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An organic zinc-chelating-peptide GQ-Zn supplementation ameliorates diarrhea through regulate gut microbiota and metabolites in pre-weaning Holstein calves

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Abstract

Newborn calf diarrhea has led to widespread overuse of antibiotics. Therefore, it is crucial to find effective solutions for calf diarrhea. In this study, we aimed to evaluate the impact of the synthetic organic zinc-chelating-peptide Glycine-Glutamine-Zn (GQ-Zn) on the microbiota and metabolites in the gut of calves with diarrhea. The results showed that GQ-Zn alleviated diarrhea in calves. Additionally, 16S rDNA sequencing and metabolomic analysis revealed that GQ-Zn improved antioxidant capacity, relieved inflammation, altered the gut microbiota by decreasing the number of harmful bacteria *Prevotella denticola*, *Fusobacterium necrophorum* and influenced metabolomic profiles via the linoleic acid metabolic pathway in calves. In conclusion, GQ-Zn supplementation alleviated diarrhea through regulating the gut microbiota and metabolites in pre-weaning Holstein calves.

Keywords: calf diarrhea; GQ-Zn; inflammation; gut microbiota; metabolomics

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1. Introduction

Ranch managers have long struggled with problems related to animal industrialization, such as diarrhea [1]. In livestock, diarrhea is typically classified as either infectious or noninfectious. Infectious agents include a variety of bacteria, viruses and parasites [2]. Causes of noninfectious diarrhea include poor management of breeding, changes in diet, and malnutrition [3]. In a pasture, an outbreak of neonatal calf diarrhea in 4-week-old calves causing 80% morbidity rate and 20% mortality rate [4]. In the Netherlands, the incidence of calf diarrhea is 24% in the first three weeks, while in Canada, the incidence of calf diarrhea is 20% during weaning [5]. Diarrhea is a major threat to cow production. Therefore, it is crucial to search for new feed additives to reduce the incidence of calf diarrhea, which could increase the number of Holstein calves that reach maturity and in turn improve the profitability of related enterprises.

Intestinal microbiota play an important role in the occurrence and treatment of calf diarrhea. Kim found that fecal microbial transplantation could improve fecal score and alleviate diarrhea of pre-weaning calves, resulting in the gradual maturation of intestinal microbiota of calves, indicating the importance of microbiota in alleviating diarrhea of calves [6]. Certain probiotics, such as Lactobacillus, have demonstrated a significant reduction in the incidence of diarrhea in cattle [7]. Certain pathogenic microbiota such as *Escherichia coli* K99 and Salmonella can lead to diarrhea in calves [8]. The fecal samples of calves were analyzed for microbiota, and there was a significant correlation between pathogenic microbiota such as *Escherichia coli* K99,

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Salmonella, *Clostridium* and diarrhea of calves [2]. *Escherichia coli* K99 and *Salmonella* are the main micobiota of antibiotic resistance [9].

Antibiotics was useful for promoting growth and curing disorders caused by microbial infections such as diarrhea, but accompanied with the reduction of absorption rate in livestock [10]. Considerable evidence showed that misuse of these antibiotics is a major contributor to current health crises [11]. Antibiotics are no longer as effective as they once were because drug-resistant microbiota proliferate [12]. Varieties of antibiotics are accumulated by humans because of biomagnification [13]. Calf diarrhea treated with medicines often relapses after a few days [14]. It is possible that antibiotics are bactericidal against a wide range of microbiota, which leads to an imbalance of gut microbiome. Furthermore, even if calves recover from diarrhea, antibiotics are not conducive to their future growth potential because they interfere with the development and colonization of rumen microbiota [15]. Overall, the excessive application of antibiotics in livestock should be restricted, and additives substitutes should be developed.

Zinc is an essential mineral for metabolism and has been widely used in animal husbandry; which was proven to promote animal growth and alleviate diarrhea [16]. Zinc supplements are divided into two main types: inorganic zinc and organic zinc. Inorganic zinc (e.g., ZnO, $ZnSO_4$) is widely used during the weaning period to alleviate diarrhea in piglets by strengthening intestinal barrier function [17]. Although ZnO is beneficial to young animals, it often results in the loss of significant amounts of zinc, which causes environmental pollution [18]. Organic zinc has higher bioavailability,

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allowing animals to digest, absorb, and utilize it better, especially when zinc is chelated with amino acids or small peptides [19]. The bioavailability of ZnO is lower than Zn-methionine and Zn-lysine in high concentrations of Zn in weanling pigs [20]. Previous study found that glycine-zinc significantly improved the weekly weight gain, feed intake, feed conversion ratio of control and inorganic zinc in broilers [21]. In our study, Zinc was chelated with glycyl-glutamine dipeptides (GQ-Zn) to form new organic zinc.

The amount of zinc added to calf milk supplements in China cannot exceed 180 mg/kg. Previous studies suggested that different doses of zinc have different effects on animals [22]. Due to the negative effects of high doses of zinc, China completely banned the addition of excessive zinc in 2017 [23]. To determine the optimal level of supplementation, a two-pronged approach was used in this study. The aim of this research was to study a novel organic zinc GQ-Zn on the growth performance, health status , microbiome of the cattle.

2. Materials and methods

2.1. Animals and Management

The trial was conducted on the Modern Farming Saibei Phase III Pasture (Guyuan County, Zhangjiakou City, Hebei, China). The experiment lasted for a total of 14 days. Thirty Holstein female calves with similar birth dates, weights, and genetic backgrounds were randomly assigned to three groups (n = 10). Every morning during the experiment, 50 mL of purified water was given to the control group (Con), 50 mL of Glycine-Glutamine-Zn (GQ-Zn) solution (80 mg/d) was given to the low-zinc group

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(Low), and 50 mL of GQ-Zn solution (160 mg/d) was given to the high-zinc group (High). During the experiment, the calves were kept on the calf island and fed 4 kg of pasteurized milk and 250 g of starter every day, with free access to food and water. If the temperature drops, a calf warmer garment will come in handy. Calves that are severely unwell or have diarrhea should be treated or euthanized.

2.2. Data and sample collection

A group of calves were fed organic zinc to further examine its impact on calves with diarrhea. The severity of diarrhea in the calves was recorded daily, and the rectal contents were collected at 0, 7, and 14 days. The weights of the calves were measured with a weighbridge on Days 0, 7, 14, 60, 365. On the mornings of Days 7 and 14, 10 mL of blood was collected from the jugular vein by using a blood collection vessel without anticoagulants. To isolate the serum, the blood was left at room temperature for one hour and centrifuged at 4000 rpm for 20 min. The upper layer of serum was extracted with a pipette gun and released into centrifuge tubes, which were stored at −20 °C until analysis of the serum biochemical indices. On Days 0, 7 and 14, 5 g of feces was collected from the calf rectum and packed into 5 mL sterile cryogenic tubes, which were subsequently stored in a -80 °C refrigerator.

2.3. Determination of diarrhea incidence

The criteria for determining the fecal score were established according to previous methods [24]. Fecal scored as 1 when firm, 2 when soft or of moderate consistency, 3 when runny or mild diarrhea, and 4 when watery and profuse diarrhea.Calf feces were observed and scored daily during the experiment. Score ≥ 3 was considered to calf

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diarrhea, and the diarrhea rate was calculated based on the score. In the control group, one calf died due to severe diarrhea on Day 9.

2.4. Analysis of biochemical indicators

Serum biochemical indicators, including the antioxidant indicators CAT, MDA, SOD, GSH-Px, and T-AOC and the immune indicators IgA, IgG, SIgA, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IL-22, IFN-γ, TGF-β and TNF-α, were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Meimian, Jiangsu, China) according to the manufacturer's instructions.

2.5. Determination of short-chain fatty acids (SCFAs)

Gas chromatography was used to determine the concentration of SCFAs in calf feces. A 1 g sample was placed into a 2 mL tube, and 1 mL of methanol was added to the tube, which was centrifuged at 12000 × g for 10 min. One milliliter of supernatant and 0.2 mL of 25% metaphosphate solution were mixed and centrifuged at 12000 × g for 10 min. Finally, the sample was filtered through a 0.45 μm membrane, and the sample was injected into a gas chromatograph [25].1 μL of sample was injected into a gas chromatograph (Trace 1300, Thermo Fisher Scientific, MA, USA), A flame ionization detector was used with an oven temperature of 75 ℃, and a inlet and detector temperature of 230 ℃, a column flow and purge flow of 3 ml/min and 5 ml/min. for determination of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate concentrations.

2.6. Determination of the fecal microbiome

Total genomic DNA was extracted from feces samples using the TGuide S96

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Magnetic Soil /Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to manufacturer's instructions. The quality and quantity of the extracted DNA were examined using electrophoresis on a 1.8% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA). The full-length 16S rRNA gene were amplified with primer pairs 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTTGTTASGACTT. Both the forward and reverse 16S primers were tailed with sample-specific PacBio barcode sequences to allow for multiplexed sequencing. We chose to use barcoded primers because this reduces chimera formation as compared to the alternative protocol in which primers are added in a second PCR reaction. The KOD One PCR Master Mix (TOYOBOLife Science) was used to perform 25 cycles of PCR amplification, with initial denaturation at 95 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min 30 s, and a final step at 72 °C for 2 min. The total of PCR amplicons were purified with VAHTSTM DNA Clean Beads (Vazyme, Nanjing,China) and quantified using the Qubit dsDNA HS Assay Kit and Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA). After the individual quantification step, amplicons were pooled in equal amounts. SMRTbell libraries were prepared from the amplified DNA by SMRTbell Express Template Prep Kit 2.0 according to the manufacturer's instructions (Pacific Biosciences). Purified SMRTbell libraries from the pooled and barcoded samples were sequenced on a PacBio Sequel II platform (Beijing Biomarker Technologies Co., Ltd., Beijing, China)using Sequel II binding kit 2.0.

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The bioinformatics analysis of this study was performed with the aid of the BMKCloud (http://www.biocloud.net/). The raw reads generated from sequencing were filtered and demultiplexed using the SMRT Link software (version 8.0) with the minPasses ≥5 and minPredictedAccuracy ≥0.9, in order to obtain the circular consensus sequencing (CCS)reads. Subsequently, the lima (version 1.7.0) was employed to assign the CCS sequences to the corresponding samples based on their barcodes. CCS reads containing no primers and those reads beyond the length range (1,200–1,650 bp) were discarded through the recognition of forward and reverse primers and quality filtering using the Cutadapt [26] (version 2.7)quality control process .The UCHIME algorithm (v8.1) [27] was used in detecting and removing chimera sequences to obtain the clean reads. Sequences with similarity > 97% were clustered into the same operational taxonomic unit (OTU) by USEARCH [28] (v10.0), and the OTUs conuts less than 2 in all samples were filtered.

Clean reads then were conducted on feature classification to output an ASVs (amplicon sequence variants) by DADA2 [29], and the ASVs conuts less than 2 in all samples were filtered. Taxonomy annotation of the OTUs/ASVs was performed based on the Naive Bayes classifier in QIIME2 [30] using the SILVA database [31] (release 138.1) with a confidence threshold of 70%. The Alpha diversity were calculated and displayed by the QIIME2 and R software, respectively. Beta diversity was determined to evaluate the degree of similarity of microbial communities from different samples using QIIME. Principal coordinate analysis (PCoA), heatmaps, UPGMA and nonmetric multidimensional scaling (NMDS) were used to analyze the beta diversity. To explore

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the dissimilarities of the microbiome among different factors, a redundancy analysis (RDA) were performed in R using the package vegan.

The qualified sequences with more than 97% similarity thresholds were allocated to one operational taxonomic unit (OTU) using USEARCH (version 10.0). Taxonomy annotation of the OTUs/ASVs was performed based on the Naive Bayes classifier in QIIME2 [30] using the SILVA database [31] (release 138.1) with a confidence threshold of 70%. Alpha was performed to identify the complexity of species diversity of each sample utilizing QIIME2 software. Beta diversity calculations were analyzed by principal coordinate analysis (PCoA) to assess the diversity in samples for species complexity. One-way analysis of variance was used to compare bacterial abundance and diversity. The online platform BMKCloud (https://www.biocloud.net) was used to analyze the sequencing data.

2.7. Determination of fecal metabolites

After processing the sample, LC–MS was used for detection. The raw data were first converted to mzXML format by MSConvert in the ProteoWizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accurate mass (< 30 ppm) and MS/MS data, which were matched with HMDB, MassBank, LipidMaps, mzCloud and KEGG data. The robust LOESS signal correction (QC-RLSC) [32] was applied for data normalization to correct for any systematic bias. After normalization, only ion peaks with relative standard deviations (RSDs) less than 30% in the QC were retained to ensure proper metabolite identification.

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Ropls software was used for all multivariate data analyses and modeling. The data were mean-centered using scaling. Models were built via principal component analysis (PCA), orthogonal partial least-square discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA). The P value, variable importance projection produced by OPLS-DA, and fold change were applied to discover the contributable variables for classification. The differentially abundant metabolites were subjected to pathway analysis via MetaboAnalyst, which combines the results from powerful pathway enrichment analysis with pathway topology analysis. The metabolites identified via metabolomics were subsequently mapped to the KEGG pathway for biological interpretation of higher-level systemic functions. The metabolites and corresponding pathways were visualized using the KEGG Mapper tool.

2.8. Statistical analysis

The data were analyzed using GraphPad Prism software with t tests and one-way ANOVA. All the figures were created with GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA). In all analyses, P values < 0.05 were considered to indicate statistical significance (*P < 0.05).

3. Results

3.1. GQ-Zn ameliorated diarrhea in pre-weaning calves

The results showed that GQ-Zn reduced the incidence of diarrhea in calves (Figure 1A-C). Evaluation of stool fluidity using the stool score method showed that the stool

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score in the high-GQ-Zn group was significantly lower than that in the control group on Days 7, 9, 10, and 12 (Figure 1D). The calves were weighed on Days 0, 7, 14, 60, 365 and the growth ability of the calves was evaluated. The results indicated that GQ-Zn had no significant effect on the growth rate of the calves (Table 1).

3.2. GQ-Zn enhanced the antioxidant capacity of calves and reduced inflammatory reactions

Several serum biomarkers have been shown to be significantly different between different treatment groups. As expected, these indicators changed after processing (Figure 2). The antioxidant capacity of the calves was assessed. GQ-Zn significantly increased the level of CAT and decreased the serum MDA concentration (Figure 2A and 2B). In terms of immunity, GQ-Zn significantly increased the level of sIgA and decreased the serum levels of IL-2 and IFN-γ (Figure 3D, 3E and 3M). However, no significant differences in the other indicators were observed between the groups (Figure 3).

3.3. SCFAs produced by the gut microbiota

We analyzed the SCFAs in rectal contents of calves in different treatment groups by GC–MS (Figure 4). We observed that after GQ-Zn supplementation, different concentrations of zinc had different effects on calves, and high concentrations of GQ-Zn tended to reduce the concentration of propionate on Day 14 (Figure 4B), while low concentrations of GQ-Zn increased the concentration of valerate on Day 7 (Figure 4E).

3.4. GQ-Zn alters the gut microbiota composition of calves

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We further investigated the gut microbiota of the three different experimental groups by 16S rDNA sequencing. A Venn diagram was constructed to display the number of OTUs annotated from the different experimental groups: control, low concentration of GQ-Zn, and high concentration of GQ-Zn had 69, 13, and 9 unique OTUs, respectively (Figure 5A). We used alpha diversity and beta diversity to assess the diversity of the gut microbiota. Alpha-diversity-based analysis revealed little microbial difference between different groups (Figure 5C-F). According to the beta diversity determined via PCoA, there was little difference in the microbial structure between the different groups (Figure 5B). After conducting a histogram analysis of the annotated OTUs, an obvious difference in the taxonomic composition was found between the three experimental groups at the phylum (Figure 5G) and genus (Figure 5H) levels. Metastats analysis was used to evaluate the differences in microbiota composition between group Con, Low and High (Figure 6) in species level. 5 highest differential relative abundance bacteria was presented. Low concentrations of GQ-Zn decreased *Cenchrus americanus*, *Enterococcus faecium*, *Lentibacillus massiliensis*, *Prevotella denticola* and *unclassified Alphaproteobacteria*. High concentrations of GQ-Zn decreased *Agathobaculum butyriciproducens*, *Cenchrus_americanus*, *Fusobacterium necrophorum*, *Prevotella denticola* and *Roseburia inulinivorans.*

3.5. GQ-Zn alters the fecal metabolomic profile of diarrheic calves

To clarify the application potential of GQ-Zn, it is important to understand how the products of microorganisms affect the incidence of calf diarrhea. We analyzed the fecal metabolome of calves in different experimental groups. After feeding, the calves

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were treated with different doses of zinc. PCoA revealed that the data points from different experimental groups became clustered, and there was significant separation between groups (Figure 7A). Moreover, the amount of fecal metabolites also changed, showing an overall upward trend (Figure 7B). The relative abundances of various metabolites were identified and annotated in rectal content samples. Enrichment of these metabolites leads to upregulation of multiple metabolic pathways, and the most significant difference is in the digestion and absorption of vitamins. Hierarchical clustering analysis was used to cluster the metabolites in calf feces from different experimental groups. Heatmap analysis revealed that the fecal metabolite profile of calves fed GQ-Zn was different from that of the other groups (Figure 7C). Most of the metabolites tended to increase, but a small portion of the metabolites were downregulated (Figure 7D). Based on these findings, it seems likely that GQ-Zn has a major impact on fecal metabolites, which may be the cause of the reversal of diarrhea in calves. The majority of these metabolites play important roles in the metabolism of linoleic acid (Figure 7E-L).

4. Discussion

GQ-Zn formed via chelation of Gly-Gln and zinc. The results of the present study indicated that GQ-Zn supplementation had no effect on ADG during days 1-365 of the experiment. Although some experiments have shown that organic zinc can promote growth, there is still controversy over whether zinc improves growth performance [33]. The reason for these conflicting results is still unclear. It may be due to the different environments in which the animals are located, or it has an impact on long-term growth.

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In a preliminary laboratory study, zinc was found to play a role in promoting growth rather than alleviating diarrhea [34]. our result showed that GQ-Zn had no significant effect on calf performance.

In the present study, GQ-Zn significantly reduced the stool score in days 7-14 of the experiment. A high concentration of GQ-Zn significantly reduced the incidence of diarrhea, indicating that GQ-Zn helped to reduce the incidence and severity of diarrhea. Several studies have shown that zinc is a guardian of immune function. Zinc alleviates diarrhea and is associated with the immune system [35]. Animal diarrhea is accompanied by intestinal inflammation [36].

Inflammation occurs when the intestine is subjected to a series of complex and harmful stimuli (e.g., pathogens), and excessive inflammation is harmful to the body [37]. Zinc induces regulatory T cells to inhibit the production of IFN-γ [38]. IFN-γ is the main inflammatory cytokine secreted by Th1 cells and promotes the upregulation of multiple proinflammatory factors to activate macrophages [39,40]. IFN-γ inhibits the replication of *Listeria*, *Salmonella*, and *Mycobacterium tuberculosis* in cells by promoting the iron ion output of pathogen macrophages [29]. GQ-Zn decrease IFN-γ in serum possibly through the action of zinc in vivo. IL-2 has a variety of biological activities and is highly important for immune regulation. IL-2 binds to specific receptors on the surface of T cells, B cells and monocytes, causes the activation and proliferation of T cells, and enhances native killer cell activity; moreover, it is an inflammatory marker that helps to determine the occurrence of inflammation [41,42]. In addition, sIgA can interfere with early pathogen infections by blocking intestinal

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epithelial cell receptors [43]. sIgA acts as a competitive inhibitor to interrupt the binding of pathogens to the intestinal epithelium [44]. Therefore, the level of sIgA in the gut is essential for assessing gut mucosal immunity. [45]. After 14 days treatment of GQ-Zn, the decrease of IL-2 and the increase of sIgA reflected a reduction in intestinal inflammation.

Oxidative stress is a normal phenomenon and has dual effects. Free radicals can prevent microbial invasion, but they can also cause cell damage during inflammation [46,47]. Under normal circumstances, free radicals in the body are maintained at a low level; however, the concentration of free radicals abnormally increases when diseases occur. Oxidative stress has been observed in many animal infectious diseases [48]. CAT is a ubiquitous enzyme in various organisms [49], and it can eliminate excess ROS produced by organisms when exposed to external stimuli [50]. Intestinal epithelial cells are shielded by CAT, hence preventing the onset of intestinal inflammation [51]. MDA is a marker of oxidative stress, and its excessive production has certain genotoxicity [52]. Infection with *Escherichia coli* and rotavirus increases the levels of MDA [53]. Several studies have shown that feeding calves different concentrations of ZnO results in a linear decrease in the serum MDA concentration [54]. These findings suggest that inflammation and oxidative stress should be controlled within a certain range during the health of calves. The concentration of MDA decreased and CAT increased after GQ-Zn treatment reflecting a reduction of oxidative stress in calves.

The concentration of SCFAs in the rectal contents also changed. SCFAs are generally produced by gut microbiota and are nutrients for intestinal epithelial cells

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[55]. SCFAs are one of the mediators through which the gut microbiota can control inflammation [56]. In a previous study, general attention was given to the functions of acetic acid, propionic acid, and butyric acid; however, a recent study suggested that valeric acid plays a certain role in immunity [57]. The decrease in the propionic acid concentration may be due to the antibacterial effect of GQ-Zn, which reduced the relative abundance of some bacteria. It was reported that zinc reduces the concentration of propionate in the intestines of piglets [58]. In the present study, the reduction in propionate concentration may have been due to changes in intestinal ecology and microbial metabolism.

After birth, various of microbiota colonize the gastrointestinal tract in mammals. This complex microbiota plays an important role in the health of newborns [59]. GQ-Zn supplementation ameliorates diarrhea may through regulate gut microbiota in pre-weaning Holstein calves. In our study, some pathogenic bacteria, such as *Enterococcus faecium*, *Prevotella denticola*, *Fusobacterium necrophorum* were decreased after treatment. *Enterococcus faecium* is considered as a pathogenic bacteria causing adult cat diarrhea [60]. But research also found that *Enterococcus faecium* decreased inflammatory responses in goat. [61]. *Prevotella denticola* infection caused necrotizing fasciitis and enhances caries-associated virulence of plaque biofilms in some research [62,63]. *Fusobacterium necrophorum* is considered as pathogenic bacteria causing empyema, mastoiditis, septic shock [64-66].

Microbiota changes can be link to metabolites in pre-weaning calves [59]. we further hypothesized GQ-Zn changed the microbiota, then altered the metabolites

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leading to the alleviation of calf diarrhea. The levels of biotin, niacinamide, riboflavin, retinol, α-tocopherol, 9-oxoODE, 13S-HODE etc. were found to increase in after treating with GQ-Zn, indicating that changes in some micobiota led to the alter of metabolites. After retinol supplementation in diarrheal mice, the expression of tight junction proteins was upregulated, indicating that the intestinal mechanical barrier was improved. Retinol can decrease the mortality and incidence rate of infectious gastrointestinal diseases [67]. α-tocopherol is a kind of Vitamin E, Vitamin E has antioxidant functions and can improve intestinal bleeding and diarrhea [68]. Calves infected with viruses will experience a decrease in vitamin E levels [69]. Supplement chicks with vitamin E can protect them from *E. coli* infection [70]. 9-OxoODE is a metabolite of conjugated linoleic acid that can inhibit the secretion of inflammatory factors by macrophages through PPARγ [71]. We hypothesized that GQ-Zn alleviates calf diarrhea by altering the structure of microbiota and thereby altering metabolites. The impact of metabolites on diarrhea and the validation of selected metabolites should be further studied.

5. Conclusion

In conclusion, supplementation with GQ-Zn alleviated diarrhea in calves, improved fecal scores, and reduced oxidative stress, intestinal inflammation, and the relative abundance of microbiome in pre-weaning Holstein calves. GQ-Zn exhibited beneficial effects on the health of calves.

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Ethics statement

The animal study was approved by Animal Welfare and Ethics Committee of Huazhong Agricultural University.

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Competing interests

The authors declare that they have no competing interests.

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Table 1. Body weight and ADG of low and high concentration of supplementation with GQ-Zn in pre-weaned calves.

Item	Con[1]	Low	High	SEM	*P*-value
BW (kg)					
d0[2]	38.15	36.60	37.00	0.61	0.5794
d7	50.80	49.95	50.05	0.68	0.8650
d14	57.17	55.45	55.80	0.72	0.6151
d60	110.20	104.20	106.40	1.63	0.3213
d365	335.10	332.50	349.40	3.53	0.0935
ADG[3] (kg/d)					
d0-7	1.81	1.91	1.86	0.10	0.9186
d8-14	0.80	0.79	0.82	0.06	0.9709
d15-60	1.04	0.96	0.99	0.03	0.3935
d60-365	0.78	0.74	0.84	0.02	0.1362

[1]Con = supplementation with 50 mL purified water, Low = supplementation with 50 mL 80 mg/d GQ-Zn solution, High = supplementation with 50 mL 160 mg/d GQ-Zn solution.

[2]Days after supplementation with GQ-Zn.

[3]ADG = average daily gain.

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Figure captions

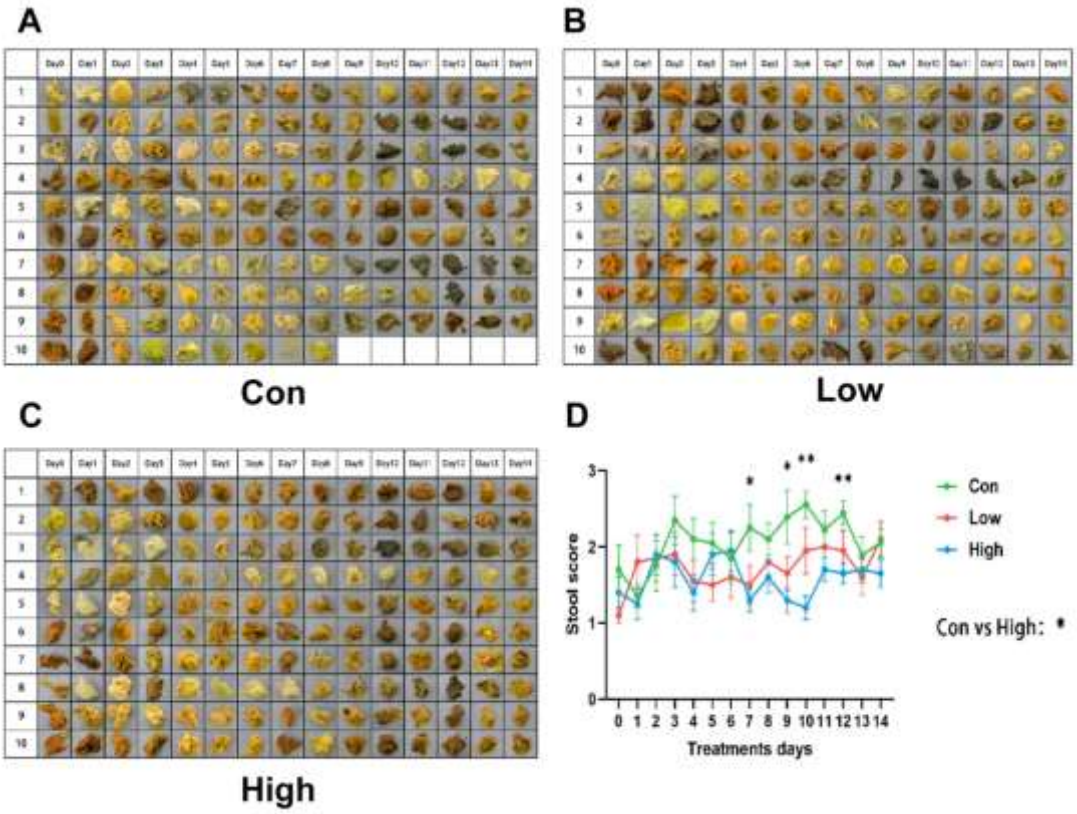

Figure 1. The effect of GQ-Zn on the incidence of diarrhea of pre-weaning Holstein calves. Images of the fecal samples collected from the recta of control (A). low (B) and high concentration of GQ-Zn (C). The incidence of diarrhea was represented by the score of fecal samples (D). The data were presented as the mean ± SEM and evaluated using One-way ANOVA (n = 10).

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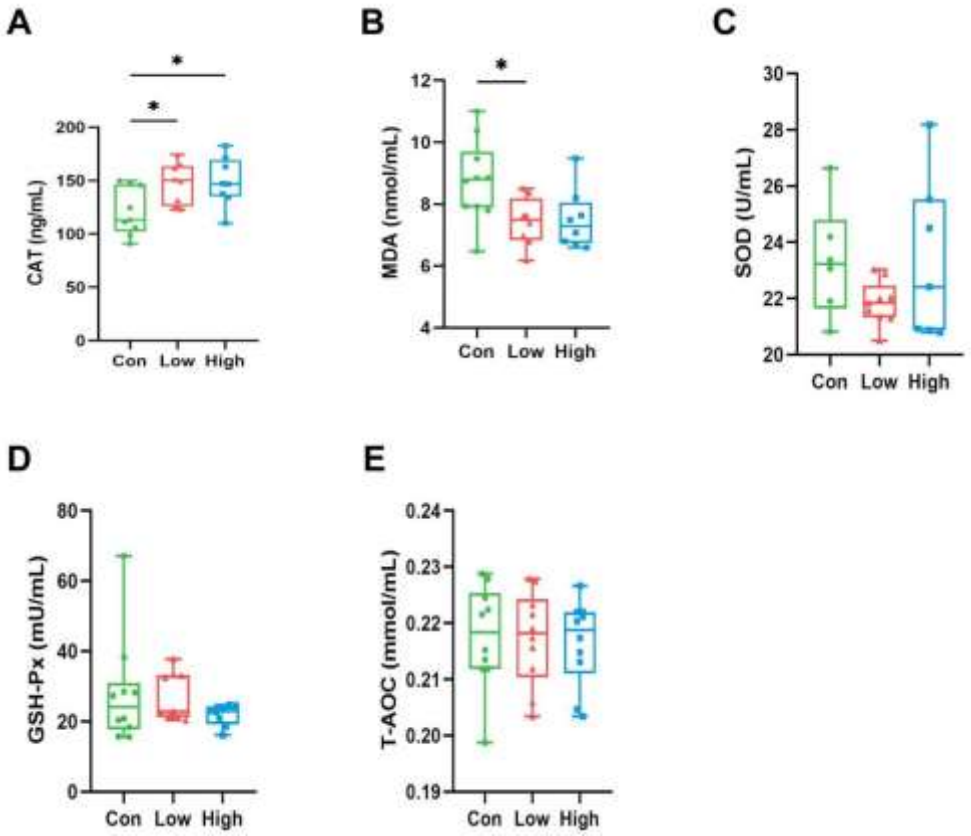

Figure 2. Effects of GQ-Zn on the level of antioxidant indicators in serum in pre-weaning Holstein calves. The concentration of catalase (CAT) (A), malondialdehyde (MDA) (B), superoxide Dismutase (SOD) (C), glutathione peroxidase (GSH-Px) (D), total antioxidant capacity (T-AOC) (E) in serum. The data were presented as the mean ± SEM and evaluated using One-way ANOVA (n = 10). *$P < 0.05$.

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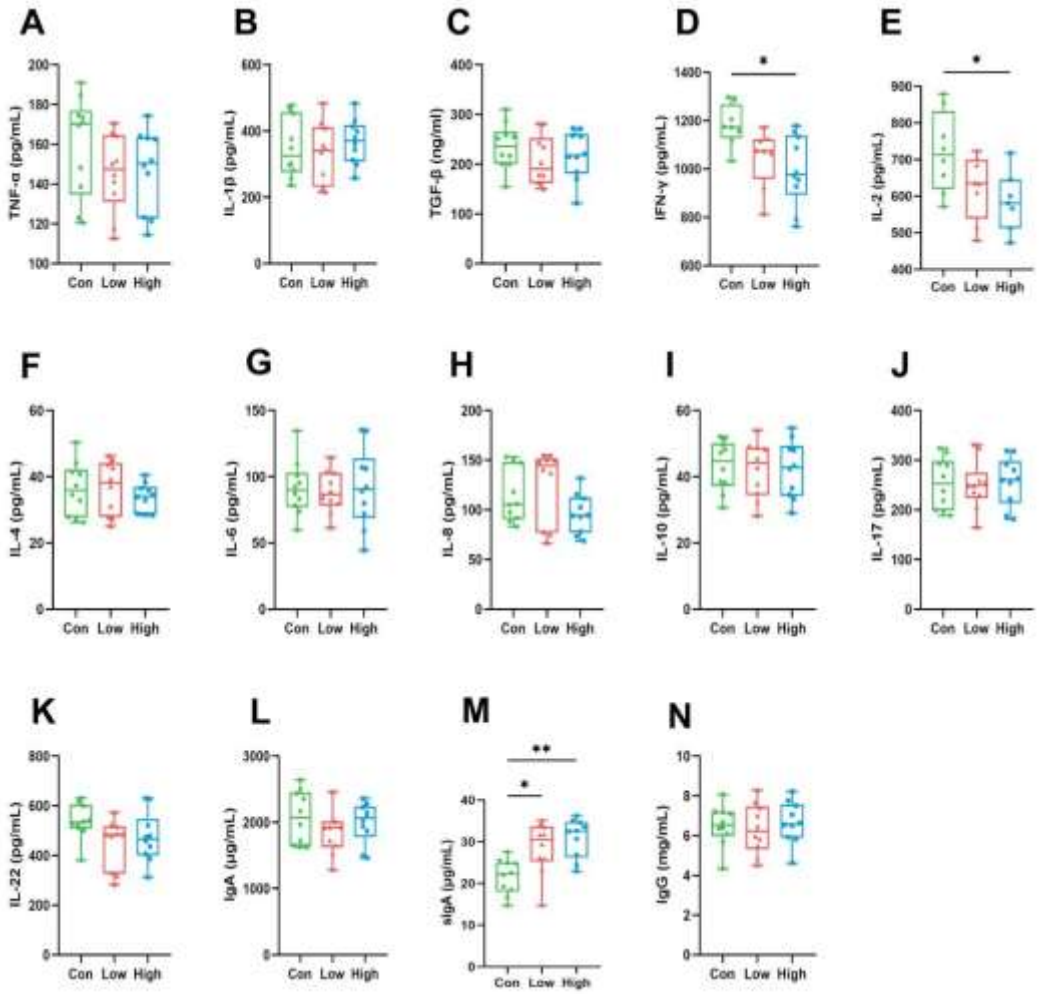

Figure 3. Effects of GQ-Zn on the level of immune indicators in serum in pre-weaning Holstein calves. The concentration of TNF-α (A), IL-1β (B), TGF-β (C), IFN-γ (D), IL-2 (E), IL-4 (F), IL-6 (G), IL-8 (H), IL-10 (I), IL-17 (J), IL-22 (K), IgA (L), IgG (N) in serum, and the concentration of sIgA (M) in feces. The data were presented as the mean ± SEM and evaluated using One-way ANOVA (n = 10). **P* < 0.05, ***P* < 0.01.

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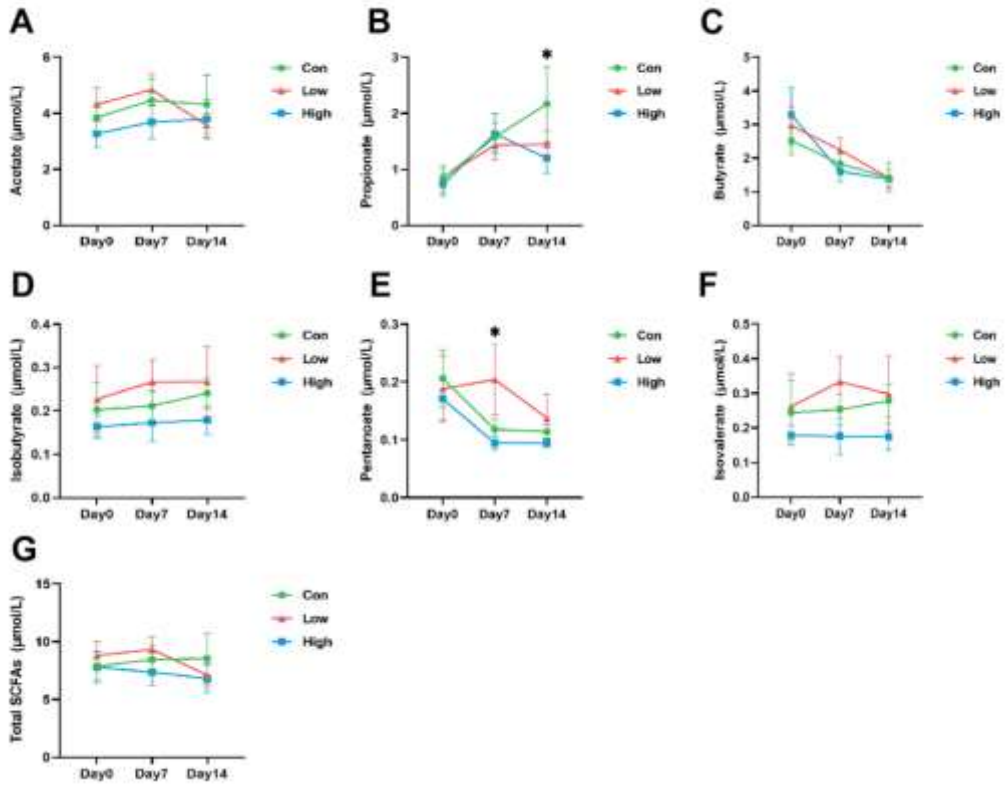

Figure 4. Effects of GQ-Zn on short-chain fatty acids in pre-weaning Holstein calves. Concentrations of acetate (**A**), propionate (**B**), butyrate (**C**), isobutyrate (**D**), pentanoate (**E**), isovalerate (**F**), and total short-chain fatty acids (SCFAs) (**G**) in the gut of calves. The data are presented as the mean ± SEM (n = 10) and evaluated using One-way ANOVA. *$P < 0.05$.

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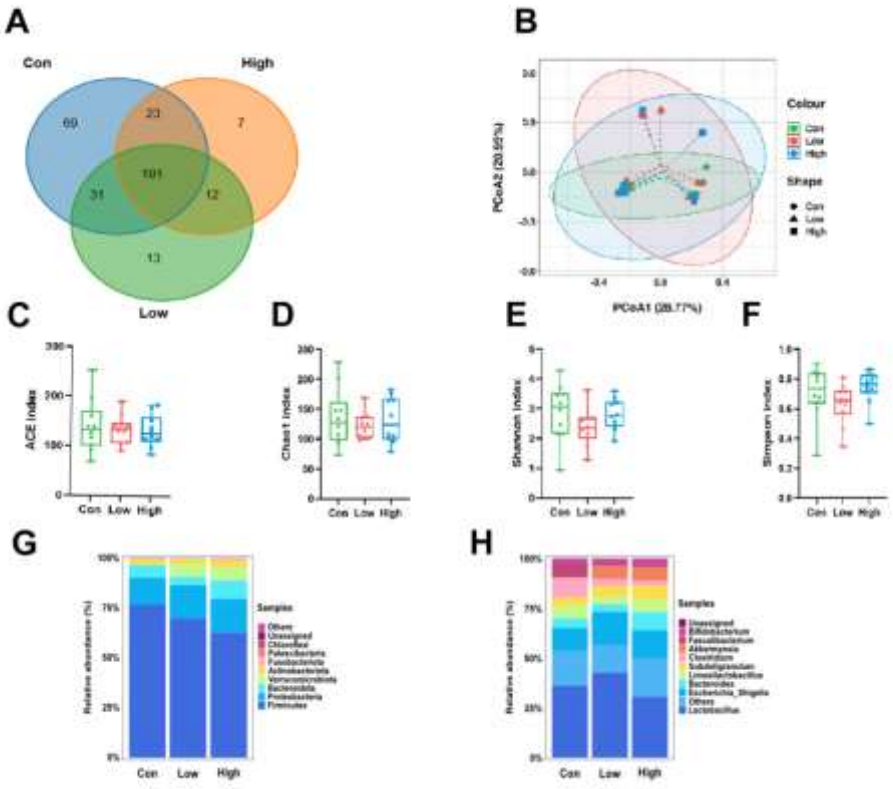

Figure 5. Effects of GQ-Zn on rectal contents microbiota in pre-weaning calves. Venn analysis based on OTUs (A). Beta-diversity visualized as principal coordinate analysis (PCoA) plots for group Con, Low and High (B). Effect of GQ-Zn supplementation on bacterial α-diversity indices: ACE (C), Chao1 (D), Shannon (E) and Simpson index (F). Histogram analysis of microbial taxonomic composition at phylum (G) and genus (H) levels. The data are presented as the mean ± SEM and evaluated using Metastats (n = 10).

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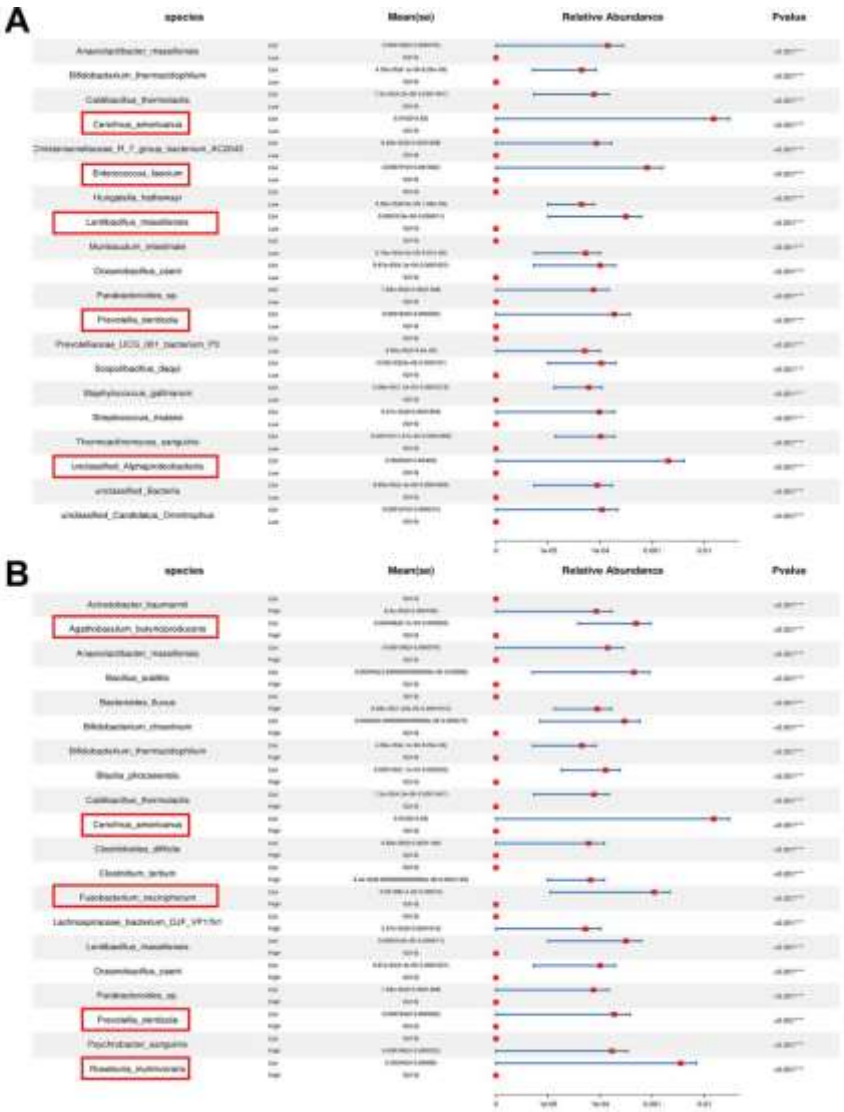

Figure 6. Metastats analysis was used to analyse the differences in microbiota abundance in feces in day 14 after treatment (n = 10), different species richness between Con and Low group (A), Con and High group (B). The red box indicates the top 5 relative abundances in Con and Low group (A) or Con and High group (B).

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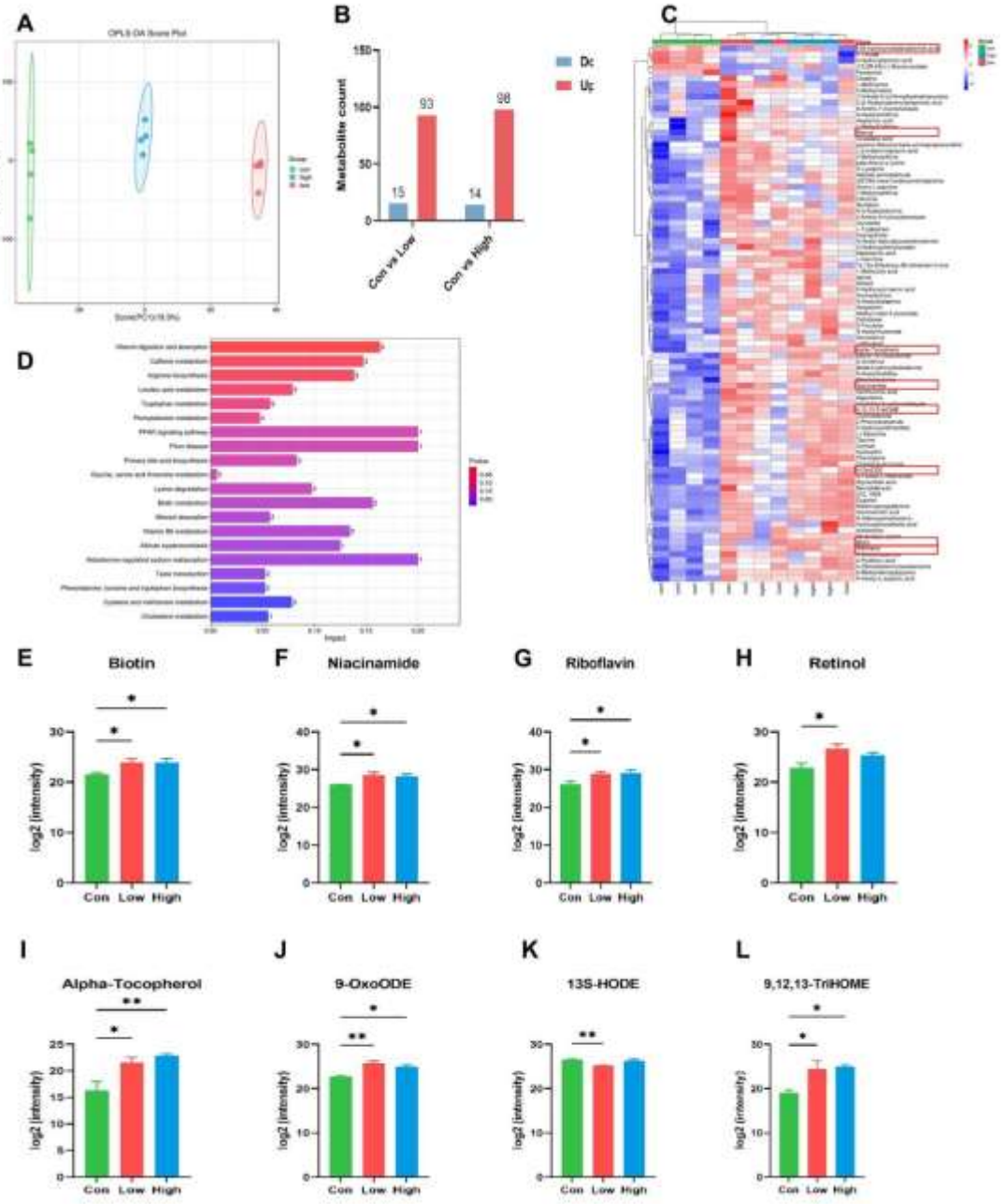

Figure 7. Metabolomics analysis of rectal contents. OPLS-DA Score Plot of metabolites (A). Statistic histograms of differently expressed metabolite (B). Metabolite sets enrichment overview (C). The enrichment analysis of KEGG pathways by differential metabolites of rectal contents (D). Metabolites that formed significant different metabolic pathways (E-L). The data are presented as the mean ± SEM and evaluated using One-way ANOVA; n = 10. **P* < 0.05, ***P* < 0.01.

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