Sodium Humate And Glutamine Combined Supplementation Alleviate Diarrhea of Weaned Calves Via Alter Intestinal Microbiota And Metabolites

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Research

Abstract

Background: Weaning is one of the most stressful periods that cause gastrointestinal tract dysfunction and diarrhea in calves. HNa and Gln were reported to exert beneficial effects on promoting growth performance, decreasing diarrhea incidence, and modulating intestinal microbiota in animals. Therefore, this study investigated the effect of HNa and Gln combined supplementation on growth performance, diarrhea incidence, serum parameters, intestinal microbiome, and metabolites of weaned calves.

Results: In Exp. 1, 40 calves at 51±3 days of age with similar body weight (66.82±4.31 kg) were randomly assigned to 4 treatments fed with a basal diet (NC group), and a basal diet supplemented with 100 mL of 1%, 3%, or 5% HNa+1% Gln, twice daily, respectively. In a 21-day trial, calves on the 5% HNa+1% Gln group had higher ADG and lower fecal score and diarrhea incidence than those in the control group (*P* < 0.05). In Exp. 2, 20 calves at 51±3 days of age with similar body weight (69.37±6.28 kg) were randomly assigned to 2 treatments fed with a basal diet (NC group) and a basal diet supplemented with 100 mL of 5% HNa+1% Gln, twice daily (H+G group, the dose was obtained from Exp. 1). In a 21-day trial, calves supplemented with HNa and Gln had higher final BW and ADG, serum IgG concentration and GSH-Px and T-AOC activities, but lower fecal score, diarrhea incidence, as well as serum DAO, D-lac, TNF-α, and MDA concentrations compared to NC group (*P* < 0.05). Analysis of intestinal microbiota indicated that supplemented with HNa and Gln significantly increased the abundance of phyla *Firmicutes* and genus of *Bifidobacterium*, *Lactobacillus*, *Olsenella*, *Ruminiclostridium* 9, *Howardella*, and uncultured organism, whereas the abundance of phyla *Bacteroidetes*, genus of *Helicobacter* and *Lachnoclostridium* were decreased as compared with NC group. Moreover, untargeted metabolomics analysis revealed that supplemented with HNa and Gln altered 18 metabolites and enriched 6 KEGG pathways (primary fatty acid biosynthesis) compared to the NC group.

Conclusions: This study showed that combined supplemented with HNa and Gln could decrease diarrhea of weaned calves, which may be associated with improved intestinal microbial ecology and altered metabolism profile.

Background

Weaning is one of the most stressful periods in calve life, which can cause gastrointestinal tract dysfunction and diarrhea [1]. High diarrhea incidence in weaned calves is the main cause of growth retardation and death, which seriously affects the welfare of calves and causes serious economic losses in the dairy industry [2]. Antibiotics have long been used in calves as growth promoters and therapeutic agents for diarrhea, but overuse of antibiotics resulted in development of antibiotic resistance and negative public health outcomes [3]. Previous studies indicated that intestinal microbiota plays an essential role in intestinal morphology, nutrient absorption, immunity response, and host health [4]. Meanwhile, intestinal microbiota participates in many metabolic activities of the host, such as amino acid and vitamin synthesis, and lipid and bile acid metabolism [5]. Studies have shown that weaning can significantly alter intestinal microbiota and metabolism in pigs, resulting in increased diarrhea incidence

and growth retarded [6]. Thus, searching for a suitable alternative to modulate intestinal microbiota and metabolites of calves at weaning stages thus improving intestinal health has gained more and more attention worldwide.

Humic acids (HAs), which are derived from the decomposition and transformation of decaying organic matter in the soil, are natural organic bioactive agents. As a type of HAs, sodium humate (HNa) has antimicrobial, antioxidant, anti-inflammatory, and antidiarrheal activities [7]. It has been reported that HAs and HNa were allowed to use in animals for dyspepsia, diarrhea, and acute intoxication [8, 9]. Wang et al. [8] confirmed the beneficial effects of HNa supplementation in finishing pigs. The growth-promoting efficacy of HNa was also confirmed in broilers [11]. Glutamine (Gln) which could maintain intestinal integrity and prevent bacterial translocation is a major fuel source for rapidly dividing cells including enterocytes, macrophages and lymphocytes [10]. The benefits of Gln supplementation on improving growth performance, repairing intestinal epithelium, enhancing nutrient digestion and absorption, activating the immune system, and modulating intestinal microbiota have been observed in rats [12], broilers [13], weaning piglets [14] and calves [15]. Furthermore, our previous study found that supplemention with HNa and Gln effectively improved the growth performance and decreased diarrhea incidence in weaned calves. Based on the studies above, we hypothesized that HNa and Gln combined supplementation might have therapeutic potential on calf diarrhea by modulating intestinal microbiota and metabolites in weaned calves.

In the present study, intestinal microbiota sequencing and fecal untargeted metabolomics were integrated to investigate the beneficial effects of HNa and Gln combined supplementation on weaned calves. Furthermore, the correlation of intestinal microbiota, metabolites and growth performance, diarrhea incidence, and serum parameters were also evaluated.

Materials And Methods

The experimental protocol was approved by the Ethics Committee of Northeast Agricultural University (Harbin, China). The study was conducted at Harbin Modern Farming (Harbin, China).

Materials

HNa (purity, 75%) was provided by the Institute of Coal Chemistry, Chinese Academy of Sciences (Shan xi, China). Glutamine (purity, 99.9%) was purchased from Sigma-Aldrich (Wisconsin, USA).

Exp. 1

Animals, diets, and experimental design

A total of 40 Holstein calves at 51 ± 3 days of age with similar body weight (66.82 ± 4.31 kg) were fed 5 L of milk replacer until weaning (58 d of age), twice daily at 08:30 am and 4:30 pm. The milk replacer used in this study contained lactose ≥ 40%, CP ≥ 22%, crude fat ≥ 19%, water ≤ 4.0%, ash ≤ 8.0%, and fiber ≤

0.3%. All calves had free access to water and starter during the entire experimental period. The ingredients and chemical composition of the starter are shown in Table 1.

Table 1
Ingredients and chemical composition of the starter concentrate diet (basal diet, Exp.1 and Exp.2)

Ingredient	Content (%)	Chemical composition	Content (%)
Corn	40.74	DM	87.78
Soybean meal	35.00	EE	4.35
Wheat bran	2.8	CP	23.56
Cottonseed meal	6.8	ADF	5.71
Molasses	4.0	NDF	9.82
Wheat middlings	7.8	Ash	3.73
$CaCO_3$	1.63	Ca	0.81
Soybean oil	0.80	Phosphorus	0.49
NaCl	0.10		
$CaHPO_3$	0.10		
MgO	0.07		
Selenium yeast	0.02		
Premix[1]	0.14		
Total	100.00		

[1]The premix provided the following per kg of diet: Fe 206.74 mg, Cu3 3.49 mg, Zn 108.81 mg, I 0.60 mg, Se 0.44 mg, Mn 79.99 mg, Co 0.36 mg, VA 9000 U, VD 24000 U, VE 47.22 mg.

All of the calves were randomly assigned to 4 treatments (n = 10): (1) NC (basal diet), (2) 1% H + G (basal diet supplemented with 100 mL of 1% HNa + 1% Gln, twice daily), (3) 3% H + G (basal diet supplemented with 100 mL of 3% HNa + 1% Gln, twice daily), and (4) 5% H + G (basal diet supplemented with 100 mL of 5% HNa + 1% Gln, twice daily). In the present study, 0, 1, 3, or 5 g of HNa and 1 g of Gln were mixed with 100 mL of milk replacer or water, respectively. The HNa and Gln were administered to each calf from a bottle before feeding (milk replacer or starter) at 08:30 a.m and 4:30 p.m. The trial lasted for 21 days.

Growth performance, fecal score, and diarrhea incidence

The calves were weighed individually at the start and the end of the experimental period, and feed consumption per calves was recorded daily to calculate average daily feed intake (ADFI), average daily gain (ADG), and the ratio of feed to gain (F: G).

The fecal scores were monitored daily before the morning feeding according to the method of Renaud et al. [16]. Fresh feces were scored by consistency: 0 = firm; 1 = loose or moderate consistency; 2 = very loose or mild diarrhea; and 3 = watery or profuse diarrhea. Diarrhea was defined as fecal scores ≥ 2 occurring for 2 or more consecutive days. The diarrhea incidence was calculated according to Renaud et al. [16]. Diarrhea incidence (%) = Number of diarrheal calves × Diarrhea days/ (Number of calves × Test days) × 100.

Exp. 2

Animals, diets, and experimental design

A total of 20 Holstein calves at 51 ± 3 days of age with similar body weight (69.37 ± 6.28 kg) were randomly assigned to 2 treatments (n = 10): (1) NC (basal diet), (2) H + G (basal diet supplemented with 100 mL of 5% HNa + 1% Gln, twice daily). The HNa concentration applied in Exp. 2 was based on the results of Exp. 1, which suggested that 5% HNa + 1% Gln resulted in lower fecal score, diarrhea incidence and higher ADG than NC group. The experimental diet, management, as well as investigation of growth performance, fecal score, and diarrhea incidence of calves were the same as Exp. 1. The trial lasted for 21 days.

Serum parameters analysis

Blood samples were obtained from the jugular vein of each calf on day 73 (before the morning feed). Samples were collected in 10-mL vacuum tubes containing anticoagulant and then centrifuged for 15 min at 3,000×g at 4°C (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany). The serum supernatants were collected and stored at -20°C for analysis of IgG, IgM, IgA, IL-6, TNF-α, diamine oxidase (DAO), and D-lactic acid (D-Lac) using ELISA kits (Jingmei Biotechnology Co., Ltd, Jiangsu, China), and determination of serum glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC) activities, and malondialdehyde (MDA) concentration according to the manufacturer's instructions (Jian cheng Bioengineering Institute, Nanjing, China).

Intestinal microbiota analysis

On day 73, fecal samples were collected from each calf before the morning feeding using sterile tubes, and then immediately frozen at liquid nitrogen until microbiota analysis. Total genome DNA from each digested sample was extracted using cetyltriethylammnonium bromide (CTAB) method, and then the integrity of extracted DNAs was detected by 1% agarose gel. The V3-V4 regions of the bacterial 16S rDNA gene were amplified by specific primers: 341F (5'- CCTACGGGNGGCWGCAG – 3') and 806R (5'- GGACTACHVGGGTATCTAAT – 3') with the following procedures: initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s, finally 72°C for 5 min. PCR products were detected by 1% agarose gel and purified with AxyPrepDNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The library was constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit (Thermo Scientific, Waltham, Mass, USA). The constructed library was quantified by Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, Mass, USA)

and Agilent Bioanalyzer 2100 system. After the library was qualified, it was sequenced by Illumina HiSeq (Illumina, San Diego, USA).

Paired-end reads from the original DNA fragments were merged using FLASH, and the sequences analysis was performed by UPARSE software package using the UPARSE-OTU and UPARSE-OTUref algorithms. In-house Perl scripts were used to analyze alpha (within samples) and beta (among samples) diversity, and PCoA (principal coordinates analysis) of weighted unifrac was generated in R project Vegan package (version 2.5.3). Sequences with ≥ 97% similarity were assigned to the same OTUs. The statistical significance of comparison in bacterial community composition between the two groups was assessed using Student's *t*-test, and STAMP software was utilized to confirm differences in the abundances of individual taxonomy between the two groups.

LC-MS/MS analysis and data processing

50 mg of fecal samples was added to 200 μL of H_2O, homogenized, and vortexed for 60 s. Then, 800 μL methanol/acetonitrile solution (1:1, vol/vol) were added to homogenized solution for metabolite extraction. The mixture was centrifuged for 15 min (14000g, 4°C). The supernatant was used for LC-MS/MS analysis.

LC-MS/MS analyses were performed using an UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (TripleTOF 6600, AB Sciex, USA) in Shanghai Applied Protein Technology Co., Ltd. For HILIC separation, samples were analyzed using a 2.1 mm×100 mm ACQUIY UPLC BEH 1.7 μm column (waters, Milford, USA). In both ESI positive and negative modes, the mobile phase contained A = 25 mM ammonium acetate + 25 mM ammonia water and B = acetonitrile. The gradient was 85% B for 1 min and was linearly reduced to 65% in 11 min, and then was reduced to 40% in 0.1 min and kept for 4 min, and then increased to 85% in 0.1 min, with a 5 min. The column temperature was 25°C, and the flow rate was 0.3 mL/min. A 2 μL aliquot of each sample was injected. The ESI source conditions were set as follows: Ion Source Gas1 (Gas1) as 60, Ion Source Gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature: 600°C, IonSpray Voltage Floating (ISVF) ± 5500 V. In MS only acquisition, the instrument was set to acquire over the m/z range 60-1000 Da, and the accumulation time for TOF MS scan was set at 0.20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25-1000 Da, and the accumulation time for product ion scan was set at 0.05 s/spectra. The product ion scan is acquired using information dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was fixed at 35 V with ± 15 eV; declustering potential (DP), 60 V (+) and − 60 V (−); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.

The collected data were used to identify the structure of metabolites using self-built MetDDA and LipDDA methods (Shanghai Applied Protein Technology Co. Ltd). The original data were converted into mzXML format by ProteoWizard MSConvert, and then the XCMS program was used for peak alignment, retention time correction, and peak area extraction. In the extracted ion features, only the variables having more

than 50% of the nonzero measurement values in at least one group were kept. The metabolite structure identification was based on accurate mass matching (< 25 ppm) and secondary spectrum matching methods and search of the laboratory's self-built commercial database (Shanghai Applied Protein Technology Co. Ltd).

After normalized to total peak intensity, the processed data were analyzed by R package (ropls), where it was subjected to multivariate data analysis, including partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The 7-fold cross-validation and response permutation testing were used to evaluate the robustness of the model. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. Metabolites with the VIP value > 1 were further applied to Student's *t*-test at univariate level to measure the significance of each metabolite, the *P* values < 0.05 were considered as statistically significant. The differential metabolites were further to cluster analysis and metabolic pathway analysis by MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) and KEGG (http://www.genome.jp/kegg). Fisher's Exact Test was used to analyze and calculate the significance level of the enrichment pathway.

Statistical analysis

Individual calves served as the experimental unit. For the growth performance in Exp.1, data were analyzed by one-way ANOVA using SPSS 20.0 software (SPSS Inc., IBM, Chicago, USA). The differences among treatments were evaluated using Turkey's test. For the growth performance, fecal score, and serum cytokines concentration and antioxidant capacity in Exp.2, data were analyzed using the independent sample *t*-test of the SPSS. Results are presented as mean ± standard error of the mean (SEM) except for the diarrhea incidence. Differences were considered significant at $P < 0.05$. The correlation analysis among intestinal microbiota, metabolites, ADG, fecal score and serum parameters was estimated by Spearman's correlation coefficient. Correlations were considered significantly different at r > 0.50 or r < − 0.50, $P < 0.05$.

Results

Growth performance and diarrhea incidence (Exp. 1)

As shown in Table 2, the initial BW, final BW, ADFI, and F: G of calves were similar among treatments ($P > 0.05$). However, the ADG of calves in 3% H + G and 5% H + G was higher ($P < 0.05$) than NC group. The decreased fecal scores and diarrhea incidence were significantly associated with the HNa and Gln supplementation. The diarrhea incidence of calves in the NC, 1% H + G, 3% H + G, and 5% H + G group was 25.81%, 17.08%, 21.42%, and 15.41%.

Table 2
Effects of HNa and Gln combined supplementation on the growth performance and diarrhea incidence of calves[1]

Item	Experimental Treatments				P-value
	NC	1% H + G	3% H + G	5% H + G	
BW, kg					
Initial (51 d)	67.70 ± 2.01	66.43 ± 1.30	65.13 ± 1.82	68.05 ± 1.01	0.430
Final (72 d)	88.00 ± 2.29	90.43 ± 1.15	90.82 ± 1.81	93.90 ± 1.69	0.092
ADG, kg	$0.96 ± 0.04^b$	$1.14 ± 0.04^{ab}$	$1.22 ± 0.06^a$	$1.23 ± 0.08^a$	0.025
ADFI, kg	2.29 ± 0.04	2.49 ± 0.05	2.61 ± 0.06	2.58 ± 0.15	0.062
F:G	2.43 ± 0.14	2.26 ± 0.17	2.20 ± 0.15	2.19 ± 0.18	0.722
Fecal score	$2.07 ± 0.12^b$	$1.50 ± 0.14^a$	$1.58 ± 0.11^a$	$1.39 ± 0.07^a$	0.001
Diarrhea incidence, %	25.81	17.08	21.42	15.41	
[a-b]Means within a row with different letters differ significantly ($P < 0.05$).					
[1]Data are the mean of 10 replicates of 1 calf per treatment.					
Data were shown as means ± SEM (n = 10).					

Growth performance and diarrhea incidence (Exp. 2)

As shown in Table 3, compared with NC group, the calves in H + G group had greater final BW ($P = 0.026$) and ADG ($P = 0.003$). No significant differences in F: G and ADFI were observed among treatments. In addition, the calves in H + G group had lower fecal scores ($P = 0.001$) and diarrhea incidence than calves in NC group.

Table 3
Effects of HNa and Gln combined supplementation on the growth performance and diarrhea incidence of calves[1]

Item	Experimental Treatments		P-Value
	NC	H + G	
BW, kg			
Initial (51 d)	68.50 ± 1.96	70.25 ± 2.07	0.548
Final (72 d)	91.17 ± 2.71[b]	101.38 ± 3.00[a]	0.026
ADG, kg	1.08 ± 0.06[b]	1.48 ± 0.06[a]	0.003
ADFI, kg	1.98 ± 0.15	2.37 ± 0.23	0.182
F:G	2.08 ± 0.27	1.63 ± 0.19	0.230
Fecal score	2.04 ± 0.15[a]	1.36 ± 0.17[b]	0.001
Diarrhea incidence, %	27.16	15.69	
[a-b]Means within a row with different letters differ significantly ($P < 0.05$).			
[1]Data are the mean of 10 replicates of 1 calf per treatment.			
Data were shown as means ± SEM (n = 10).			

Serum parameters (Exp. 2)

The concentration of serum DAO and D-lac are shown in Fig. 1A and 1B. Compared with NC group, supplemented with HNa and Gln significantly decreased the concentration of serum DAO and D-lac ($P < 0.05$). As shown in Table 4, compared with NC group, supplemented with HNa and Gln increased the IgG ($P = 0.018$) concentration, as well as activities of GSH-Px ($P = 0.003$) and T-AOC ($P = 0.005$) in the serum of calves. Furthermore, lower concentrations of serum TNF-α ($P = 0.047$) and MDA ($P = 0.002$) were observed in the H + G group compared with the NC group. There was no significant difference among treatments in serum IgA, IgM, IL-6, and T-SOD concentration ($P > 0.05$).

Table 4
Effects of HNa and Gln combined supplementation on the concentrations of cytokines and antioxidant capacity of calves in the serum[1]

Item	Experimental Treatments		P-Value
	NC	H + G	
IgA, µg/mL	89.51 ± 3.62	78.32 ± 4.61	0.073
IgG, µg/mL	1245.86 ± 41.61[a]	1083.57 ± 46.06[b]	0.018
IgM, µg/mL	74.24 ± 7.50	69.33 ± 6.79	0.295
IL-6, ng/L	8.64 ± 0.51	8.17 ± 0.62	0.569
TNF-α, ng/L	211.22 ± 8.34[a]	186.46 ± 8.10[b]	0.047
GSH-Px, U/L	128.65 ± 7.00[b]	167.48 ± 9.15[a]	0.003
T-SOD, pg/mL	59.64 ± 7.76	66.52 ± 4.70	0.458
T-AOC, U/mL	6.58 ± 0.27[b]	7.94 ± 0.33[a]	0.005
MDA, mmol/mL	3.08 ± 0.43[a]	2.29 ± 0.51[b]	0.002
[a–b]Means within a row with different letters differ significantly ($P < 0.05$).			
[1]Data are the mean of 10 replicates of 1 calf per treatment.			
Data were shown as means ± SEM (n = 10).			

Analysis of intestinal microbiota in weaned calves (Exp. 2)

In the microbiome study, 5,116,836 effective tags were acquired after filtering the data quality, with an average number of 255,842 tags per sample. Based on the 97% identity level, these sequences were decomposed into 1,912 operational taxonomic units (OTUs), while 1,035 and 884 specific OTUs were observed in H + G and NC groups, respectively (Fig. 2B). The Chao1, Ace, Shannon, and Simpson indexes associated with bacterial richness and diversity were similar among groups (Fig. 2A). The principal coordinate analysis (PCoA) plots showed an overlap of partial samples between NC and H + G groups.

The relative abundances of different phyla were shown in Fig. 3. The microbial community was dominated by *Firmicutes* (76.28–30.67%), *Bacteroidetes* (45.46–15.45%), *Proteobacteria* (13.88 – 0.68%), *Spirochaetes* (11.91 – 0.08%), *Fusobacteria* (4.22 – 0.02%), and *Actinobacteria* (1.96 – 0.01%), which were more than 97% (Fig. 3A). Compared with NC group, HNa and Gln supplementation significantly increased ($P < 0.05$) the ratio of *Firmicutes* to *Bacteroidetes* (Fig. 3B) and the relative abundance ($P < 0.001$) of *Firmicutes* (Fig. 3C), but decreased ($P < 0.05$) the relative abundance of *Bacteroidetes* (Fig. 3D).

At the genus level (Fig. 4A), *Ruminococcaceae.*UCG-005 (40.38–3.03%), Others (55.06–23.37%), *Succinivibrio* (26.07–0.64%), *Bacteroides* (29.19–1.12%), *Ruminococcaceae.*UCG-010 (20.29–0.44%), *Treponema.2* (17.99 – 0.03%), *Rikenellaceae.RC9.gut.group* (9.41 – 0.05%), *Ruminococcaceae.*UCG-014 (10.62 – 0.48%), *Lachnospiraceae.AC2044.group* (19.32–0.35%), *Eubacterium.coprostanoligenes.group* (6.45 – 0.07%), and uncultured bacterium (26.69 – 0.18%) were the most predominant genera in all the samples. Compared with NC group (Fig. 4B), H + G group had higher relative abundance of *Bifidobacterium* ($P < 0.001$), *Lactobacillus* ($P < 0.001$), *Olsenella* ($P < 0.05$), *Ruminiclostridium* 9 ($P < 0.01$), *Howardella* ($P < 0.05$), and uncultured organism ($P < 0.05$), but lower relative abundance of *Helicobacter* ($P < 0.05$) and *Lachnoclostridium* ($P < 0.05$).

Analysis of metabolic profiling in weaned calves (Exp. 2)

In the present study, the untargeted metabolomics analysis was generated based on fecal samples by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS). There were overall 289 and 199 metabolites identified in the feces of weaned calves under the positive and negative mode, respectively. Fecal metabolic profiling was analyzed by PLS-DA, which showed clear segregation in the positive ion mode (R^2 = 0.912, Q^2 =−0.326) (Fig. 5A) and negative ion mode (R^2 = 0.893, Q^2 =−0.248) (Fig. 5B) between the NC and H + G groups, and suggesting the high reliability and predictive power. As a supervised method for pattern recognition, OPLS-DA analysis was performed on the data between the NC and H + G groups. As shown in Fig. 5C-D, the positive ion mode (R2 = 0.968, Q2 =−0.116) and negative ion mode (R2 = 0.873, Q2 =−0.222) for NC and H + G groups were distinctly separated in the OPLA-DS scatter plots, and illustrating the significant differences in the fecal metabolism between NC and H + G groups..

Metabolites with VIP values > 1.0 and *P*-value < 0.05 were considered significantly change. As shown in Table 5, a total of 18 (10 positive ion mode and 8 negative ion mode) significantly changed metabolites in fecal samples of weaned calves were detected among NC and H + G groups according tom ultivariate statistical analysis. Additionally, there were 16 (1-Palmitoylglycerol, Terbutaline, 3-Aminobutanoic acid, Leu-Ala, Thr-Arg, Nitrosobenzene, 1-Palmitoyl-sn-glycero-3-phosphocholine, Tyr-Met, N6-Methyladenine, Oleic acid, Sebacic acid, 13(S)-HODE, D-Mannose, Nname, cis-9,10-Epoxystearic acid, cis-9-Palmitoleic acid, and L-Malic acid) significantly upregulated metabolites and 2 (2-Methylbenzoic acid and N-Acetyl-D-lactosamine) significantly downregulated metabolites in the H + G group compared to NC group.

Table 5

Identified differentially expressed metabolites between H + G and NC group calves[1]. (Exp. 2)

Adduct	Metabolite	VIP	FC	*p*-value	m/z	Rt (s)	Trend
(M + H-H2O)+	1-Palmitoylglycerol	2.50	2.43	0.007	313.27	72.26	↑
(M + CH3CN + Na)+	Terbutaline	1.07	1.67	0.007	289.15	195.19	↑
(M + H)+	3-Aminobutanoic acid	2.38	3.07	0.01	104.07	45.25	↑
(M + CH3CN + H)+	Leu-Ala	1.01	3.12	0.012	244.16	254.23	↑
(M + H)+	Thr-Arg	2.01	2.01	0.019	276.16	398.92	↑
(M + NH4)+	Nitrosobenzene	1.83	1.3	0.02	125.07	67.04	↑
(M + H)+	1-Palmitoyl-sn-glycero-3-phosphocholine	4.94	1.67	0.037	496.34	190.05	↑
(M + H-H2O)+	Tyr-Met	1.2	1.41	0.044	295.11	129.32	↑
(M + H-H2O)+	N-Acetyl-D-lactosamine	1.16	0.54	0.045	366.14	364.75	↓
(M + H)+	N6-Methyladenine	1.28	1.55	0.05	150.08	126.98	↑
(M-H)-	Oleic acid	31.47	1.56	0.001	281.25	37.72	↑
(M-H)-	2-Methylbenzoic acid	1.04	0.74	0.005	135.045	155.13	↓
(M-H)-	Sebacic acid	1.23	2.03	0.008	201.11	321.5	↑
(M-H)-	13(S)-HODE	1.92	1.39	0.016	295.23	37.64	↑
(M + CH3COO)-	D-Mannose	3.22	1.58	0.018	239.08	369.75	↑
(M-H)-	Nname,cis-9,10-Epoxystearic acid	7.68	2.07	0.018	297.24	37.53	↑
(M-H)-	cis-9-Palmitoleic acid	5.98	1.43	0.032	253.22	37.69	↑
(M-H)-	L-Malic acid	1.55	1.62	0.045	133.01	405.99	↑

Difference metabolites identified by positive and negative ion mode. (multi-dimensional statistical analysis of VIP > 1 and univariate statistical analysis of *P* value < 0.05). VIP = variable importance in the projection, FC = Fold change, m/z = mass-to-charge ratio, Rt(s) = retention time,↑= the compound is up-regulated,↓= the compound is down-regulated.

To organize and cluster the significantly different metabolites, two-way hierarchical cluster analysis was performed for comparisons between NC and H + G groups under positive ion mode (Fig. 6A) and negative ion mode (Fig. 6B), which indicated that the metabolites were highly differentiated among the groups.

To reveal the underlying mechanism, these changed metabolites were further performed by KEGG enrichment analysis. The data of pathway analysis reflected that there were 6 significant enriched pathways of metabolites in weaned calves supplemented with HNa and Gln, which included: 1) Fatty acid biosynthesis, 2) Proximal tubule bicarbonate reclamation, 3) C-type lectin receptor signaling pathway, 4) PPAR signaling pathway, 5) Lysosome, 6) Renal cell carcinoma (Fig. 6C).

Correlation analysis (Exp. 2)

To further investigate the correlation of the altered intestinal microbiota and altered metabolites in weaned calves supplemented with HNa and Gln, we performed a Spearman's correlation analysis. In detail, as shown in Fig. 7A, *Bifidobacterium* was positively correlated ($r > 0.52$, $P < 0.05$) with Leu-Ala, Sebacic acid, and 1-Palmitoylglycerol. *Lactobacillus* was positively correlated ($r = 0.56$, $P < 0.01$) with Oleic acid, and negatively correlated ($r = -0.50$, $P = 0.04$) with N-Acetyl-D-lactosamine. *Olsenella* was positively correlated ($r > 0.51$, $P < 0.01$) with Oleic acid, Sebacic acid and negatively correlated ($r = -0.50$, $P = 0.02$) with 2-Methylbenzoic acid. *Lachnoclostridium* was positively correlated ($r = 0.52$, $P = 0.01$) with Nitrosobenzene. *Ruminiclostridium 9* was positively correlated ($r = 0.53$, $P = 0.01$) with Sebacic acid.

The correlation of altered intestinal microbiota, metabolites and ADG, fecal score, and serum parameters in weaned calves are shown in Fig. 7B, *Bifidobacterium*, *Lactobacillus*, Leu-Ala, and Oleic acid was positively correlated ($r > 0.51$, $P < 0.02$) with ADG. *Helicobacter* was positively correlated ($r = 0.53$, $P < 0.01$) with fecal score, and *Bifidobacterium*, *Lactobacillus*, *Olsenella*, Oleic acid, and 1-Palmitoylglycerol was negatively correlated ($r < -0.50$, $P < 0.04$) with fecal score. *Lachnoclostridium* was positively correlated ($r = 0.50$, $P = 0.01$) with DAO and *Bifidobacterium* was negatively correlated ($r = -0.51$, $P < 0.01$) with DAO. *Bifidobacterium* was positively correlated ($r = 0.51$, $P = 0.02$) with IgG. *Lachnoclostridium* was positively correlated ($r = 0.56$, $P = 0.01$) with TNF-α. *Olsenella* and Oleic acid was positively correlated ($r > 0.57$, $P < 0.03$) with GSH-Px. *Lachnoclostridium* was positively correlated ($r = 0.50$, $P = 0.02$) with MDA and *Bifidobacterium* was negatively correlated ($r = -0.55$, $P = 0.01$) with MDA.

Discussion

Diarrhea, which is one of the most prevalent diseases of weaned calves, could result in growth retardation, reduced feed utilization, and increased mortality [17]. The ban on antibiotic growth promotion (AGP) has further intensified the search for suitable alternatives. Thus, seeking some alternatives for antibiotics to maintain the health status and decrease diarrhea incidence of weaned calves is necessary. In the present study, the ADG of weaned calves was significantly improved by HNa and Gln combined supplementation. Another interesting discovery is that the diarrhea incidence and fecal score were dramatically decreased in weaned calves supplemented with HNa and Gln. This was consistent with the previous report in piglets [18], broilers [19], and growth-retarded yaks [20].

Calve diarrhea induced by weaning is associated with impaired intestinal epithelial barrier [21]. DAO and D-lac can be used as useful biomarkers for monitoring the integrity of intestinal mucosa barrier [22, 23]. When the intestinal barrier function is impaired, the serum levels of DAO and D-lac will increase. In the

present study, weaned calves supplemented with HNa and Gln significantly decreased the concentration of serum DAO and D-lac. The results are similar to Yasar et al. [24], who reported that dietary supplementation with HAs decreased serum D-lac concentration in rats. In addition, the positive effects of dietary Gln supplementation on intestinal barrier function have been reported in broilers [25], piglets [26], and growth-retarded yaks [20].

To the best of our knowledge, some investigators have indicated that HNa or Gln could boost immunity by improving host antioxidant function and immunoglobulin concentration [27–29]. Weaning is one of the most severe early-life stresses for calves which could induce inflammatory response, oxidative stress, intestinal dysfunction, and diarrhea. In our study, we found that calves in NC group had higher diarrhea incidence and concentration of TNF-α and MDA in the serum, nevertheless, these indicators were lower in H + G group, and serum concentration of IgG, GSH-Px, and T-AOC were increased, which indicated that HNa and Gln supplementation may alleviate diarrhea caused by weaning via improving the antioxidant and anti-inflammatory capacity of calves. Consistent with our results, previous research found that dietary supplementation with HAs improved serum activities of T-SOD and GSH-Px, but decreased serum MDA levels in juvenile broilers [30]. Rensburg and Constance [31] indicated that HAs could inhibit the release of pro-inflammatory cytokines by inhibiting the activation of classical inflammatory pathways. Moreover, the anti-inflammatory and immunomodulatory activities of Gln have been widely reported. For example, Zhou et al. [32] indicated that intravenously administered Gln increased the concentration of serum IgA and IgG, intestinal mucosal sIgA in early-weaned calves. Ma et al. [20] demonstrated that dietary supplementation with Gln significantly decreased the mRNA expression of IL-1β and TNF-α in growth-retarded yaks.

It is well known that intestinal microbiota plays an important role in nutrient utilization, intestinal morphology and immunity [33–35]. To clarify the mechanism of beneficial effects of HNa and Gln supplementation in weaned calves, the 16S rRNA sequencing and untargeted LC – MS metabolomics analysis were performed. From the results of phylum analysis, we found that the intestinal microbiota was dominated by *Firmicutes* and *Bacteroidetes*, and supplemented with HNa and Gln significantly increased the abundance of *Firmicute* but decreased the abundance of *Bacteroidetes* of weaned calves, which is consistent with previous studies conducted by Zhang et al. [6]. *Firmicutes* are considered as one of the producers of short-chain fatty acid, and more efficient in promoting nutrition absorption than *Bacteroidetes* [36]. At the genus level, weaned calves supplemented with HNa and Gln had higher abundance of *Bifidobacterium*, *Lactobacillus*, *Olsenella*, *Ruminiclostridium 9*, *Howardella*, and uncultured organism but lower abundance of *Helicobacter* and *Lachnoclostridium* as compared with NC group. It is well known, *Bifidobacterium* and *Lactobacillus* were probiotics, which have excellent efficacy in reducing gastrointestinal infections [33, 37]. The results of the present study indicated that supplemented with HNa and Gln could increase the abundance of probiotics in intestinal microbiota. In addition, Spearman's correlation analysis also indicated that *Bifidobacterium*, *Lactobacillus*, and *Olsenella* were positively correlated with ADG, serum IgG level and T-AOC, while negatively correlated with fecal score and concentration of serum DAO, D-lac, TNF-α, and MDA. This observation might explain that supplemented

with HNa and Gln could improve growth performance, anti-inflammatory, and antioxidative status and alleviate diarrhea of weaned calves via increasing the abundance of beneficial intestinal microbiota.

In addition, feces metabolites may also reflect the physiological status of calves [38, 39]. Currently, LC-MS based metabolomics analyses are being increasingly performed to explore the alteration of metabolites [40]. Based on that, we examined the effect of HNa and Gln combined supplementation on metabolites of weaned calves. The results showed that the levels of fatty acid metabolites (Oleic acid, Sebacic acid, 1-Palmitoylglycerol, Nname,cis-9,10-Epoxystearic acid, cis-9-Palmitoleic acid), amino acid metabolites (3-Aminobutanoic acid, Leu-Ala, Thr-Arg, Tyr-Met), and carbohydrates metabolites (D-Mannose) were significantly up-regulated by HNa and Gln inclusion. Furthermore, the results of KEGG enrichments suggested that weaned calves supplemented with HNa and Gln primarily up-regulated the fatty acid biosynthesis pathway. Surprisingly, Spearman's correlation analysis found that up-regulated metabolites were positively correlated with increased beneficial intestinal microbiota. For example, *Bifidobacterium* was positively correlated with Leu-Ala and Sebacic acid, *Lactobacillus* was positively correlated with 3-Aminobutanoic acid and Oleic acid, *Olsenella* was positively correlated with 1-Palmitoylglycerol and Oleic acid. This indicated that the improved growth performance and decreased diarrhea incidence may be attributed to the modulatory role of HNa and Gln on intestinal microbiota and metabolites.

An interesting result obtained by Spearman's correlation analysis was that Oleic acid, Sebacic acid, and 1-Palmitoylglycerol was negatively correlated with the fecal score, while, *Bifidobacterium*, *Lactobacillus*, *Olsenella*, *Ruminiclostridium 9*, and *Howardella* was positively correlated with Oleic acid, Sebacic acid, and 1-Palmitoylglycerol, which indicated that decreased diarrhea incidence is closely related to increased abundance of beneficial intestinal microbiota and altered metabolites. This might provide a new evidence for the mechanism of decreased diarrhea of weaned calves supplemented with HNa and Gln. Currently, information on the mechanism of HNa and Gln supplementation on the intestinal health of weaned calves is limited, further investigations required to be conducted.

Conclusion

Weaned calves supplemented with HNa and Gln had a higher ADG, antioxidant status and intestinal barrier function thereby lower diarrhea incidence. Moreover, Analysis of intestinal microbiota and metabolic profile revealed that weaned calves supplemented with HNa and Gln increased the relative abundance of intestinal beneficial microbiota and enriched many lipid metabolites and KEGG pathway of fatty acid biosynthesis. These findings provide a better understanding of the mechanism of decreasing diarrhea of HNa and Gln supplementation, which could further provide useful information for developing an effective and safe non-antibiotics alternative in the dairy calve industry to prevent and treatment calves diarrhea.

Abbreviations

BW: Body weight; ADG:Average daily gain; ADFI:Average daily feed intake; F:G:Feed to gain ratio; HNa:Sodium humate; Gln:Glutamine; DAO:Diamine oxidase; D-lac:Diamine lactic acid; Ig:immunoglobulin; IL-6:Interleukin6; TNF-α:Tumour necrosis factor-α; GSH-Px:Glutathione peroxidase; MDA:Malondialdehyde; T-SOD:Total superoxide dismutase; T-AOC:Total antioxidant capacity; OTUs:Operational taxonomic units; PCoA:principal coordinate analysis; PLS-DA:Partial least-squares discriminant analysis; OPLS-DA:Orthotopic partial least-squares discriminant analysis;KEGG:Kyoto Encyclopedia of Genes and Genomes.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

Dong Wang: writing-original draft, formal analysis, investigation. Yuanyi Du: Conceptualization. Siqi Huang: investigation. Haotian Jia: investigation. Lei Lei: formal analysis. Yun Liu: project administration, editing, validation, funding acquisition.

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Availability of data and materials

The data produced or analyzed during the current study are available from the corresponding author by reasonable request.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of Northeast Agricultural University (Harbin, China). The study was conducted at Harbin Modern Farming (Harbin, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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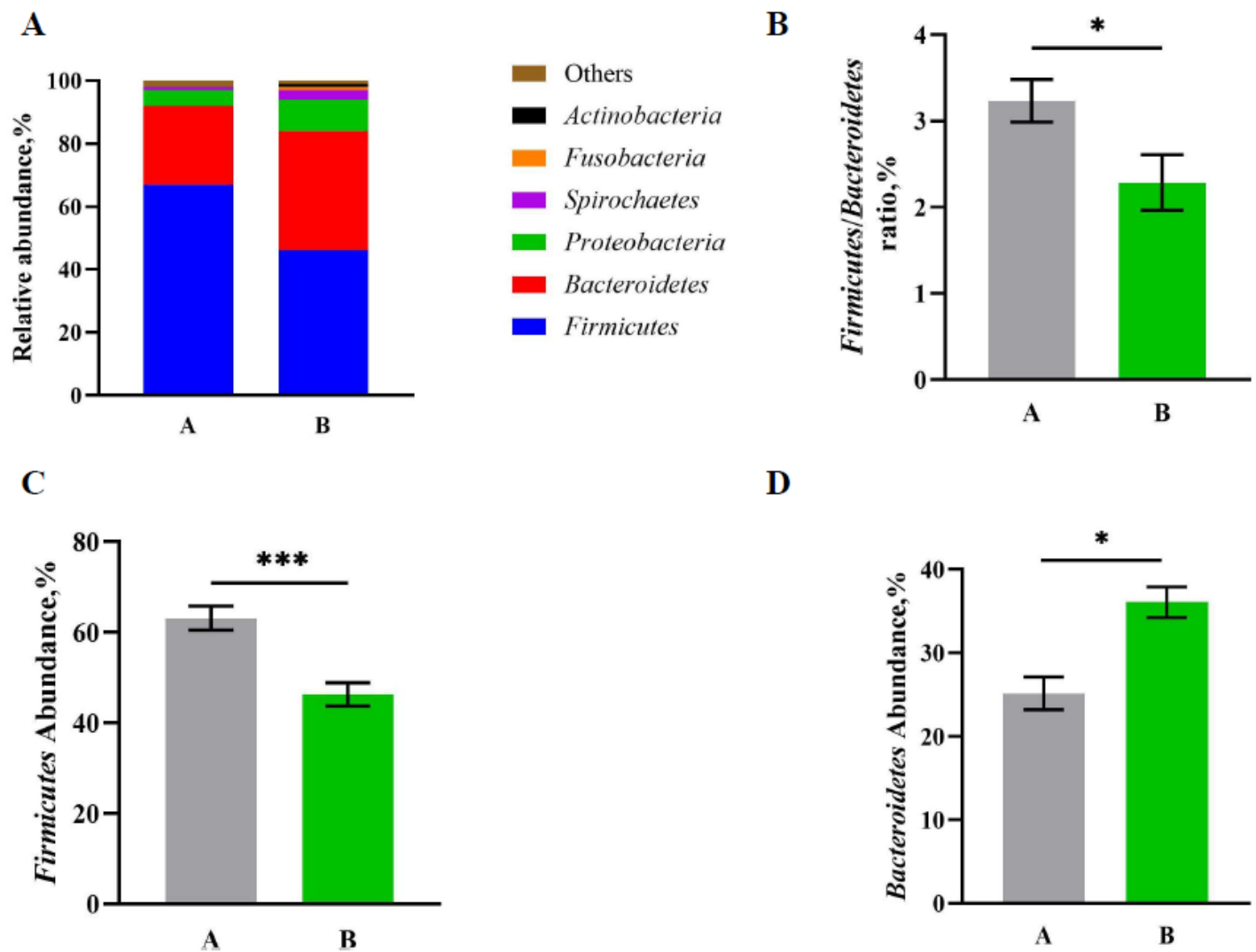
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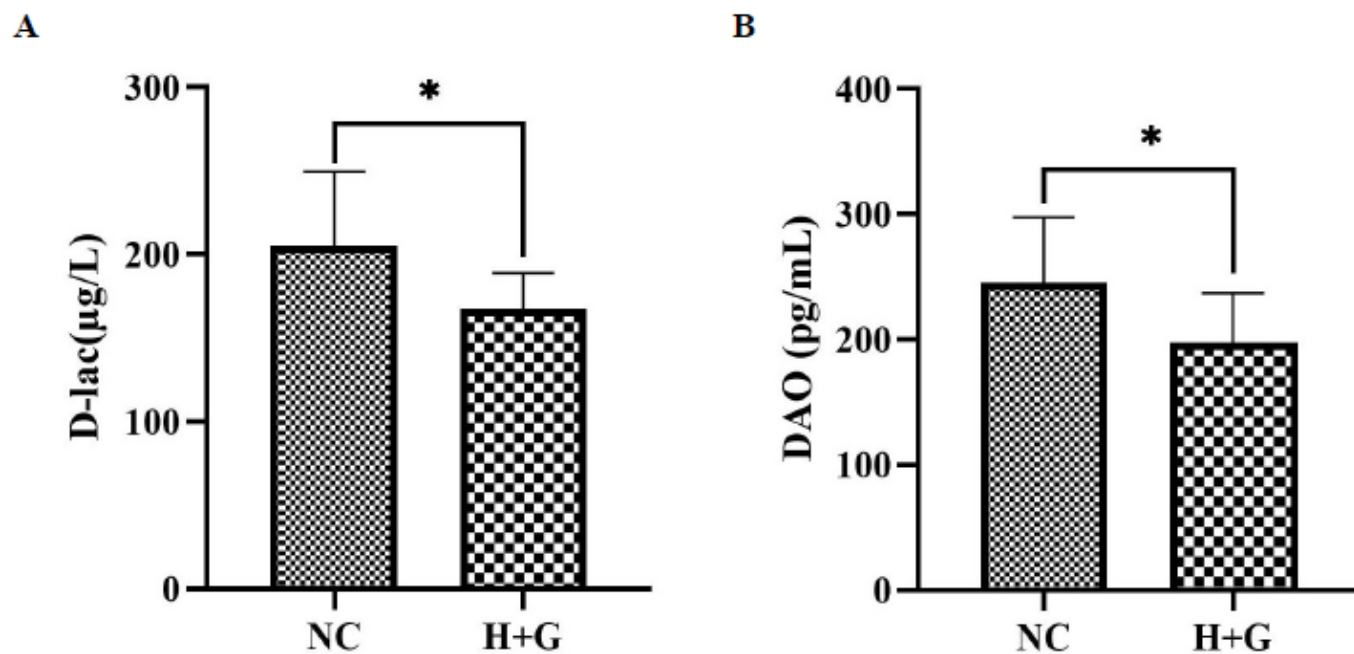
Figure 1

Effects of HNa and Gln combined supplementation on the serum concentration of DAO and D-lac of calves, Data were shown as means ± SEM (n = 10), *P < 0.05
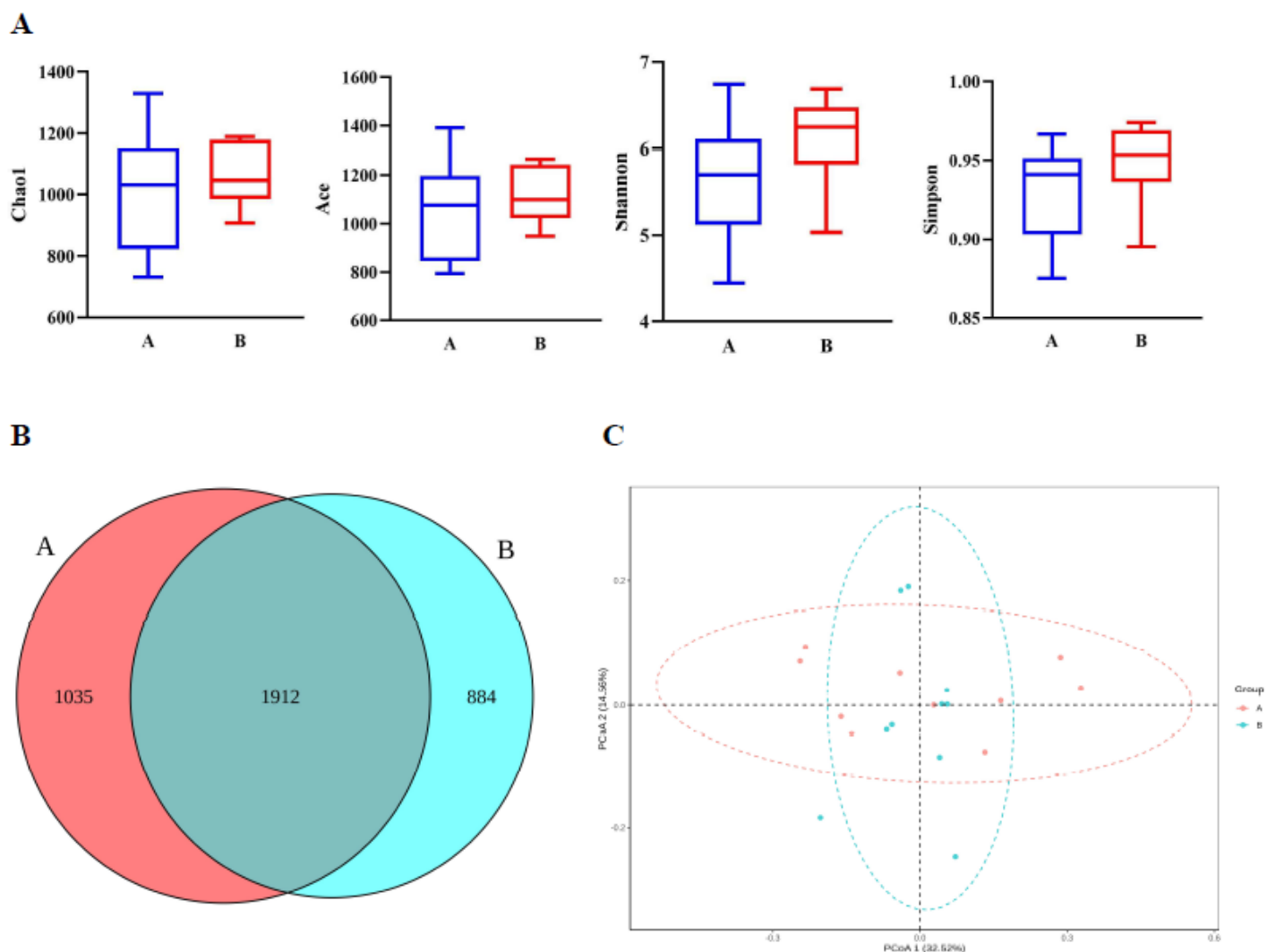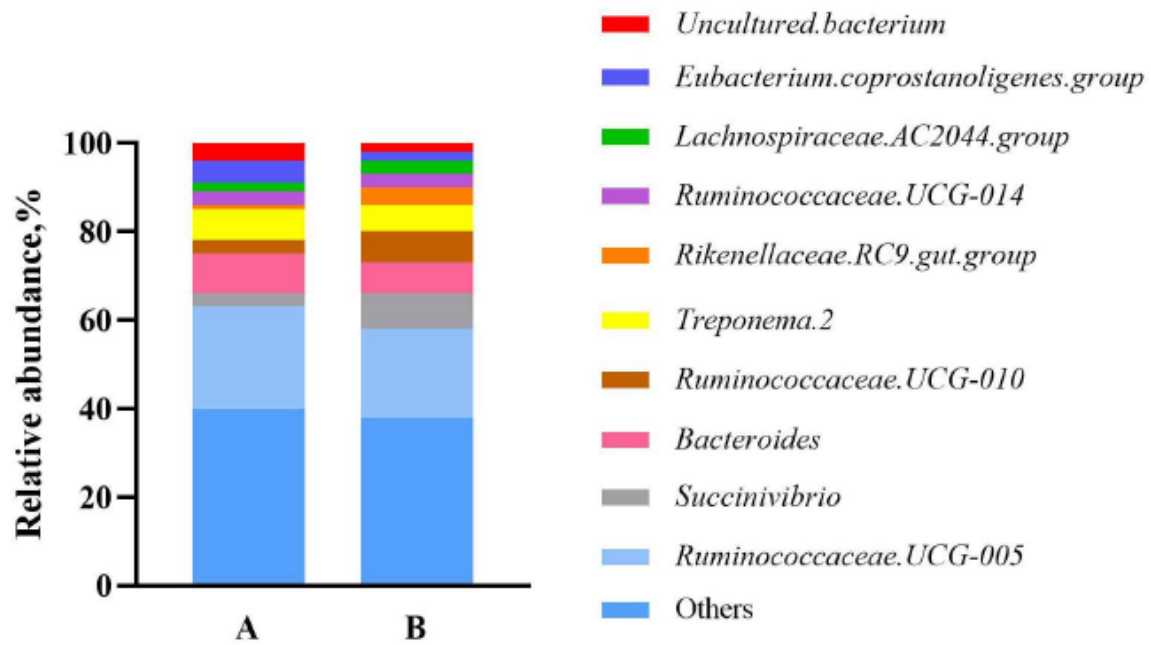
Figure 2

Differences in bacterial community diversity, richness, and structures in the intestinal of calves without or with HNa and Gln of supplementation. (A) The Chao 1, Ace, Shannon, and Simpson indexes of α-diversity analysis at the OTU level were calculated and compared among two groups. (B) Venn diagram of the operational taxonomic unit (OTU) distribution shows unique and shared OTUs between the two groups. (C) β-Diversity principal coordinate analysis (PCoA) was performed to calculate the different intestinal microbiota structures among the two groups. Data were shown as means ± SEM (n = 10). A = H+G group, basal diet+5% HNa and 1% Gln; B = NC group, basal diet.

Figure 3

Characterization of communities on phylum level. (A) Average relative abundances of microbial community composition for each group are shown by bar plots for the phylum level. (B) The ratio of Firmicutes to Bacteroidetes in each group based on their relative abundance. The effect of HNa and Gln combined supplementation on Firmicutes (C) and Bacteroidetes (D) abundance. Data were shown as means ± SEM (n = 10). A = H+G group, basal diet+5% HNa and 1% Gln; B = NC group, basal diet. Data were shown as means ± SEM (n = 10). *P < 0.05, ***P < 0.001.

A

B

Figure 4

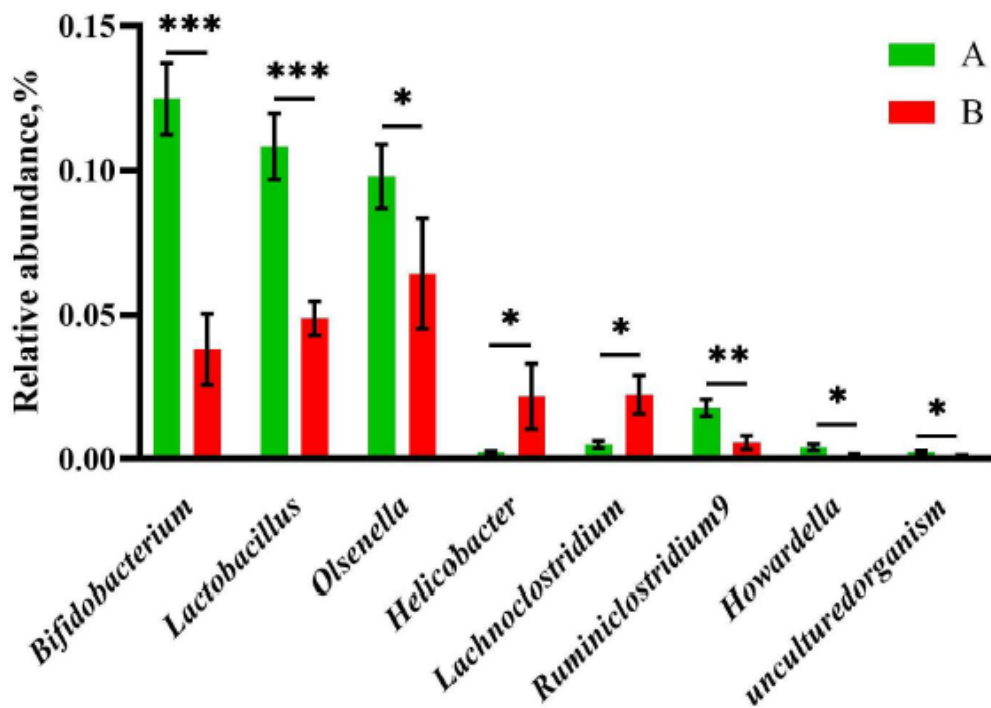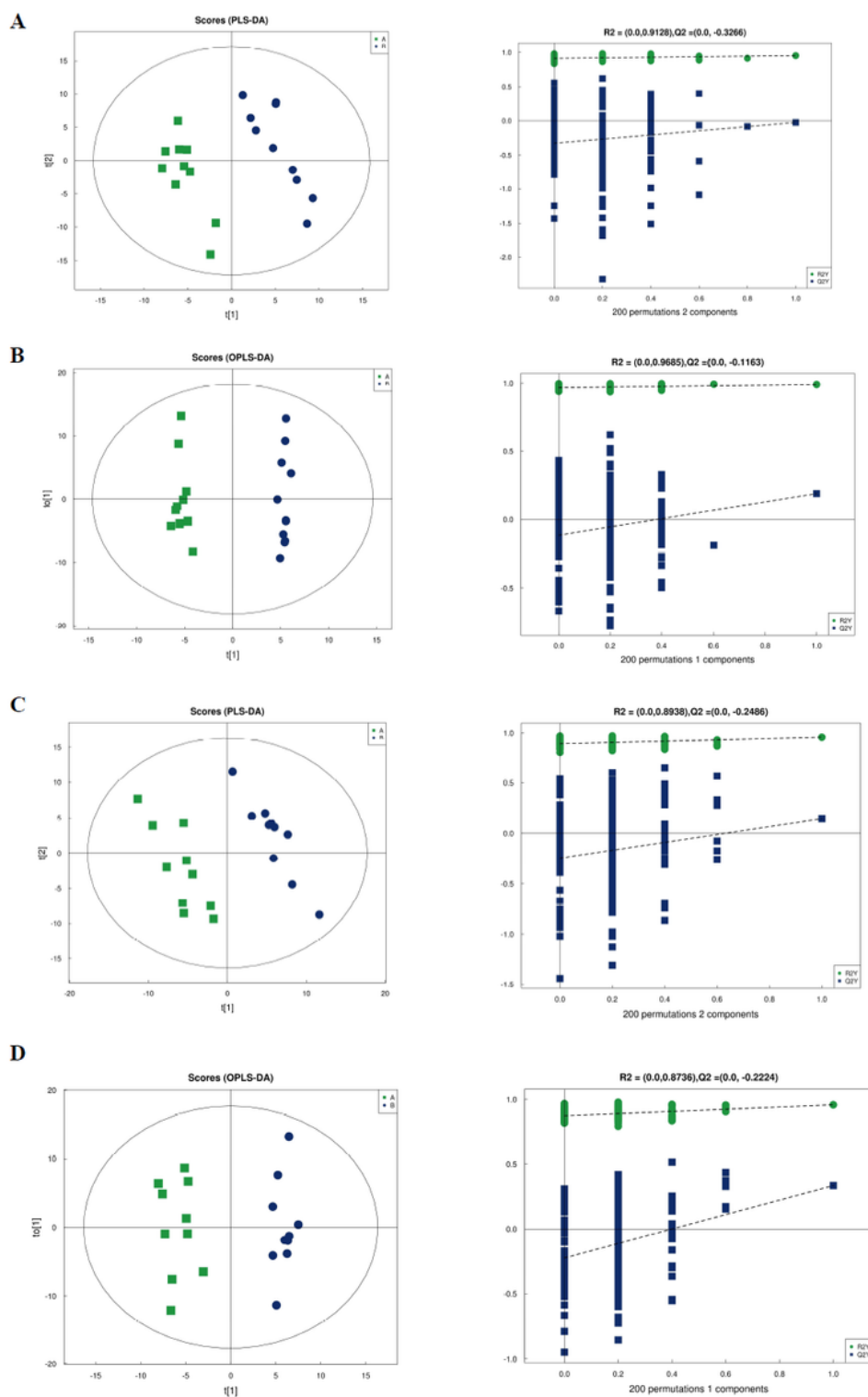Characterization of communities on genus level. (A) Average relative abundances of microbial community composition for each group are shown by bar plots for the genus level, and the 8 abundant genera (B) significant differences are shown among the two groups. Data were shown as means ± SEM (n = 10). *P < 0.05, **P < 0.01, ***P < 0.001. A = H+G group, basal diet+5% HNa and 1% Gln; B = NC group, basal diet.

Figure 5

Metabolomic analysis of calves treated with or without HNa and Gln of supplementation. Partial least-squares discriminant analysis (PLS-DA) score plots among the NC and H+G groups in positive (A) and negative (B) ion modes. Orthotopic partial least-squares discriminant analysis (OPLS-DA) score plots among the NC and H+G groups in positive (C) and negative (D) ion mode. A = H+G group, basal diet+5% HNa and 1% Gln; B = NC group, basal diet.
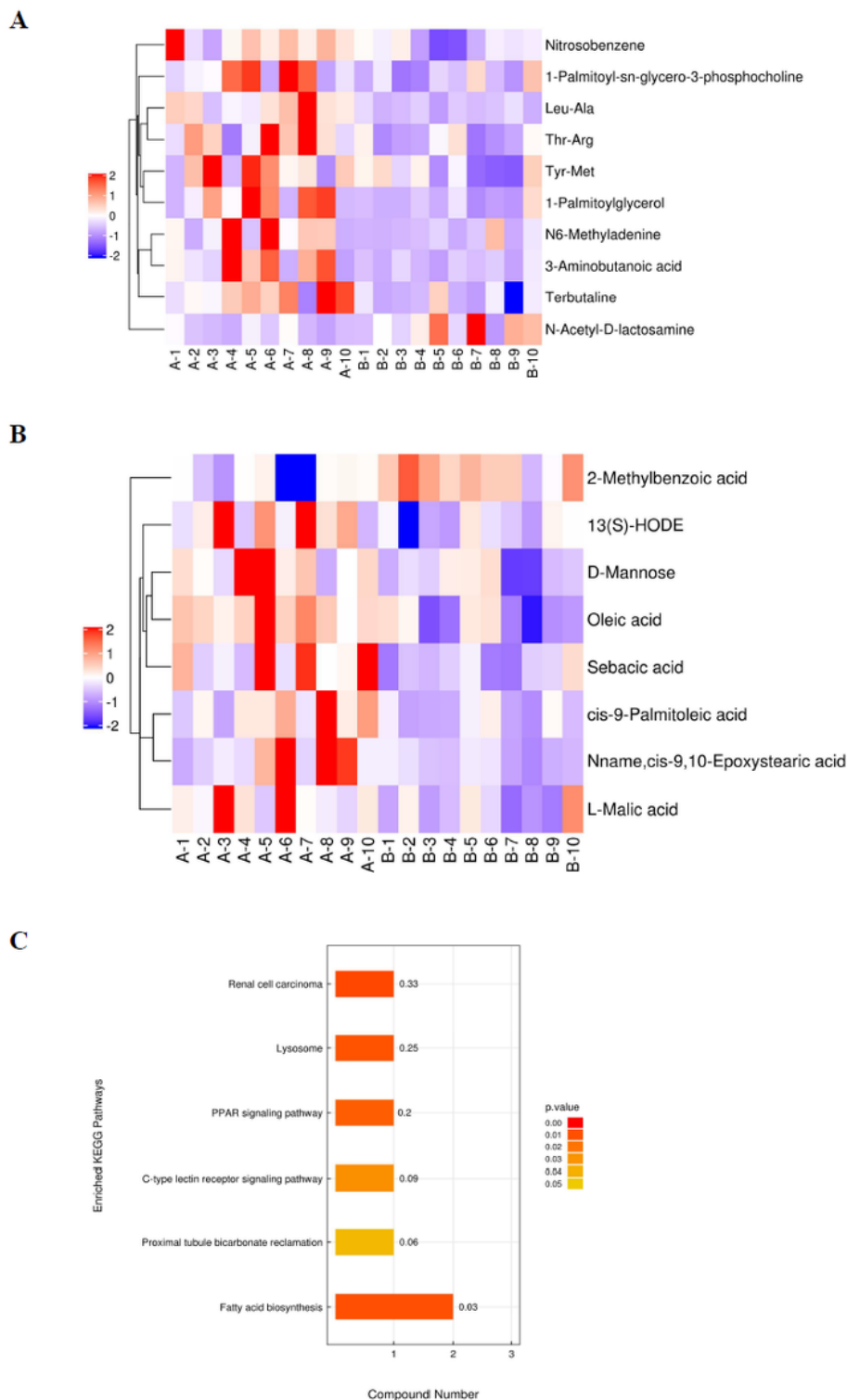
Figure 6

Altered metabolites and metabolic pathways in calves supplemented with HNa and Gln. Heat plot of the differentially expressed metabolites in calves between H+G group and NC group (A, positive ion; B, negative ion). (C) Enriched KEGG pathways based on significantly different metabolites between NC and H+G groups. A = H+G group, basal diet+5% HNa and 1% Gln; B = NC group, basal diet.

A

	Bifidobacterium	Lactobacillus	Olsenella	Helicobacter	Lachnoclostridium	Ruminiclostridium 9	Howardella	uncultured organism
1-Palmitoylglycerol	0.55	0.40	0.45	-0.29	-0.04	0.38	0.29	-0.33
Terbutaline	-0.42	-0.26	-0.35	-0.35	-0.26	0.20	0.21	-0.14
3-Aminobutanoic acid	0.04	0.46	-0.48	0.28	0.44	0.48	0.45	-0.11
Leu-Ala	0.52	0.33	0.36	-0.10	0.08	0.45	-0.26	-0.46
Thr-Arg	0.31	-0.16	0.49	0.20	-0.07	-0.23	0.34	0.22
Nitrosobenzene	-0.30	0.39	0.18	0.17	0.52	0.26	0.17	-0.25
1-Palmitoyl-sn-glycero-3-phosphocholine	0.15	0.47	0.44	-0.27	-0.22	-0.40	-0.18	-0.00
Tyr-Met	0.04	-0.32	-0.31	0.03	-0.03	-0.22	-0.37	-0.24
N-Acetyl-D-lactosamine	-0.21	-0.50	0.43	0.44	-0.31	0.04	0.17	0.20
N6-Methyladenine	-0.21	0.27	-0.42	0.27	0.35	-0.41	-0.41	0.01
Oleic acid	0.37	0.57	0.59	0.08	-0.23	0.40	0.23	-0.34
2-Methylbenzoic acid	-0.37	-0.37	0.50	0.31	-0.28	0.17	-0.18	-0.32
Sebacic acid	0.60	-0.30	0.51	-0.32	0.12	0.53	0.36	-0.31
13(S)-HODE	-0.27	-0.40	-0.41	0.01	0.31	-0.01	-0.23	-0.35
D-Mannose	0.41	0.45	-0.45	0.05	0.38	-0.43	-0.29	-0.20
Nname,cis-9,10-Epoxystearic acid	0.31	-0.36	0.28	0.48	-0.14	-0.36	0.46	-0.14
cis-9-Palmitoleic acid	-0.38	0.30	0.45	-0.27	-0.16	0.24	-0.32	-0.43
L-Malic acid	-0.03	-0.21	-0.13	0.23	0.24	-0.29	-0.44	-0.16

B

	ADG	Fecal score	DAO	D-lac	IgG	TNF-α	GSH-Px	T-AOC	MDA
Bifidobacterium	0.53	-0.50	-0.51	-0.34	0.51	-0.49	0.38	0.46	-0.56
Lactobacillus	0.53	-0.65	-0.34	-0.32	0.45	-0.38	0.37	0.30	-0.30
Olsenella	0.43	-0.63	-0.13	-0.16	0.27	-0.26	0.57	0.47	-0.45
Helicobacter	-0.41	0.54	0.41	0.25	-0.39	0.11	-0.28	-0.16	0.35
Lachnoclostridium	-0.39	0.30	0.50	0.42	-0.29	0.56	-0.28	-0.33	0.50
Ruminiclostridium 9	0.23	-0.38	-0.07	-0.20	0.36	0.15	0.19	0.30	-0.38
Howardella	0.42	-0.42	-0.15	-0.16	0.22	0.12	0.44	0.42	0.35
uncultured organism	0.15	-0.37	-0.01	-0.16	-0.08	0.21	0.15	0.21	-0.16
1-Palmitoylglycerol	-0.41	-0.50	0.22	0.06	0.39	0.05	-0.39	-0.29	-0.21
Terbutaline	-0.17	0.41	-0.07	0.28	-0.32	-0.32	0.45	0.47	-0.36
3-Aminobutanoic acid	0.17	-0.42	0.12	-0.45	0.33	-0.05	0.29	0.38	-0.27
Leu-Ala	0.53	-0.10	0.07	0.01	0.25	-0.09	0.31	0.30	0.26
Thr-Arg	-0.31	0.11	-0.27	-0.20	0.42	0.09	-0.46	-0.34	-0.33
Nitrosobenzene	0.20	-0.31	0.31	0.32	-0.11	-0.27	0.05	0.31	0.34
1-Palmitoyl-sn-glycero-3-phosphocholine	-0.33	0.46	0.07	-0.31	0.40	-0.35	0.27	0.19	-0.25
Tyr-Met	0.24	-0.38	0.35	0.05	-0.06	-0.19	-0.38	-0.18	0.48
N-Acetyl-D-lactosamine	-0.21	0.48	-0.17	-0.18	-0.15	0.35	0.16	0.31	0.38
N6-Methyladenine	-0.14	0.21	0.13	0.36	-0.25	0.08	-0.29	-0.36	0.39
Oleic acid	0.51	-0.53	-0.41	-0.29	0.28	-0.07	0.58	0.35	-0.41
2-Methylbenzoic acid	-0.38	0.31	0.41	0.37	-0.39	0.15	-0.33	-0.25	0.40
Sebacic acid	0.40	-0.34	0.14	-0.15	0.46	-0.49	0.22	0.37	-0.27
13(S)-HODE	-0.16	0.37	0.35	0.34	-0.16	-0.14	-0.21	-0.33	-0.24
D-Mannose	0.33	0.11	0.28	0.26	0.18	0.33	-0.22	-0.38	0.36
Nname,cis-9,10-Epoxystearic acid	-0.37	-0.39	0.01	-0.18	-0.43	0.19	-0.38	-0.32	-0.36
cis-9-Palmitoleic acid	0.30	0.24	-0.03	-0.18	-0.41	0.03	-0.43	-0.24	-0.22
L-Malic acid	-0.22	0.18	0.39	0.02	0.09	-0.09	-0.42	-0.39	0.16

Figure 7

Heatmap of Sperman's correlation between significantly different intestinal microbiota and differential metabolites (A), ADG, fecal score, and serum parameters (B). The significant correlations ($r > 0.50$ or $r <= -0.50$, $p < 0.05$) were shown in the correlation heatmaps. The intensity of the colors represented the degree of correlation (red, positive correlation; blue, negative correlation).