism and ABC transporters (Fig. 6D, Table S11). DEMs in Combination II vs. Combination III were significantly enriched in histidine metabolism, aminoacyl-tRNA biosynthesis, arginine biosynthesis, valine, leucine and isoleucine biosynthesis, ABC transporters, valine, leucine and isoleucine degradation and glycerophospholipid metabolism (Fig. 6E, Table S12). The pathways most enriched for DEMs in Combination I vs. Combination III were valine, leucine and isoleucine biosynthesis, valine, leucine and isoleucine degradation, glycerophospholipid metabolism, aminoacyl-tRNA biosynthesis, glycerolipid metabolism, glycine, serine and threonine metabolism and phenylalanine metabolism (Fig. 6F, Table S13). Based on these results, we found that Combination II was significantly enriched in histidine metabolism, ABC transporters and glycospholipid metabolism compared to Combination III and Combination I, respectively. By GSEA enrichment analysis, glycerophospholipid metabolism was found to be significantly enriched in both groups, and the pathway consisted of 37 metabolites, including three down-regulated DEMs for PC (14:0/P-18:1(11Z)), PC (16:0/0:0) and PC (17:0/0:0) (Fig. 6G, H).

Correlation analysis of dominant microorganisms, differential metabolites and growth performance

The effect of rumen-dominant microorganisms and its DEMs and plasma DEMs on growth performance was further explored by Pearson correlation analysis for Combination II. First, a correlation heat map was constructed using Pearson correlation coefficient to analyse the relationship between dominant microorganisms and DEMs. We found that the dominant microorganisms Mycoplasma, Succinivibrio, Anaerostipes, Methanosphaera, Aspergillus, and Acidomyces were positively correlated with each other. The dominant microorganisms were all negatively correlated with the DEMs, with Aspergillus being significantly negatively correlated with PC (14:0/P-18:1 (11Z)) and 9(S)-Hpode ($P<0.05$). Succinivibrio was significantly negatively correlated with 9(10)-Epome,

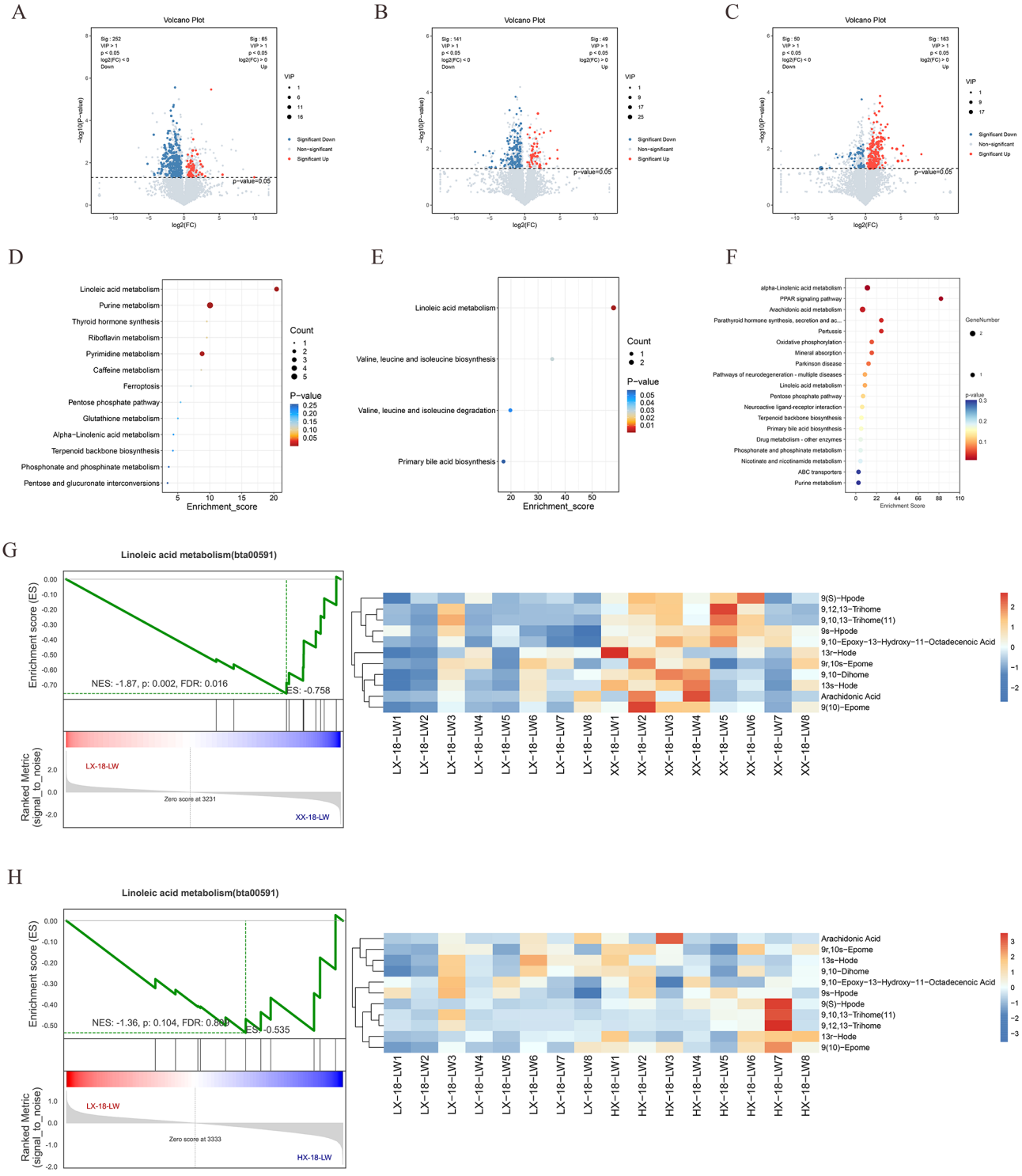

Fig. 5 Characterisation of the rumen fluid metabolome ($n=8$). **(A-C)** Volcano plots of combination II vs. combination I, combination II vs. combination III, and combination I vs. combination III. Blue dots represent down-regulated differential metabolites, red dots represent up-regulated differential metabolites, and grey dots represent non-differential differential metabolites. **(D-E)** The KEGG pathway significantly enriched for differentially expressed metabolites in Combination II vs. Combination I, Combination II vs. Combination III, and Combination I vs. Combination III. **(G-H)** GSEA enrichment analysis of Combination II vs. Combination I, Combination II vs. Combination III

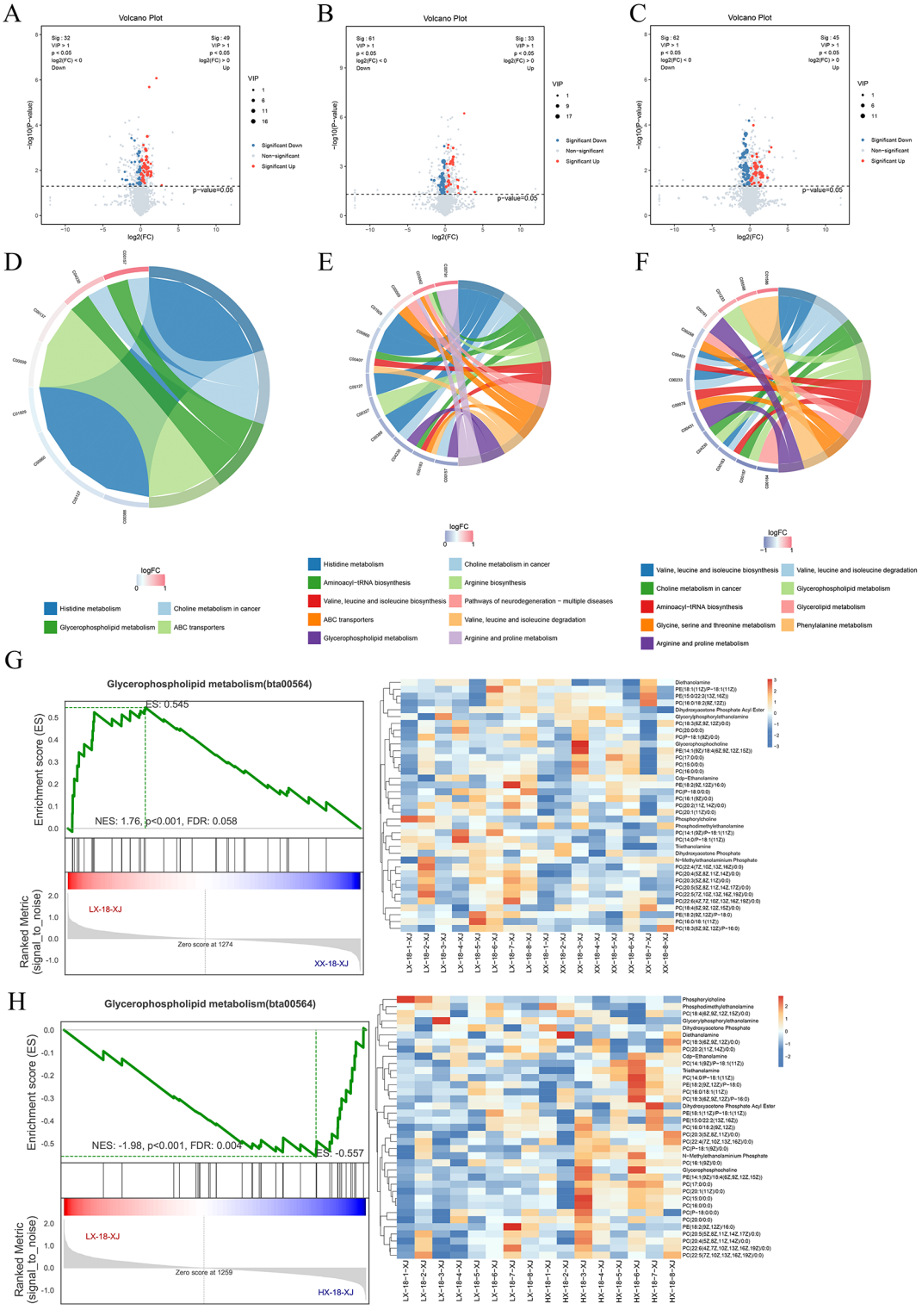

Fig. 6 Characterisation of plasma metabolome ($n=8$). **(A-C)** Volcano plots of Combination II vs. Combination III, Combination II vs. Combination I, and Combination I vs. Combination III. Blue dots represent down-regulated differential metabolites, red dots represent up-regulated differential metabolites, and grey dots represent non-differential metabolites. **(D-E)** KEGG pathway significantly enriched for DEMs in Combination II vs. Combination III, Combination II vs. Combination I, Combination I vs. Combination III. **(G-H)** GSEA enrichment analysis of Combination II vs. Combination III, Combination II vs. Combination I

9,10,13-Trihome (11) and 9(S)-Hpode ($P<0.05$). Hpode were significantly negatively correlated ($P<0.05$). Anaerostipes and Methanosphaera were significantly negatively correlated with 9(10)-Epome, 9,10,13-Trihome (11), 9,10,13-Trihome, 9(S)-Hpode and PC (17:0/0:0) ($P<0.05$, Fig. 7A). We also correlated the dominant microorganisms and DEMs with growth performance. The results showed that dominant microorganisms were positively correlated with growth performance, with Anaerostipes being significantly positively correlated with BW, BH, CSH and TC ($P<0.05$). Methanosphaera and Succinivibrio were significantly positively correlated with BW, BH and TC ($P<0.05$). DEMs were negatively correlated with growth performance, with PC (16:0/0:0) being significantly negatively correlated with CSH and TC ($P<0.05$). PC (17:0/0:0) was significantly negatively correlated with CSH ($P<0.05$). 9,10,13-Trihome (11) was significantly negatively correlated with BH, CSH and TC ($P<0.05$). 9(10)-Epome PC and 9,12,13-Trihome were significantly negatively correlated with BH ($P<0.05$). 9(S)- Hpode was significantly negatively correlated with BH, CSH and TC ($P<0.05$, Fig. 7B).

Discussion

The hybrid vigor of beef cattle is typically manifested in aspects such as superior growth rate, meat quality, and feed digestion efficiency. Research indicates that after crossbreeding purebred Goudali with Italian Simmental, the hybrid offspring significantly outperform the parents in terms of slaughter performance and have lower fat content [26]. Similarly, the offspring of crossbreeding Red Steppe cattle with Simmental and Limousin showed an average increase of 10.8% in carcass weight, 9.0% in carcass meat weight, a 2.7% increase in first-class meat yield, and an increase of 0.31 in the protein quality index [27]. Another study showed that the offspring of crossbreeding Simmental and Holstein cattle exhibited significant advantages in pre-slaughter body weight, skin weight, carcass weight, and dressing percentage [28]. Additionally, this study found that the hybrid offspring of Simmental cows as the dam and Belgian Blue bulls as the sire exhibited distinct advantages in indicators such as BW and BH.

We hypothesize that the differences in growth performance of hybrid cattle may be related to digestive efficiency [29], with the composition and function of the rumen microbiota playing a crucial role in the digestion efficiency of ruminants [30]. To test this hypothesis, we analyzed the rumen microbiota of different hybrid combinations. As previously pointed out in other studies [31], the core microbiota of ruminants includes Bacteroidota, Bacillota, and Pseudomonadota, and at the genus level, *Prevotella*, *Ruminococcus*, and *Hallella* are the major representative genera, which is consistent with our findings [32]. The diversity and abundance of rumen microorganisms directly or indirectly affect the growth performance of cattle in terms of body weight gain, feed conversion ratio, and health status [33]. In this study, there was no significant difference in Alpha diversity and Beta diversity among the three combinations. We hypothesised that the better growth performance observed in Combination

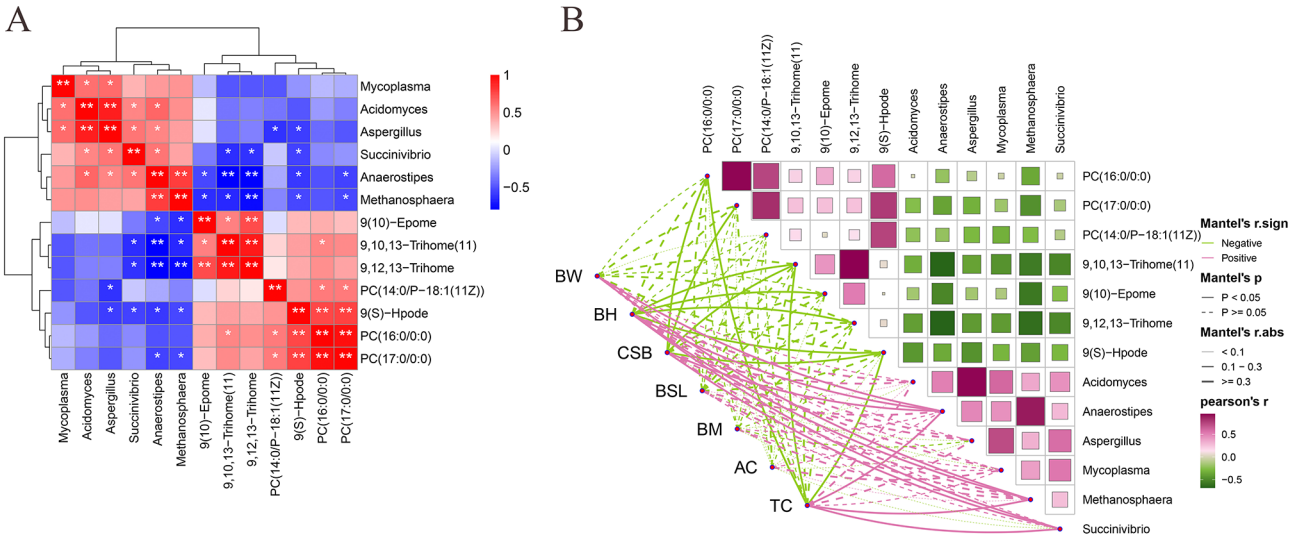

Fig. 7 Correlation analysis of rumen-dominant microorganisms, its differential metabolites and plasma differential metabolites with growth performance ($n=8$). **(A)** Heatmap of the correlation network of rumen-dominant microorganisms, its DEMs and plasma DEMs. Red colour indicates positive correlation and blue colour indicates negative correlation. **(B)** Correlation of rumen-dominant microorganisms, its DEMs and plasma DEMs with growth traits. Units are based on red for positive correlation and green for negative correlation. Red lines indicate positive correlation and green indicates negative correlation. Solid lines indicate significant correlation and dotted lines indicate correlation but not significant

II may be related to microbial variability rather than microbial diversity.

Worth noting is that all microorganisms were screened for a total of 210 differential Phylum among the three com cattlefenbbinations by the Kruskal-Wallis test with a threshold of $P<0.05$. Here, we focus on the dominant microorganisms in Combination II. Elusimicrobiota and Candidatus_Riflebacteria are two significantly different microbial Phylum with higher relative abundance in Combination II. Elusimicrobiota is thought to be involved in the degradation of lignocellulose in ruminants [34]. The role of Candidatus_Riflebacteria in the rumen is uncertain [35]. In this study, Combination II showed better growth performance, suggesting that Elusimicrobiota and Candidatus_Riflebacteria could enhance the growth advantage. In addition, a total of 658 differential Genus were screened at the Genus level, and the significantly different microbial abundance in Combination II was in the unclassified state. However, the relationship between microorganisms in the unclassified state and bovine growth performance cannot be clearly articulated or confirmed at this time [36]. In addition, we analysed the composition of differential microorganisms using LEfSe, and found that the composition of microorganisms in Combination II included Mycoplasma, Succinivibrio, Anaerostipes, Methanosphaera, Aspergillus, and Acidomyces. Nowadays, the study of Mycoplasma has mostly been studied in the context of disease diagnosis in cattle [37]. Bovine Mycoplasma is the most common disease-causing pathogen and is highly contagious, causing a variety of illnesses [38]. Some studies have shown that Succinivibrio ferments glucose to produce acetic and succinic acids, which contribute to the metabolism of different types of fatty acids [39]. Analysis of the gut microbial composition of Kasaragod Dwarf and Holstein crossbred cattle revealed that Succinivibrio was identified in the faecal microflora of Kasaragod Dwarf cattlefenb, and has been shown to be present in higher abundance in animals with higher feed efficiency, suggesting that Succinivibrio may play a role in metabolic regulation and cellulose degradation [40]. Reduced relative abundance of Succinivibrio in the intestinal microbiota of omnivorous cattle is closely associated with reduced grass consumption [41]. Related studies have shown that Anaerostipes are observed in higher abundance during the cold spring and winter periods, which helps yaks to overcome the climatically harsh highland conditions [42]. The microbial community in the faeces of cattle from semi-finisher production systems is associated with the immune system, nutrient metabolism, and energy production compared to Brazilian cattle from conventional production systems and includes Anaerostipes. The development of a faecal microbiome that promotes healthier and more efficient cattle by increasing beneficial bacteria [43]. Methanosphaer usually plays an important role in bovine methane emissions [44]. In a study examining the relationship between residual methane emissions (RME) and rumen flora of fattening cattle populations, an increase in the abundance of Methanosphaera and Methanobrevibacter RO clade was found to be negatively correlated with RME in the low RME group ($P<0.05$), emphasising that Methanosphaera can be used as a potential microbial biomarker of methanogenic potential in cattle [45]. In the last few decades, Aspergillus has been found to be a threat to animal health and productivity [46]. It has been reported that testing of silage and concentrate feed on 21 cattle farms revealed that both types of feed were contaminated with Aspergillus, which increased the risk of disease in cattle [47]. The risk of Aspergillus contamination has also been found to be higher in cattle than in other cattle farms. CtC72T belongs to Acidomyces, a specialised anaerobic, rod-shaped, Gram-stain-positive, non-spore-forming, non-motile bacterial strain. The strain hydrolyses cellulose and xylan and utilises a range of monosaccharides, disaccharides and oligosaccharides as a source of carbon and energy. Glucose fermentation produces acetic acid and formic acid as primary metabolites, while propionic acid, lactic acid and ethanol are used as secondary products as well as to produce CO_2 [48]. Related studies have indicated that Acidomyces and other microorganisms act synergistically to induce bovine mastitis [49]. However, the specific mechanisms of action and effects of these microorganisms still need to be further investigated and confirmed.

Based on the Kruskal-Wallis test ($P<0.05$), 34 biological pathways were significantly different between the three groups. LEfSe analyses of unique pathways in the three combinations, including the insulin signaling pathway and steroid hormone biosynthesis, were considered significantly enriched pathways in Combination II. Related studies have shown that the involvement of the insulin signaling pathway in Src homology 2 B 2 (SH2B2) enhances potentiating Janus kinase 2 (JAK2) activation and promotes insulin signaling pathway to positively affect growth traits [50]. Several studies have found that the insulin signaling pathway is closely related to the formation of marbling in cattle [51]. Kai found that steroid hormone biosynthesis was highly enriched in the large intestine by analysing the gastrointestinal tract of Simmental × 17 17-month-old Holstein crossbred heifers for digestion, which greatly affected the cattle's performance [52]. Meanwhile, CAZy analysis revealed that GTs and PLs were most enriched in Combination II. It was suggested that GTs and PLs are encoded by rumen Butyrivibrio and are involved in the depolymerisation and transport of insoluble phytopolysaccharides in the rumen of ruminants [53]. Therefore, we hypothesized that these CAZymes may affect the rumen's ability to degrade

cellulose and complex polysaccharides to some extent in cattle growth.

We analysed metabolomic data on rumen fluid, and hybridisation induced not only changes in the species of the rumen microbiota, but also altered the abundance of rumen metabolites. We found that DEMs were significantly enriched in Linoleic acid metabolism in both Combination II vs. Combination I and Combination II vs. Combination III. Meanwhile, GSEA analysis identified four DEMs from linoleic acid metabolism that were all down-regulated, including 9,10,13-Trihome (11), 9,12,13-Trihome, 9(10)-Epome, and 9(S)-Hpode. It has been reported that, by feeding monensin to cattle, not only did it regulate the function of rumen microbiota, but also the metabolic pathways related to linoleic acid and amino acid metabolism, thus revealing that monensin regulates rumen fermentation to improve feed utilisation efficiency [54]. Exogenous addition of a certain percentage of linoleic acid increased body weight, feed intake, and feed efficiency in pigs [55]. Therefore, we postulated that secondary metabolites produced by rumen microorganisms may have a positive impact on regulating growth in cattle. Metabolites produced by rumen microorganisms can enter the blood circulation through the rumen wall and affect metabolites in the plasma [56], so we further analysed the plasma metabolome. KEGG and GSEA integration analyses of the DEMs among the triple combinations revealed a significant enrichment of glycerophospholipid metabolism, including three down-regulated DEMs, PC (14:0/P-18:1(11Z)), PC (16:0/0:0) and PC (17:0/0:0). It has been suggested that glycerophospholipid metabolism plays an important role in regulating the growth and development of skeletal muscle in cattle [57]. PC (16:0/0:0) has been considered as a biomarker for the formation of marbling in the hybrid offspring of Luxi Yellow cattle and Japanese Wagyu cattle [58]. PC (14:0/P-18:1(11Z)) and PC (17:0/0:0) have not been reported and more in-depth analyses are needed to explore their roles.

More evidence suggests that phenotypic traits in animals are driven by rumen microorganisms [59]. Therefore, we further explored the effects of Combination II rumen-dominant microorganisms, rumen DEMs, and plasma DEMs on growth performance by Pearson correlation analyses. In this study, we found that Mycoplasma, Succinivibrio, Anaerostipes, Methanosphaera, Aspergillus, and Acidomyces were positively correlated with each other. According to previous studies, rumen microbiota affects the abundance of metabolites [60]. Similarly, the present study found that rumen and plasma DEMs abundance was reduced in Combination II and was negatively correlated with the dominant microorganisms. The study showed an increasing trend in the mean daily weight gain of geese by feeding certain doses of Honeysuckle extract, which was associated with intestinal enrichment of Anaerostipes and Succinivibrio [61]. Addition of dietary tannic acid (TAN) to the ration altered the abundance of the rumen-dominant microorganisms Methanosphaera, Methanobacteriaceae, Ruminococcus and Saccharomonas in cattle, which in turn had an impact on gas production, growth performance, antioxidant capacity, rumen flora and fermentation functions were affected [62]. Another study confirmed that rumen dominant microorganisms were notably associated with DEMs (PC(16:0/0:0), PC(18:3/0:0), uridine 3′-monophosphate, and adenosine monophosphate) in yaks, which had a major impact on improving their growth performance [63]. In the present study, Anaerostipes, Methanosphaera and Succinivibrio were found to be significantly and negatively correlated with PC (14:0/P-18:1 (11Z)), 9(10)-Epome, 9,10,13-Trihome (11), 9(S)-Hpode, 9,10,13-Trihome, PC (17:0/0:0) and PC (16:0/0:0), and significantly positively correlated with growth traits [64]. This is in agreement with previous findings indicating that changes between microbiota and metabolites are closely related to growth performance. Overall, these variations and relationships reveal important features of differences in cattle growth performance across crossbreeding combinations. However, given that little is known in this study about the possible causes of the negative and positive correlations between microbes and metabolites, their pathways of bovine growth regulation cannot yet be elucidated. Therefore subsequent additional work is required for us to assess the causes and mechanisms driving the interactions between rumen microbes and rumen metabolism.

Conclusion

In summary, this study integrated macrogenomics and metabolomics analysis to investigate the association of growth performance differences with rumen-dominant microorganisms, rumen metabolites, and plasma metabolites in hybrid offspring of different combinations. The results showed that Combination II had more advantageous growth performance. Our analysis obtained that differences in the abundance of the dominant microorganisms Succinivibrio, Anaerostipes, Methanosphaera may affect the abundance of the rumen metabolites 9(10)-Epome, 9(S)-Hpode, 9,10,13-Trihome(11) and 9,12,13-Trihome, which are absorbed through the rumen wall, causing down-regulation of the plasma lipid molecules PC(14:0/P-18:1(11Z)), PC(16:0/0:0), and PC(17:0/0:0) and ultimately affecting the growth performance of crossbred cattle (Fig. 8). Our study reveals the role of rumen microorganisms and their metabolites with host metabolism in the regulation of growth performance of crossbred cattle, which will contribute to the development of modern cattle breeding.

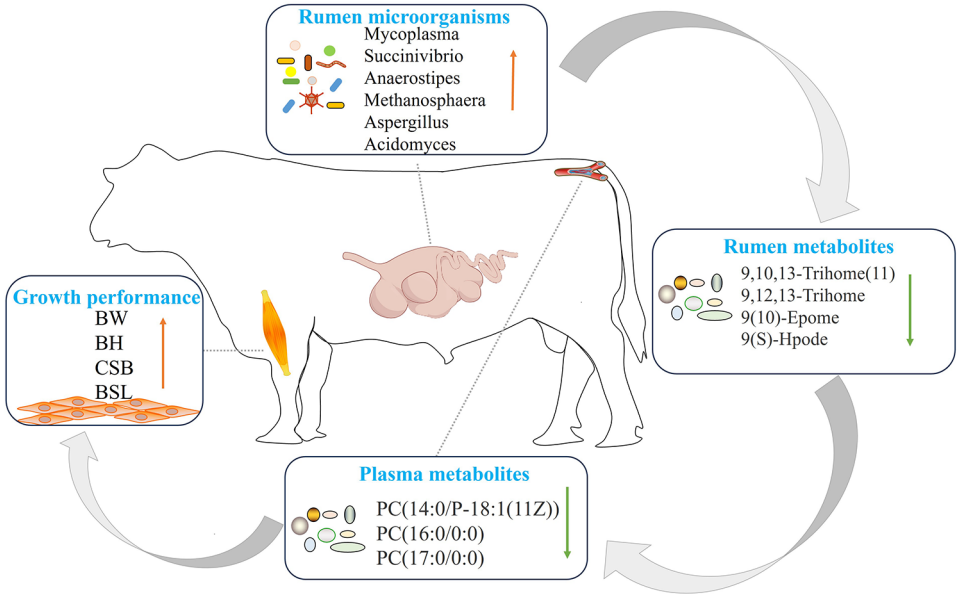

Fig. 8 Mechanisms of action of rumen microorganisms and its metabolites in combination with host metabolites in regulating growth performance of crossbred cattle

Abbreviations

DEMs	Differentially expressed metabolites
PCR	Polymerase chain reaction
PCA	Principal component analysis
PCoA	Principal coordinate analysis
NMDS	Nonmetric multidimensional scaling
LEfSe	Linear discriminant analysis effect size
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
HMDB	Human Metabolome Database
CAZy	Carbohydrate-Active enzymes database (
VIP	Variable importance projection
GSEA	Gene set enrichment analysis
BH	Body weight
BW	Body height
CSH	Cross-sectional height
BSL	Body slant length
BM	Breast measurement
AC	Abdominal circumference
TC	Tube circumference
RME	Residual methane emissions
SH2B2	Src homology 2 B 2
JAK2	Janus kinase 2

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11465-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

Supplementary Material 12

Supplementary Material 13

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Author contributions

W.Z. and L.M. were responsible for conceptualization, methodology, writing–original draft, and writing–review and editing. L.X. and S.W. were responsible for conceptualization and resources. Y.F. and S.W. were responsible for methodology and supervision. Q.J. and J.T. were responsible for resources and software. W.Z. and X.T. were responsible for investigation and software. J.Z. and Y.G. were responsible for project administration, funding acquisition, and resources.

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Data availability

The datasets generated during the current study are available in the NCBI short read archive (SRA) under BioProject ID PRJNA1181273.

Declarations

Ethical approval

This experiment was approved by the Animal Welfare Committee of Ningxia University and was conducted according to the Guidelines of Animal Use of the Committee of the Ministry of Agriculture of China (Beijing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details
[1]Ningxia Key Laboratory of Ruminant Molecular and Cellular Breeding, College of Animal Science and Technology, Ningxia University, Yinchuan 750021, China
[2]Ningxia Hui Autonomous Region Animal Husbandry Workstation, Yinchuan 750021, China

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