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ORIGINAL RESEARCH

Muscone Ameliorates High-Altitude Hypoxia Gastrointestinal Stress via Modulation of Lactobacillus Murinus and Mitochondrial Metabolism

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Purpose: This study is to investigate the previously unexamined mechanism through which muscone alleviates acute high-altitude gastrointestinal stress. Specifically, it focuses on the modulation of gut microbiota, emphasizing its role in promoting the abundance of *Lactobacillus murinus* and optimizing mitochondrial metabolic pathways.

Patients and Methods: A high-altitude hypoxia model mouse treated with muscone was established to assess routine blood indices, inflammatory factors, and inflammatory cell counts. Additionally, alterations in intestinal flora were analyzed using macrogenomics. To further investigate the relationship between muscone's mechanism of action and intestinal flora, the ABX hypoxia mice model was employed, alongside in vitro experiments to evaluate muscone's effect on the growth of *Lactobacillus murinus*. The ameliorative effects of *Lactobacillus murinus* on acute gastrointestinal stress induced by high-altitude conditions were validated through various methods, such as HE staining, immunohistochemistry, transmission electron microscopy, ELISA, and flow cytometry. Furthermore, the underlying mechanism was explored through transcriptomics and qPCR.

Results: Muscone markedly alleviated hypoxia-induced inflammation improved hematological parameters, and reshaped gut microbiota composition, with a notable increase in the abundance of *Lactobacillus murinus*. In vitro, muscone directly stimulated the proliferation of *Lactobacillus murinus*. However, the protective effects of muscone were significantly diminished in ABX-treated mice, highlighting the critical role of the gut microbiota. Supplementation with *Lactobacillus murinus* alone effectively reduced serum inflammatory cytokines, alleviated intestinal oxidative stress, and restored mucosal integrity. Transcriptomic analysis and RT-qPCR findings suggest that these effects may be linked to the mitochondrial metabolic pathway.

Conclusion: Muscone may alleviate acute high-altitude gastrointestinal stress by enhancing the abundance of *Lactobacillus murinus* within the intestinal tract. Its mechanism of action appears to be associated with mitochondrial metabolic pathways.

Keywords: high-altitude hypoxia, oxidative stress, muscone, gut microbiota, lactobacillus murinus

Introduction

The growing global population exposed to high-altitude environments (>2500 m, currently exceeding 81.6 million)[1] faces unique physiological challenges stemming from distinct environmental stressors, including hypobaric hypoxia, extreme temperature fluctuations, and intense ultraviolet radiation.[2] Of particular clinical concern is the strong correlation between acute hypoxic exposure and gastrointestinal (GI) pathology. Notably, 80% of acute mountain sickness (AMS) cases are accompanied by significant GI symptoms, such as anorexia,

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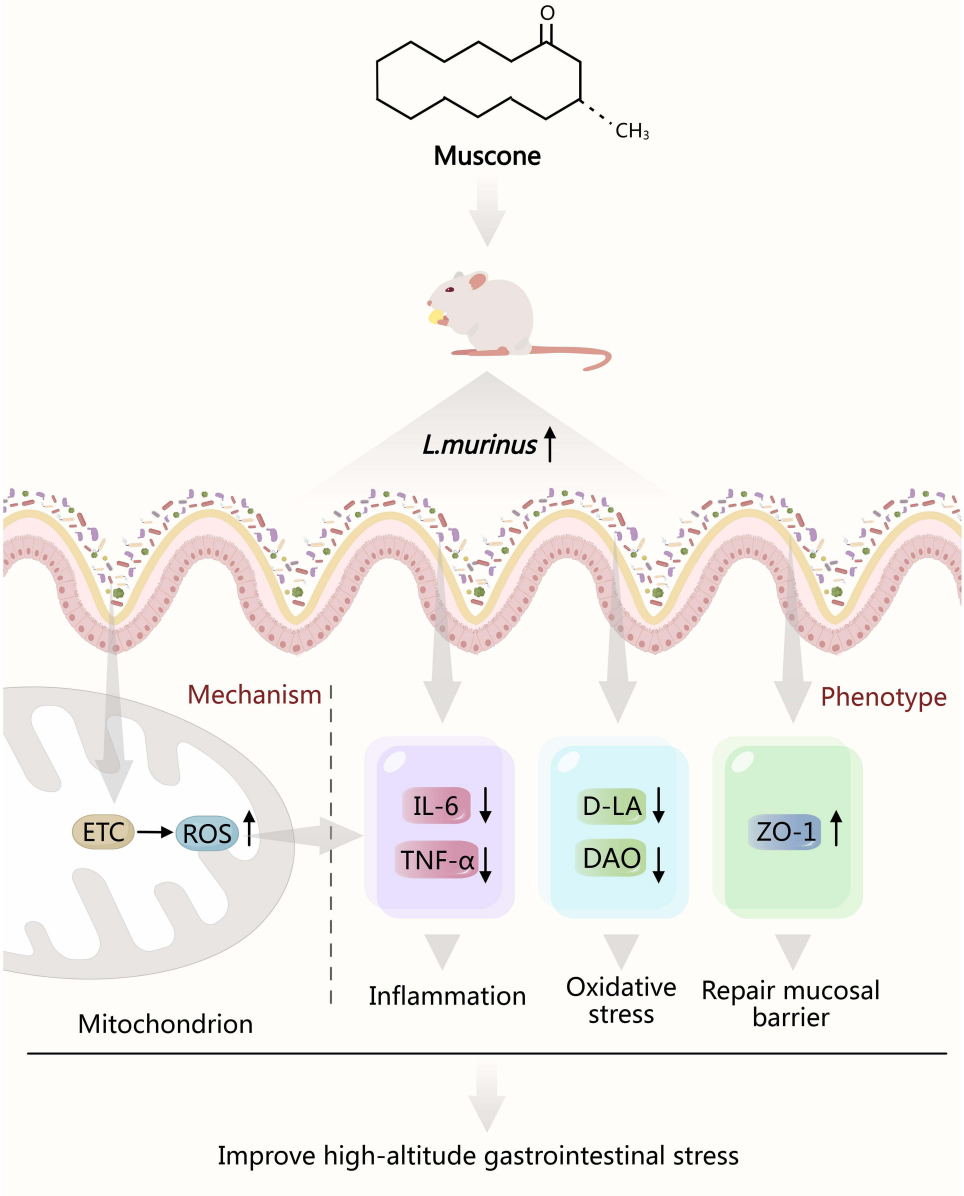
Graphical Abstract



nausea, vomiting, and diarrhea.[3] Endoscopic studies have demonstrated mucosal lesions in 61% of healthy individuals following acute high-altitude exposure.[4] Furthermore, there is an increased incidence of altitude-exacerbated peptic ulcer bleeding.[5] Despite these compelling clinical findings, the pathophysiological mechanisms driving high-altitude-induced gastrointestinal stress remain poorly understood, posing significant challenges to the development of targeted therapeutic interventions.

Increasing evidence indicates that acute exposure to high altitude disrupts intestinal flora, alters microbial diversity, compromises intestinal intestinal barrier function, triggers bacterial translocation, and amplifies intestinal inflammation through a cascade effect.[6–8] Studies have revealed that hypoxia promotes the overgrowth of anaerobic bacteria in the gut,[9] while reducing the population of aerobic bacteria in rats subjected to acute hypoxic exposure, coupled with an increase in specialized anaerobic bacteria.[10] Moreover, a recent study found

that administering an antibiotic cocktail significantly mitigates hypoxia-induced gastrointestinal injuries at high altitudes.[8] Together, these findings highlight gut microbiota alterations induced by hypoxia as a critical pathogenic factor in high-altitude gastrointestinal injury. Notably, emerging evidence continues to emphasize that acute hypoxic exposure uses profound gut microbiota disturbances. These disruptions are not only closely linked to altitude-related digestive disorders,[8,11] but may also have far-reaching systemic effects on cardiovascular[12] and neurological health.[13] Collectively, this growing body of research positions gut microbiota-targeted therapies as innovative and promising strategies for managing high-altitude gastrointestinal stress syndrome.

Although certain probiotic strains have shown efficacy in mitigating altitude-induced gastrointestinal stress in animal models,[11,14] the lack of clinical validation remains a significant limitation. In recent years, active monomers extracted from traditional Chinese medicines (TCMs) have garnered widespread attention as novel therapeutic agents due to their remarkable efficacy. Emerging evidence suggests that TCM compounds may act as potential prebiotics by selectively promoting the growth of probiotics.[15] Creating new research opportunities for exploring TCM-derived prebiotics as innovative treatments for acute high-altitude gastrointestinal stress responses. Musk, an aromatic substance derived from the secretions of the abdominal glands of male musk (Mus musculus), is a rare TCM ingredient extensively utilized in China for the treatment of various common and complex diseases. It is particularly esteemed for its ability to enhance blood circulation, reduce inflammation and relieve pain. On the Tibetan Plateau, musk is commonly employed to address gastrointestinal disorders. Muscone (3-methylcyclopentanol, $C_{16}H_{30}O$), the primary active ingredient in musk, has demonstrated broad applications in treating neurological, cardiovascular, chronic inflammatory conditions and cancer.[16–19] Nevertheless, the mechanisms underlying its gastrointestinal protective effects, particularly its interaction with gut microbiota remained largely unexplored until recent years. This gap in knowledge prompted us to hypothesize that muscone may function as a novel prebiotic agent, alleviating altitude-induced gastrointestinal stress through gut microbiota modulation.

Among potential probiotic candidates, *Lactobacillus murinus* has emerged as a particularly promising strain, displaying therapeutic effects in various gastrointestinal disorders,[20–23] pulmonary hypertension,[24,25] and metabolic diseases.[26] However, its role in altitude-associated gastrointestinal pathology remains entirely unexplored, highlighting a critical gap in current research.

This study investigates the link between muscone, gut microbiota, and high-altitude gastrointestinal stress, addressing a critical gap in understanding. Our research reveals that muscone modifies the composition of intestinal flora, enhances the abundance of *Lactobacillus murinus* and alleviates gastrointestinal stress is caused by acute high-altitude hypoxia in mice. Further exploration delves into the association between muscone, intestinal flora and *Lactobacillus murinus*. Additionally, the potential of Lactobacillus *murinus* as a probiotic to mitigate acute gastrointestinal stress at high-altitude was confirmed, offering novel insights into the application of natural compounds and probiotics in managing high-altitude gastrointestinal stress.

Materials and Methods

Materials

Muscone ($C_{16}H_{30}O$, Pubchem CID: 10947, purity≥97%) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China), Carboxymethylcellulose sodium (CMC-Na, CAS# 9004–32-4) was ordered from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

Experimental Animals

Six- to eight-week-old BALB/c male mice (20–25 g) were supplied by Jiangsu Huachuang Xinnuo Pharmaceutical Science and Technology Co. Ltd (License No.: SCXK; Su, 2020–0009). The mice were housed under standard conditions in a barrier environment animal room and the experiment commenced one week after the animals were acclimated to their surroundings. The study protocol followed the National Research Council's

Guide for the Care and Use of Laboratory Animals and was approved by the Qinghai University Experimental Animal Ethics Committee(Approval No.2022–43).

Bacterial Strain Culture

Lactobacillus murinus lyophilized powder (BeNa Culture Collection, Beijing, China) was dissolved with 0.5 mL of Man Rogosa Sharpe (MRS) medium (HKM, Guangdong, China), and then the bacterial solution was applied to the plate, and bacterial colonies appeared after about 24 hours. Individual colonies were picked into MRS medium and incubated at 37°C under anaerobic conditions, and the OD600 = 0.6–0.7 of the culture was measured until mid-logarithmic after 12–16 h of growth, at which time the colony count was 6.8×108 CFU/mL. Preparation of a frozen stock solution of *Lactobacillus murinus* (on the MRS medium containing 25% glycerol) and stored at - 80 °C for further experiments. Then, 50 μL of *Lactobacillus murinus* cryoproduct was added to 5 mL of MRS medium and incubated under aerobic conditions for 12 h at 37°C before being used for tube feeding method in mice.

Animal Experimental Design

Twenty-four mice were randomly assigned to three groups, with eight mice in each group: the normoxic control group (NC), the hypoxic control group (HC), and the hypoxic muscone group (HM). Muscone was prepared as a suspension by dissolving it in 0.5% sodium carboxymethylcellulose, and the HM group received a daily gavage of muscone at a dose of 2 mg/kg. Meanwhile, the NC and HC groups were administered an equivalent amount of sodium carboxymethylcellulose via gavage. The mice in the HC and HM groups were then housed in a hypobaric oxygen chamber at a simulated altitude of 6,000 meters for seven days (16°C; 55% humidity; PaO2: 10.2 kPa).

Twenty-four mice were randomly assigned to three groups, with each group consisting of eight mice: the antibiotic cocktail normoxic control group (A-NC), the antibiotic cocktail hypoxic control group (A-HC), and the antibiotic cocktail hypoxic muscone group (A-HM). Mice in the HC and HM groups were treated with broad-spectrum antibiotic cocktails (ABX) composed of vancomycin (0.5 mg/mL), ampicillin (1 mg/mL), metronidazole (1 mg/mL), and neomycin (1 mg/mL) at a dose of 0.2 mL once daily for one week. Following a two-day acclimatization period, mice in the HC and HM groups were placed in a low-pressure oxygen chamber simulating an altitude of 6,000 m for seven days (16 °C; 55% humidity; PaO2, 10.2 kPa). During this time, mice in the A-HM group received muscone via gavage at a dose of 2 mg/kg/day, whereas mice in the A-NC and A-HC groups were administered an equivalent volume of sterile water intragastrically each day.

Twenty-four mice were randomly assigned to three groups, with eight mice in each group: the normoxic control (NC) group, the hypoxic control (HC) group, and the hypoxic Lactobacillus murinus (HL) group. Mice in the HC and HL groups were housed in a low-pressure oxygen chamber, simulating an altitude of 6000 m, for seven days (16°C; 55% humidity; PaO2: 10.2 kPa). The NC and HC groups received daily gavage doses of 0.2 mL MRS medium, whereas the HL group was gavaged with 0.2 mL of Lactobacillus murinus at a concentration of 6.8×10^8 CFU/mL. The chamber's altitude was returned to sea level for 30 minutes each day to clean the cages, replenish food and water, and administer treatment. All animals had unrestricted access to food and water throughout the study.

Sample Collection

At the conclusion of the experiment, the mice were anesthetized via intraperitoneal injection of sodium pentobarbital (30 mg/kg). Blood samples were then collected from the ophthalmic venous plexus, and the animals were humanely euthanized through cervical dislocation. The collected blood was centrifuged at 3,500 revolutions per minute (RPM) for 15 minutes to separate serum samples, which were subsequently stored at −80°C. Additionally, a segment of the mouse ileal tissue was extracted, rinsed with PBS, and preserved in 4% paraformaldehyde solution, 2.5% glutaraldehyde solution, and liquid nitrogen for freezing. Fresh fecal samples were also collected from the mice and stored at −80°C.

In Vitro Bacterial Assay

Twelve tubes of *Lactobacillus murinus* culture medium were prepared by adding 150 μL of *Lactobacillus murinus* frozen stock solution to 15 mL of MRS medium and divided into four groups (three tubes in each group): the blank control group, the low-dose muscone group (muscone, 2μg/mL), the middle-dose muscone group (muscone, 5μg/mL), and the high-dose muscone group (muscone, 10μg/mL). mL). The incubation was carried out at 37 °C for 24 h. The OD values were measured every 2 h. The growth curves were plotted by taking the average of the OD values of each group at different time intervals.

Histopathologic and Immunohistochemical Examinations

Ileal tissues were fixed in 4% formaldehyde 4C for 24 h and embedded in paraffin. The tissues were serially cut into 4μm thick sections and stained with H&E. The gastric tissue was observed under the microscope. Meanwhile, the ileal tissue sections were taken into antigen repair solution (EDTA), placed in a microwave oven for 15 min for antigen repair, 3% H_2O_2 solution was added dropwise, and after washing, fetal bovine serum (BSA) blocking solution was added dropwise, after which ZO-1 antibody (Wuhan Servicebio Technology Co., Ltd, Wuhan, China) was added (1: 200) and incubated overnight. After washing, horseradish (HRP)-labeled goat anti-rabbit IgG (1:200) was added dropwise and incubated at room temperature for 50 min. Finally, the antibody and nuclei were stained with immunohistochemical DAB chromogenic kit and hematoxylin, respectively.

Transmission Electron Microscopy (TEM)

Ileal tissue was collected and fixed in 2.5% glutaraldehyde solution for 4 h (4°C), rinsed 4 times with 0.1 mol/L phosphate buffer for 15 min. 1% osmium solution was post-fixed for 2 h at 4°C, rinsed twice with 0.1 mol/L phosphate buffer for 5 min each acetone dehydration was carried out in gradients of 50%, 70%, 90%, and 100% for 15 min, then infiltrated with 100% acetone:resin=1:12, 100% acetone:resin=1:2 for 2 h, pure resin overnight, embedded, and oven polymerized (37°C for 12 h; 45°C for 12 h; 60°C for 48 h). Ultrathin sections with a thickness of 60–70 nm (section model: Ultracut R, Leica) were stained with uranyl acetate and lead nitrate and visualized by transmission electron microscopy. The morphology of the obtained biomaterials was studied using a JEM1200EX transmission electron microscope (JEOL, CO., Ltd, Japan).

Serum Biochemical Indicators

Serum samples were taken from mice and operated according to the kit instructions to determine the serum levels of IL-6 (Wuhan Servicebio Technology Co., Ltd, Wuhan, China), TNF-α (Wuhan Servicebio Technology Co., Ltd, Wuhan, China), IL-17A(Wuhan Servicebio Technology Co., Ltd, Wuhan, China), D-ALA (Elabscience Biotechnology Co., Ltd, Wuhan, China) and DAO activity (Elabscience Biotechnology Co., Ltd, Wuhan, China). LA levels (Elabscience Biotechnology Co., Ltd, Wuhan, China) and DAO activity (Elabscience Biotechnology Co., Ltd, Wuhan, China). And the blood routine indexes were detected by blood routine analyzer.

Flow Cytometry

Fresh mice spleens were harvested and processed to prepare single-cell suspensions. The cells were subsequently stained with anti-mouse CD3-APC, anti-mouse CD4-PE, anti-mouse INF-γ -FITC and anti-mouse IL-17- BV421 antibodies (all purchased from BioLegend Co., Ltd, Beijing, China). Stained samples were analyzed using a flow cytometer, and the data were processed with FlowJo software.

Macro-Genomics

Mice fecal samples were taken for on-line testing, and all raw macro-genome sequencing data were quality controlled by MOCAT2 software.[27] First, all raw sequencing reads were de-joined by Cutadapt software, and then SolexaQA package was used to filter out the reads with quality lower than 20 and length less than 30 bp,[28] and clean reads were obtained by quality control, and finally the filtered reads were compared with the host

genome using SOAPaligner to remove contaminated host reads, and high quality reads were obtained by quality control host genome to remove contaminated host reads and obtain high quality clean data.[29] After comparing the clean reads to their customized databases using HUMAnN to obtain the relative abundance at the metabolic pathway level from the gene families in the UniProt Reference Cluster and MetaCyc databases,[30] the annotation results of individual samples were then merged using the humann_regroup_table operation, and finally the KEGG data results of HUMAnN were selected.

Transcriptomics

Ileal tissues were weighed at 100 mg per sample, and total RNA was extracted using TRIzol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The extracted RNA was assessed for concentration, purity, and integrity. Subsequently, mRNA was fragmented, reverse transcribed, and amplified to construct a cDNA library. Sequencing procedures were outsourced to Jiangsu SanShu Biotechnology Co. Differential expression at both the transcript and gene levels was analyzed using DESeq2. The criteria for identifying differentially expressed genes were set as $|\log2(\text{fold change})| > 1$ and an FDR value < 0.05. Identified differentially expressed genes (DEGs) were then imported into the EggNOG-mapper software for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

RT-qPCR

Total RNA was extracted from the tissues following the protocol provided with the RNA extraction kit. The extracted RNA was subsequently reverse transcribed into cDNA using the reverse transcription kit as per the manufacturer's instructions. The resulting cDNA served as the template for PCR amplification, and real-time fluorescence quantitative PCR was performed to measure the transcript levels of target gene mRNAs in the tissue samples. The primers, designed by BGI Genomics Co., Ltd. (China), are detailed in Supplementary Table 1.[31]

Statistical Analysis

All analyses were conducted using GraphPad Prism software (version 9.51). The data are presented as the mean ± standard error of the mean (SEM). Before performing statistical analyses, assumptions of normality and homogeneity of variance were evaluated using the Shapiro–Wilk test and Levene's test, respectively. Unpaired t-tests were applied for comparisons between two groups, while one-way analysis of variance (ANOVA) was employed for comparisons among multiple groups. When ANOVA identified significant differences, post hoc multiple comparisons were carried out using Tukey's honestly significant difference (HSD) test. Alongside reporting p-values, effect sizes and 95% confidence intervals (CIs) were calculated to provide a more nuanced understanding of the observed effects.

Results

Effects of Muscone on Inflammation and Immune Response

The blood routine analysis revealed marked differences between the HC group and both the NC and HM groups (Figure 1). Compared to the HC group, the NC and HM groups displayed significantly lower levels of WBC and MCHC, alongside higher HCT, MCV, RDW, and RDW-SD. No other parameters showed significant differences. Similarly, serum inflammatory markers and immune cell profiles varied among the groups (Figure 2). The HC group exhibited elevated levels of IL-6, TNF-α, Th17+ T cells, and the $CD4^+/CD8^+$ ratio compared to the NC and HM groups. Conversely, levels of IL-17A, Th1 T cells, $CD3^+$, and $CD8^+$ cells were significantly reduced in the NC group relative to the HC group.

Impact of Muscone on Gut Microbiota

To investigate the mechanism by which muscone alleviates acute high-altitude gastrointestinal stress, we utilized metagenomics to examine the characteristics of gut flora in mice subjected to different treatments. Results from

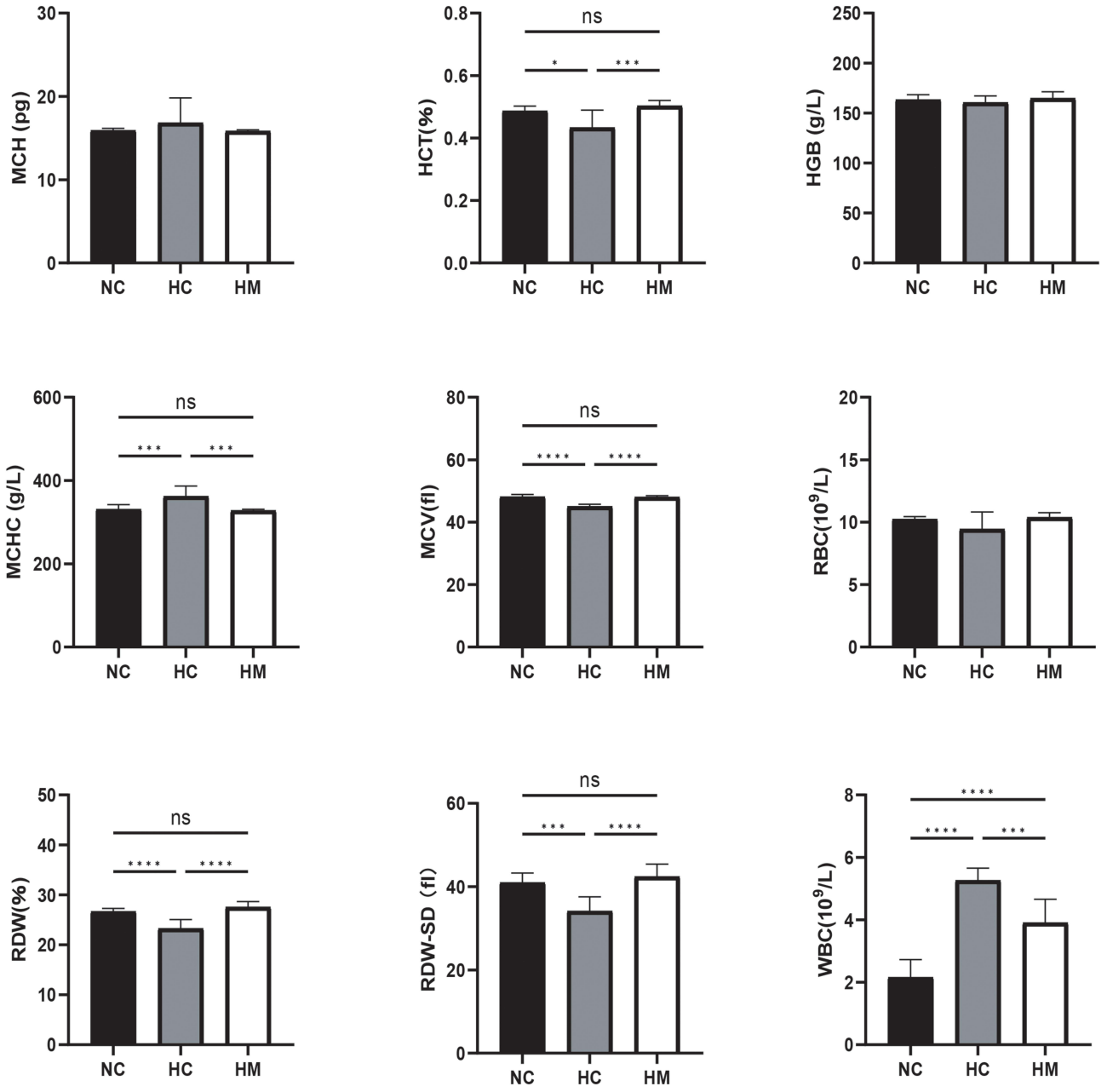


Figure 1 Blood routine examination results. Statistical significance is denoted as follows: ****$p < 0.001$, ***$p < 0.001$, *$p < 0.05$.
Abbreviation: ns, not significant.

the α-diversity analysis revealed that the Shannon index, Simpson index, Inverse Simpson index, and evenness index were higher in the NC and HM groups compared to the HC group. Additionally, species abundance was elevated in the NC and HC groups relative to the HM group (Figure 3A). PCoA (Principal Coordinates Analysis) demonstrated clear separation between the NC, HC, and HM groups (Figure 3B). Further analysis of gut flora at the phylum level showed an increased presence of Firmicutes in the NC and HM groups, whereas Bacteroidetes were more prevalent in the HC group (Figure 3C). At the species level, hypoxic conditions significantly reduced the abundance of *Lactobacillus murinus, Anaerotruncus sp. G3 2012*, and *Mucispirillum schaedleri*. Muscone treatment restored their abundance to levels observed under normoxic conditions. Notably, several studies suggest that *Lactobacillus murinus*, as a potential probiotic, can markedly improve intestinal inflammation (Figure 3D).

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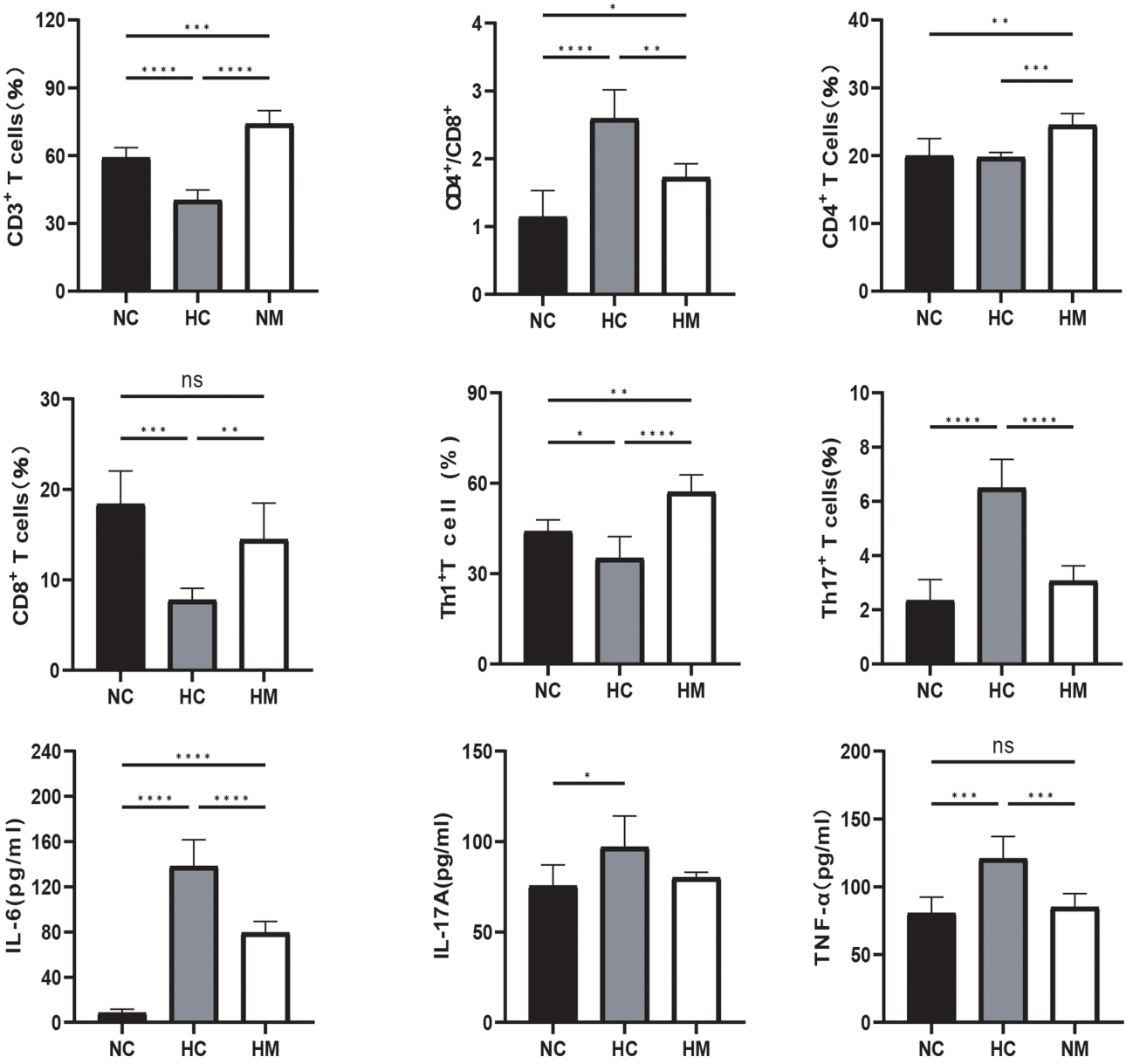

Figure 2 Inflammatory Factors and Immune Cells in Serum. Statistical significance is indicated as follows: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.
Abbreviation: ns, not significant.

Impact of Muscone in ABX Mice

To investigate whether muscone could alleviate high-altitude gastrointestinal stress via modulation of the gut microbiota in mice, we subjected ABX-treated mice to acute hypoxic exposure. Results indicated that, compared to the A-NC group, no significant improvement was observed in intestinal tissue pathological damage within the A-HC and A-HM groups. Moreover, mitochondrial swelling and the disappearance of cristae structures were evident. In terms of protein expression, ZO-1 levels were significantly higher in the A-NC group compared to the A-HC and A-HM groups (Figure 4). Additionally, an analysis of serum inflammatory factors and oxidative stress indicators revealed that IL-6, TNF-α, D-LA levels, and DAO activity in the A-NC group were significantly lower than those in the A-HC and A-HM groups ($P < 0.05$) (Figure 5). Furthermore, flow cytometric analysis (Figure 6) demonstrated that $CD4^+/CD8^+$ ratios were substantially higher in the A-HC and A-HM groups compared to the A-NC group, while the proportion of $CD8^+$ T cells in the A-NC group exceeded that observed

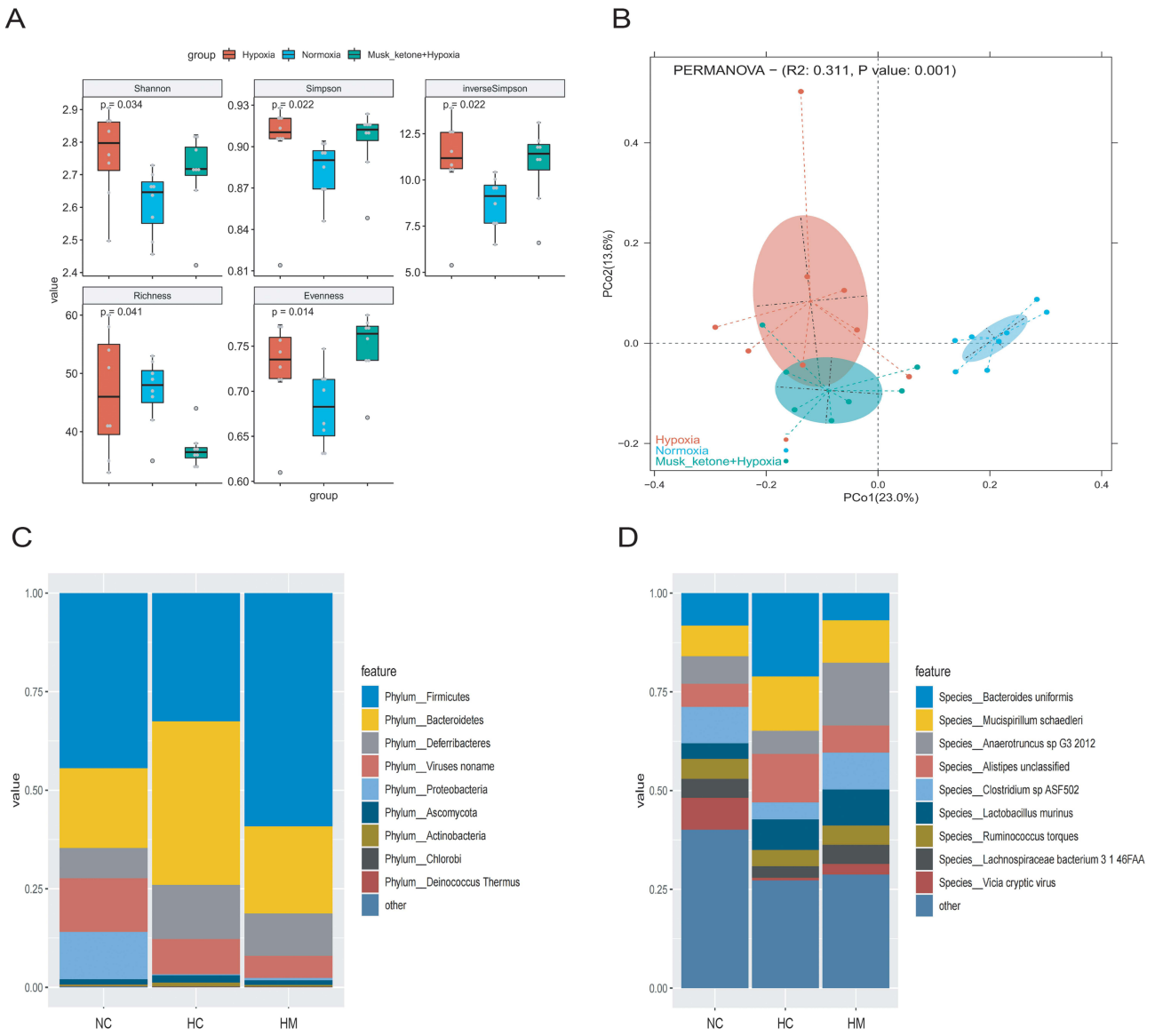

Figure 3 Analysis of Macrogenomics Results. (**A**) Analysis of α-diversity indices reveals higher Shannon, Simpson, inverse Simpson, and evenness values in the NC and HM groups compared to the HC group. (**B**) Principal Coordinates Analysis (PCoA) results show clear and significant separation among the NC, HC, and HM groups. (**C**) Differential species abundance analysis at the species level identifies notable changes in *Lactobacillus murinus, Anaerotruncus sp. G3 2012*, and *Mucispirillum schaedleri* across the NC, HC, and HM groups. (**D**) Differential phylum abundance analysis at the phylum level highlights variations in Firmicutes and Bacteroidetes among the NC, HC, and HM groups.

in the A-HC and A-HM groups ($P < 0.05$). In conclusion, the experimental findings suggest that the therapeutic effects of muscone on high-altitude gastrointestinal stress in ABX-treated mice are closely tied to alterations in the gut microbiota.

Lactobacillus Murinus In Vitro Bacterial Assay

Previous studies have demonstrated a notable increase in the abundance of *Lactobacillus murinus* in the intestines of mice after muscone administration under hypoxic conditions. To determine whether muscone directly affects bacterial growth, we conducted anaerobic in vitro cultures and examined the dose-dependent effects of muscone on *Lactobacillus murinus* proliferation.

Supplementation with 2 μg/mL and 5 μg/mL muscone significantly boosted the growth of *Lactobacillus murinus*, while 10 μg/mL exhibited mild inhibitory effects (Figure 7). This biphasic response indicates that

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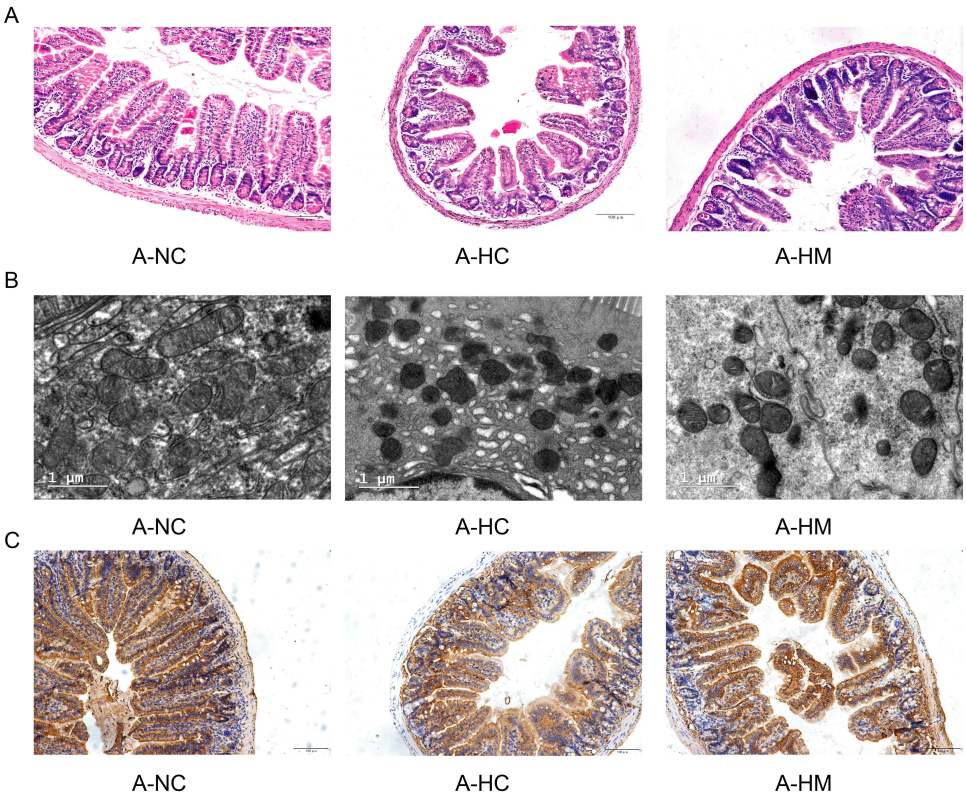

Figure 4 Pathological Results of Ileal Tissues in ABX Mice. (**A**) HE staining revealed that the intestinal villi and epithelial cells in the A-HC group were more sparsely arranged and disorganized compared to the A-NC group. Furthermore, no notable improvement was observed in the A-HM group compared to the A-HC group. (**B**) Transmission electron microscopy demonstrated signs of mitochondrial swelling and disappearance of the cristae structure in the A-HM group, similar to the changes detected in the A-HC group. (**C**) Immunohistochemical analysis indicated the expression of the tight junction protein ZO-1. ZO-1 appeared brown in staining, with darker coloration corresponding to higher protein expression levels. The A-NC group exhibited significantly greater expression of ZO-1 compared to both the A-HC and A-HM groups.

muscone functions as a growth modulator, with its beneficial effects being concentration dependent. The promotion observed at lower concentrations may be attributed to muscone's role in enhancing nutrient uptake, regulating bacterial metabolism, or interacting with membrane receptors to stimulate proliferation. In contrast, higher concentrations might inhibit growth by compromising cell membrane stability due to muscone's hydrophobic properties. Future research should delve deeper into the mechanisms behind these effects, including the potential involvement of signaling pathways, nutrient metabolism, and microbial interactions. A deeper understanding of these mechanisms could offer valuable insights into the development of muscone-based strategies for modulating gut microbiota and enhancing gastrointestinal health.

Role of 'Lactobacillus Murinus' in Mitochondrial Metabolism

H&E analysis revealed that the intestinal villi and epithelial cells in the NC and HL groups were more uniformly arranged and exhibited no noticeable abnormalities compared to the HC group. Immunohistochemistry results indicated that the protein expression of ZO-1 was significantly elevated in the NC and HL groups relative to the HC group. Furthermore, transmission electron microscopy was utilized to examine the intestinal mitochondria, revealing normal morphology and size in the HL and NC groups, whereas the HC group exhibited visibly swollen mitochondria with a loss of ridge structure (Figure 8). Serum enzyme-linked immunosorbent assay (ELISA) demonstrated significant increases in IL-6, TNF-α, DAO activity, and D-LA levels under hypoxia conditions ($P < 0.05$), which were markedly reversed by the administration of *Lactobacillus murinus* ($P < 0.05$) (Figure 9). Additionally, flow cytometry analysis showed that the $CD4^+/CD8^+$ ratio was considerably higher in the HC group compared to the NC and HL groups (Figure 10).

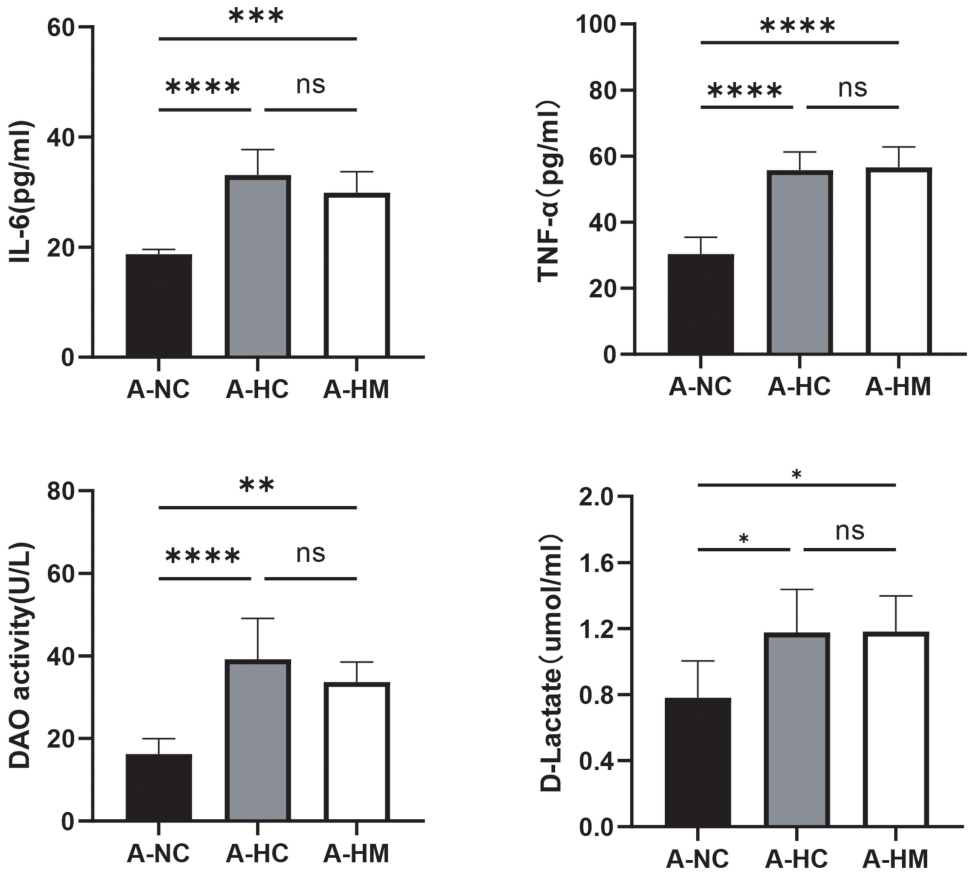

Figure 5 Serum ELISA analysis of ABX mice. Statistical significance is indicated as follows: ****$p < 0.001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.
Abbreviation: ns, not significant.

To explore the molecular mechanisms underlying the protective effects of *Lactobacillus murinus* against high-altitude gastrointestinal stress, we conducted RNA sequencing on ileal tissues from two groups of mice: the hypoxic control (HC) group and the hypoxic *Lactobacillus murinus* (HL) group. Our analysis identified 1,978 differentially expressed genes (DEGs), with 898 genes upregulated and 1,090 genes downregulated in the HL group compared to the HC group. KEGG enrichment analysis revealed that these DEGs were predominantly associated with pathways such as the reactive oxygen species (ROS) pathway and the receptor activation pathway (Figure 11). To validate the transcriptomic findings, we selected three DEGs—mt-Nd2, mt-Nd4, and mt-Nd5—involved in the ROS pathway, and performed RT-qPCR analysis. As shown in Figure 12, the RT-qPCR results confirmed that *Lactobacillus murinus* significantly downregulated the expression of mt-Nd2, mt-Nd4, and mt-Nd5. These results were consistent with the RNA sequencing data, further substantiating the role of *Lactobacillus murinus* in modulating mitochondrial metabolism. Taken together, this suggests that *Lactobacillus murinus* may alleviate high-altitude gastrointestinal stress by influencing the mitochondrial metabolic pathway.

Discussion

The rising number of high-altitude travelers has heightened awareness of acute gastrointestinal stress associated with such environments. Various dietary supplements and medications, including acetazolamide, non-steroidal anti-inflammatory drugs (NSAIDs), and dexamethasone, have been investigated for their potential to mitigate high-altitude gastrointestinal stress. However, research findings on these interventions remain inconsistent. For example, acetazolamide has been reported to inhibit carbonic anhydrase and reduce mucus secretion, potentially impairing gastrointestinal function.[32] Conversely, other studies have highlighted its protective effects against

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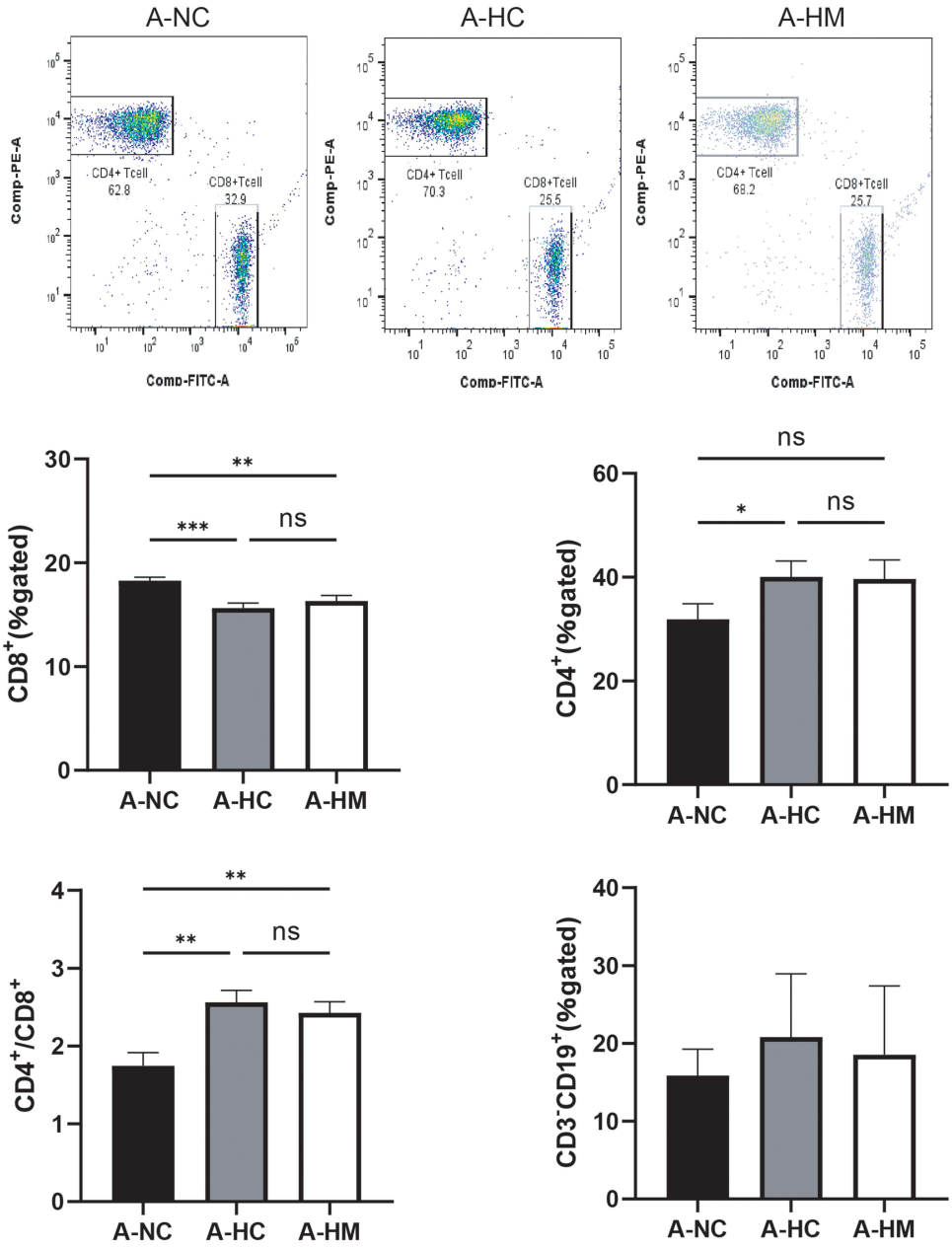

Figure 6 Flow cytometry analysis results for ABX mice. Statistical significance is indicated as follows: ***$p < 0.001$, **$p < 0.01$; *$p < 0.05$.
Abbreviation: ns, not significant.

gastric ulcers through the stimulation of prostaglandin synthesis.[33] These conflicting results may stem from differences in study designs, such as variations in participant susceptibility, the methods of acetazolamide administration and the dosage regimens employed. Moreover, the effects of such drugs on intestinal barrier integrity under hypoxic conditions remains poorly understood.[34] In addition, while glutamine, vitamin E, probiotics, and synbiotics have demonstrated promising results in alleviating high-altitude gastrointestinal stress in some studies, the interplay between these drugs and the gut microbiota remain inadequately explored.[35–37] Given the limitations and uncertainties surrounding conventional pharmacological treatments for high-altitude illnesses, our study aimed to evaluate the therapeutic potential of muscone, a bioactive natural compound, in addressing high-altitude gastrointestinal stress. Widely used in traditional medicine for its anti-inflammatory and immunomodulatory properties, muscone has garnered attention for its potential applications. Emerging evidence

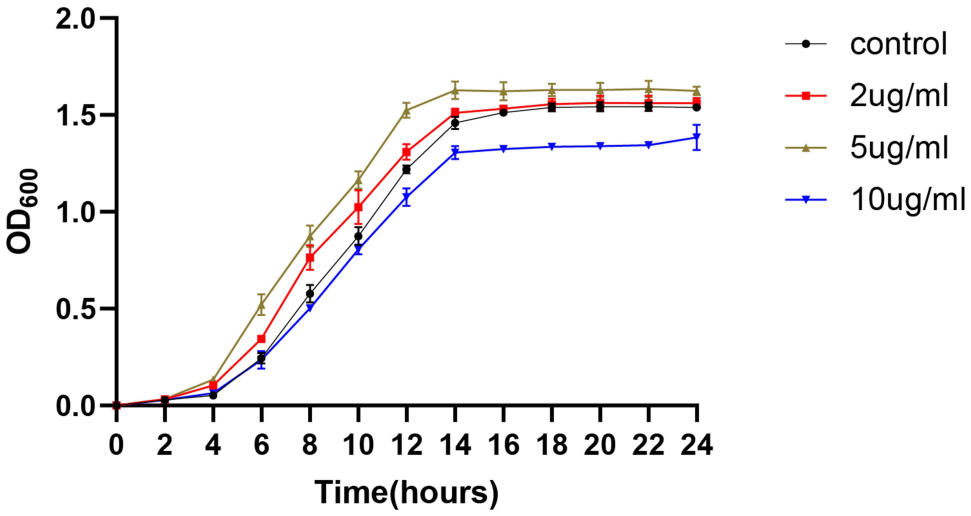

Figure 7 Growth curve of Lactobacillus murinus over 24 hours under muscone treatment.

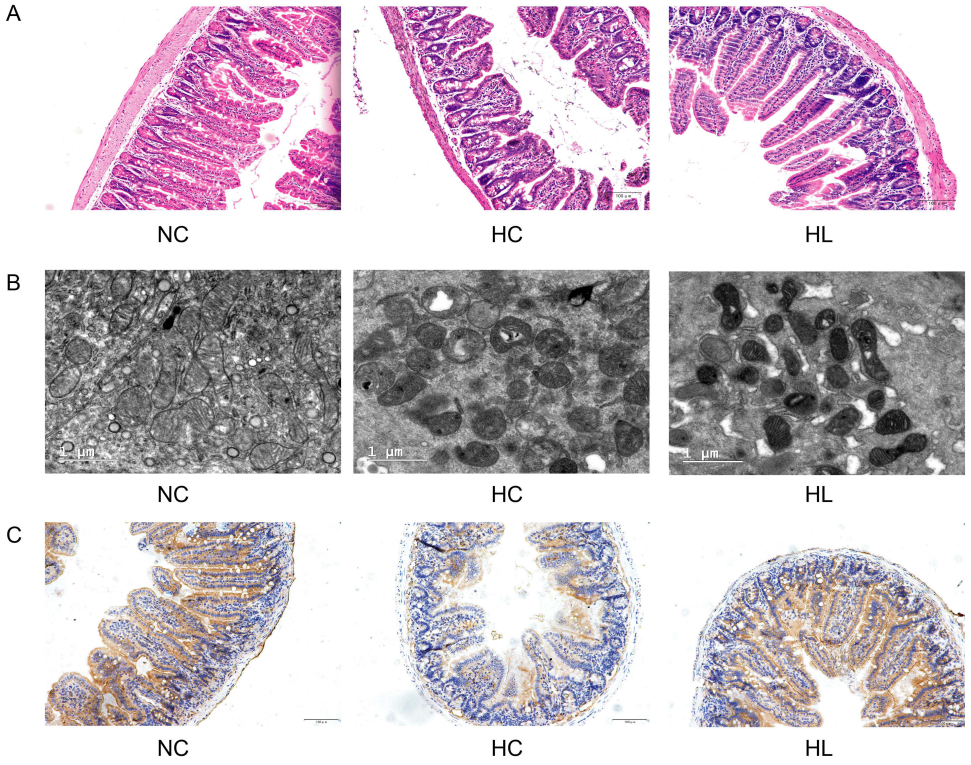

Figure 8 Pathological Analysis of Ileal Tissues. (**A**) HE Staining Results. In comparison to the HC group, the intestinal villi and epithelial cells in both the NC and HL groups demonstrated a more uniform arrangement, with no significant abnormalities observed. (**B**) Transmission Electron Microscopy Results. The morphology and size of the mitochondria in the NC and HL groups appeared normal. In contrast, the HC group exhibited noticeably swollen mitochondria accompanied by the loss of cristae structure. (**C**) Immunohistochemical Analysis of ZO-1 Expression. The expression of the tight junction protein ZO-1, indicated by brown staining, was more pronounced in areas with darker coloration.

further suggests that bioactive monomers derived from traditional Chinese medicine may contribute to gut microbiota homeostasis,[15] providing a rationale for investigating muscone's efficacy in mitigating gastrointestinal dysfunction under hypoxic conditions.

In this study, we demonstrated that hypoxia triggers an inflammatory response in mice and significantly impacts the composition and diversity of the gut microbiota. Muscone effectively alleviated high-altitude gastrointestinal stress by reducing TNF-α and IL-6 levels, aligning with previous findings on its anti-inflammatory properties in cardiac

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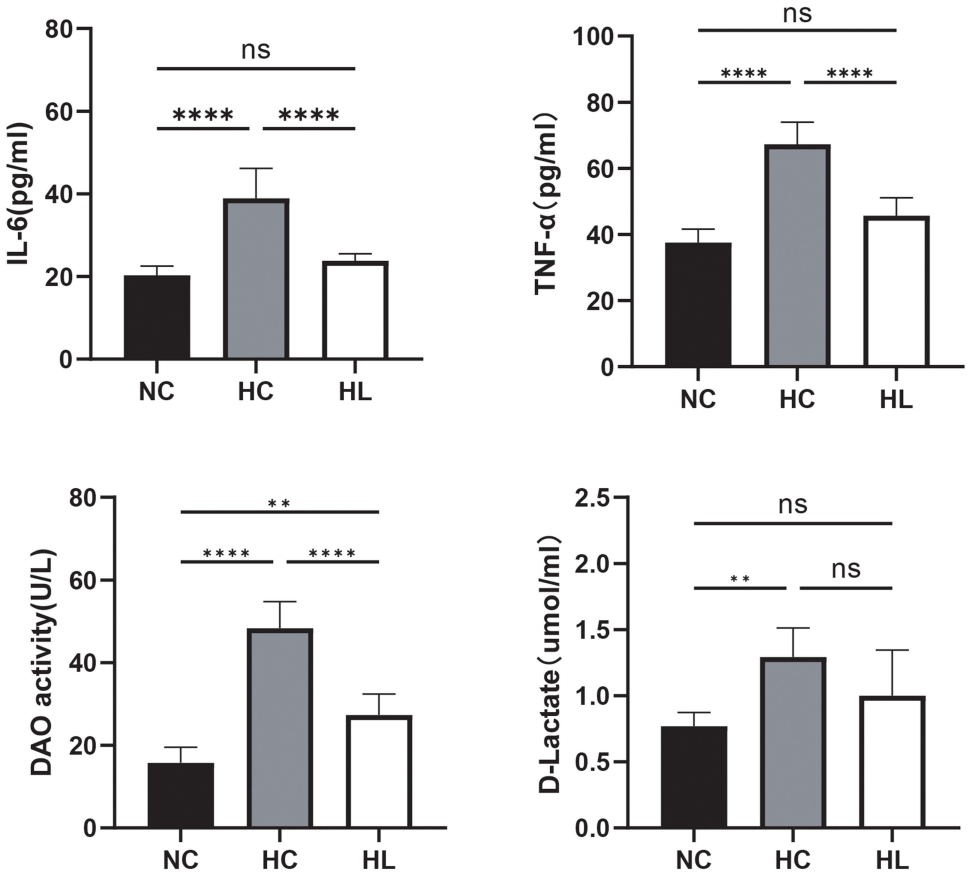

Figure 9 Serum ELISA Analysis. Statistical significance is represented as follows: ****$p < 0.001$, **$p < 0.01$.
Abbreviation: ns, not significant.

macrophages.[38] Additionally, muscone modulated immune responses by lowering TH17 cell counts and the $CD4^+/CD8^+$ ratio, while simultaneously improving blood parameters.

Macrogenomic analysis demonstrated that muscone reshaped the gut microbiota in hypoxic mice, by increasing the abundance of *Firmicutes*, reducing *Bacteroidetes*, and significantly enriching *Lactobacillus murinus* at the species level. To explore the role of gut microbiota in muscone's protective effects, we administered muscone to antibiotic-treated hypoxic mice. Interestingly, muscone did not alleviate hypoxia-induced intestinal tissue damage, mitochondrial dysfunction, alterations in ZO-1 expression, or changes in inflammatory markers (IL-6, TNF-α, D-LA, DAO), and immune cell proportions, indicating its effects are microbiota-dependent. In vitro, muscone was found to promote the growth of *Lactobacillus murinus*, highlighting its potential as a prebiotic.

Meanwhile, this probiotic strain demonstrated the ability to enhance intestinal barrier function and mitigate inflammation, as evidenced in various studies. For example, *Lactobacillus murinus* was found to alleviate deoxynivalenol-induced intestinal barrier disruption[21] and reduce intestinal ischemia-reperfusion injury by promoting IL-10 secretion from macrophages.[22] Additionally, it inhibited *enterogenic Candida albicans* infections in mice and safeguarded rats against necrotizing small bowel colitis by effectively colonizing the neonatal intestine.[20,23]

Based on these findings, we hypothesize that muscone alleviates high-altitude stress by promoting the enrichment of *Lactobacillus murinus*. Validation experiments supported this hypothesis, demonstrating that *Lactobacillus murinus* gavage improved intestinal histology, enhanced mitochondrial structure, and increased ZO-1 expression. Additionally, it reduced serum levels of IL-6, TNF-α, D-LA, and DAO activity, consistent with the results reported by Hu et al.[22] Moreover, the proportions of Immune cells were restored to normal levels.

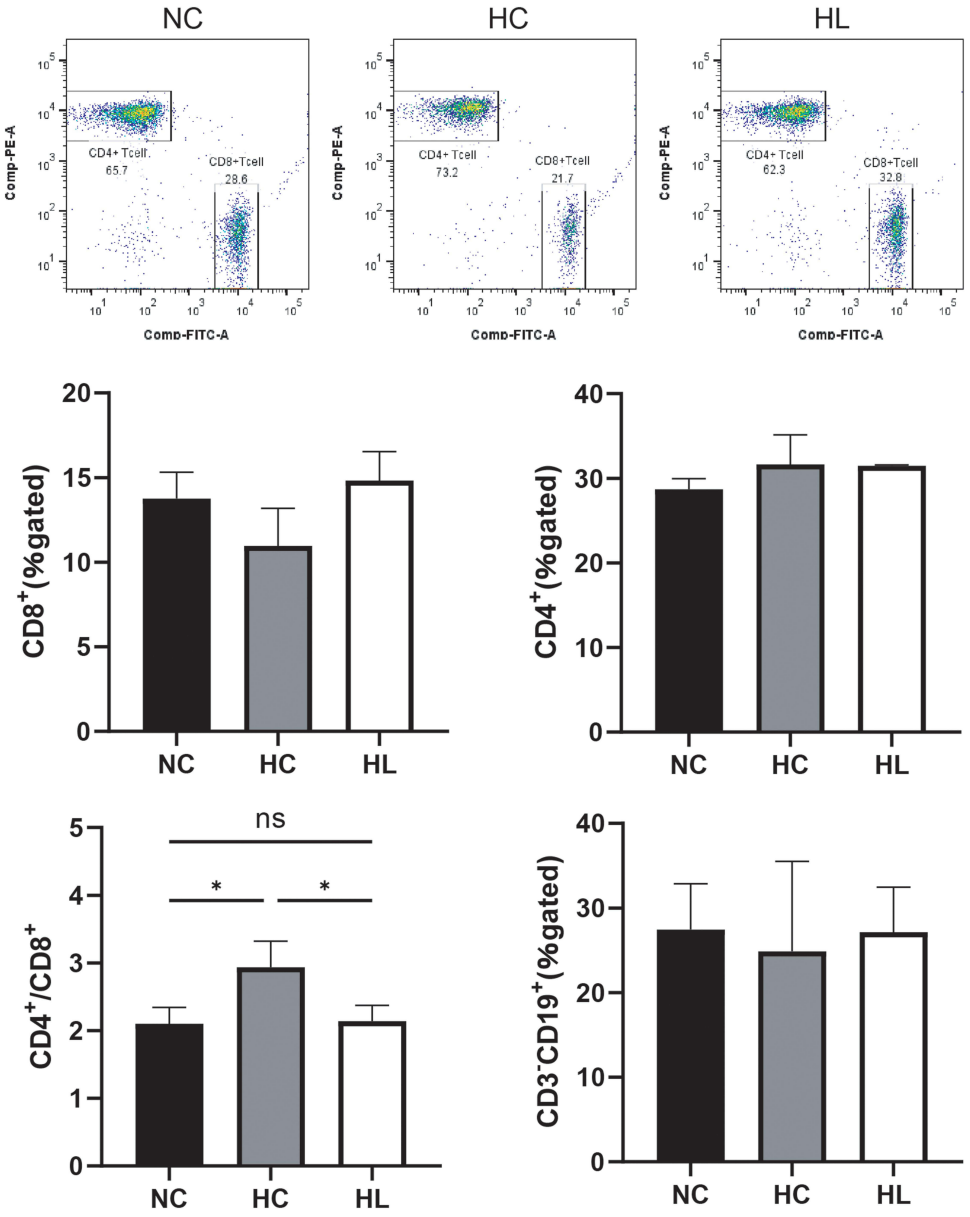

Figure 10 Results of flow cytometry analysis. Statistical significance is indicated as follows: *$p < 0.05$.
Abbreviation: ns, not significant.

Transcriptomic analysis revealed that the genes differentially expressed after *Lactobacillus murinus* treatment were primarily enriched in the reactive oxygen species (ROS) pathway. RT-qPCR experiments validated that the expression of mt-Nd2, mt-Nd4, and mt-Nd5, key genes in the ROS pathway, was significantly downregulated following *Lactobacillus murinus* treatment. These findings suggest that *Lactobacillus murinus* may alleviate oxidative stress by inhibiting the activation of essential enzymes in the ROS-generating pathway, thereby improving intestinal function under high-altitude hypoxic conditions. Furthermore, ROS imbalance can compromise lipid membranes, proteins, and DNA through processes such as peroxidation, enzymatic dysfunction, and structural damage.[39–41] Conversely, probiotics like *Lactobacillus murinus* may help restore redox balance, strengthen antioxidant defenses, and enhance barrier integrity, all critical for maintaining gut homeostasis.[42]

Our study offers fresh insights into the role of muscone in regulating gut microbiota to alleviate gastrointestinal stress caused by high-altitude environments. By identifying *Lactobacillus murinus* as a critical mediator, we underscore the

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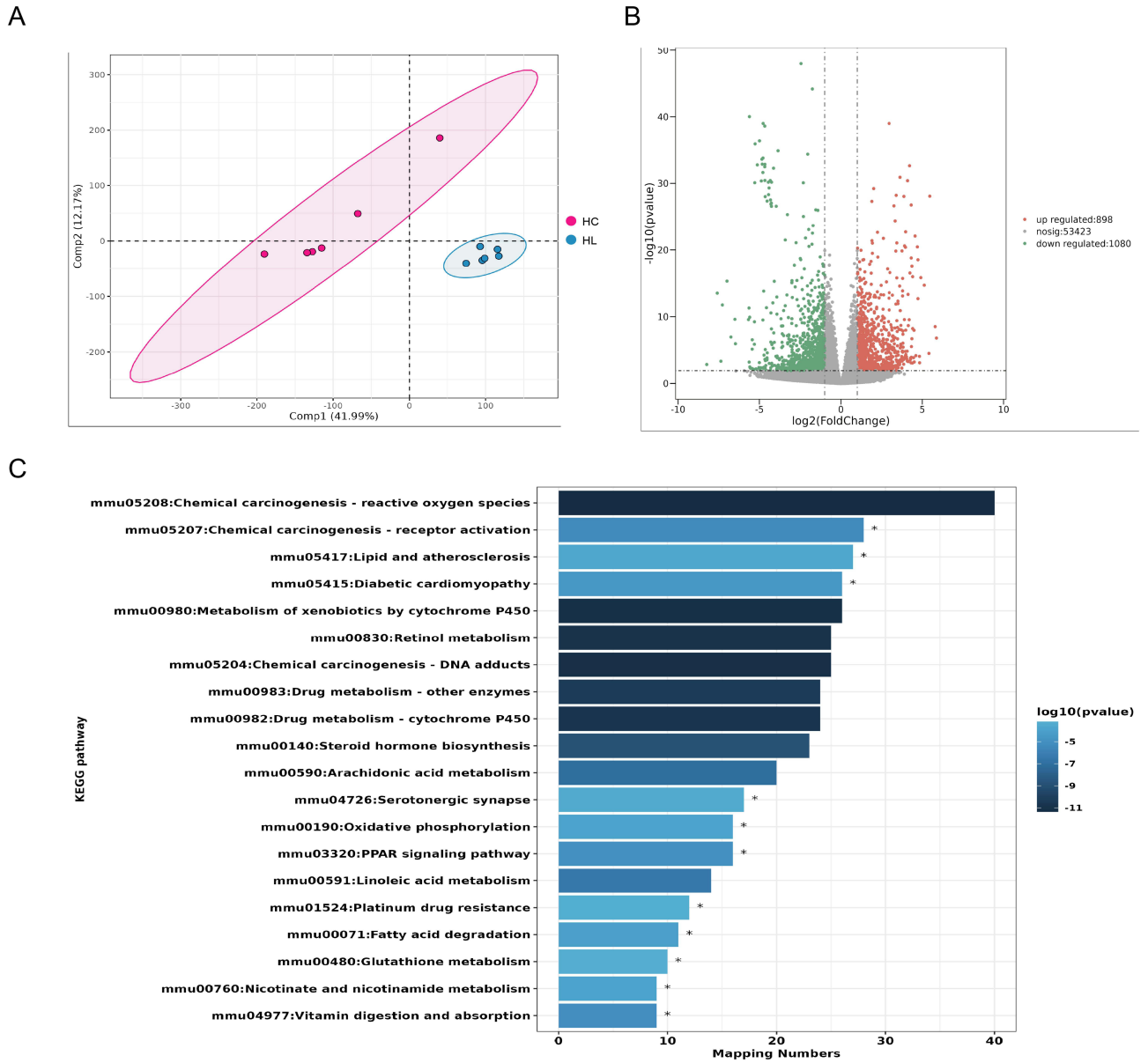

Figure 11 Results of Transcriptomics Analysis. (**A**) Principal Coordinate Analysis (PCoA) outcomes. (**B**) Differential Gene Volcano Plot. Each point in the figure represents a gene. The X-axis indicates the logarithmic value of the fold change in gene expression between the two samples. The Y-axis represents the negative logarithm of the p-value for the change in gene expression. The larger the absolute value of the X-axis, the greater the fold change in expression between the two samples. The higher the value of the Y-axis, the more significant the differential expression. Genes with significant differential expression are indicated by red and green points (red indicates upregulated expression, and green indicates downregulated expression), while genes without significant differential expression are represented by gray points. (**C**) KEGG Enrichment Analysis. The Y-axis represents KEGG pathways, while the X-axis indicates the number of differentially expressed genes enriched in each pathway. The color represents the significance of enrichment, as indicated by the p-value. **p.adjust < 0.05*.

potential of targeting gut microbiota as a therapeutic avenue. These findings hold significant promise for the development of innovative strategies to mitigate high-altitude gastrointestinal stress and its associated conditions.

Despite our findings, several limitations must be acknowledged. First, the discrepancies between animal models and human high-altitude gastrointestinal stress may impact the applicability of our results. Second, the specific molecular targets of muscone remains inadequately understood. Future research should prioritize verifying the causal relationship between changes in gut microbiota and the observed effects, potentially through fecal microbiota transplantation experiments. Furthermore, additional studies are essential to uncover the precise molecular mechanisms through which muscone influences gut microbiota and enhances intestinal barrier function.

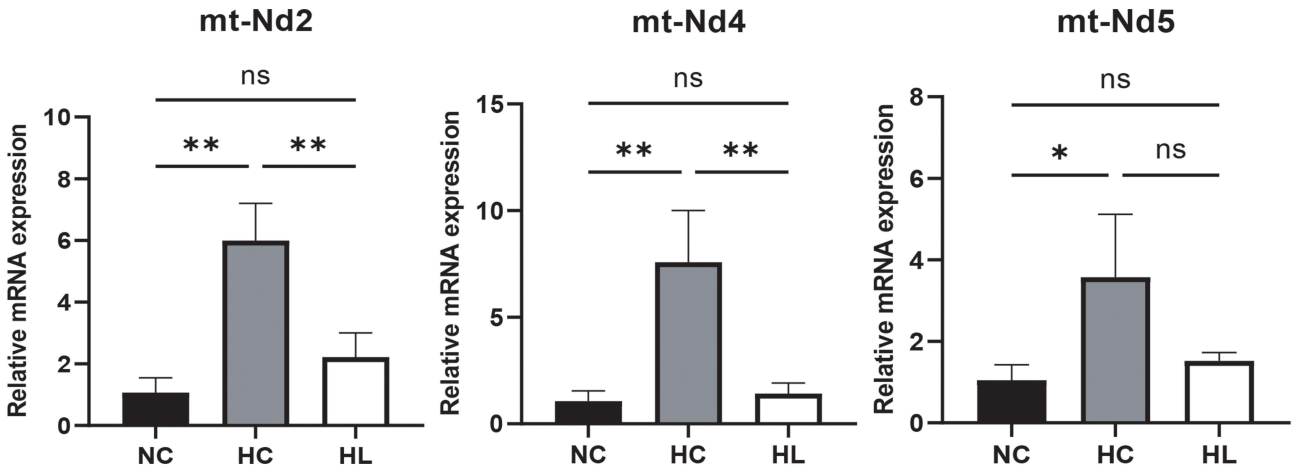

Figure 12 Results of RT-qPCR. Statistical significance is indicated as follows: $^{**}p < 0.01$; $^{*}p < 0.05$.
Abbreviation: ns, not significant.

Conclusion

Muscone can improve acute gastrointestinal stress in high-altitude, reduce inflammatory response, regulate disordered intestinal flora and increase the abundance of *Lactobacillus murinus*. And its mechanism of action is closely related to the intestinal flora, and *Lactobacillus murinus* as the key flora of muscone, muscone can promote the growth of *Lactobacillus murinus* in vitro and in vivo. And *Lactobacillus murinus* could ameliorate acute gastrointestinal stress in high-altitude through the reactive oxygen pathway. In conclusion, muscone may improve high-altitude acute gastrointestinal stress by increasing the abundance of *Lactobacillus murinus* in the intestinal flora, thereby providing a novel therapeutic strategy involving natural compounds and probiotics for mitigating acute gastrointestinal stress at high altitudes.

Abbreviations

TCM, traditional Chinese medicine; NC, normoxic control group; HC, hypoxic control group; HM, hypoxic muscone group; A-NC, antibiotic cocktail normoxic control group; A-HC, antibiotic cocktail hypoxic control group; A-HM, antibiotic cocktail hypoxic muscone group; HL, hypoxic Lactobacillus murinus group; Lactobacillus murinus, L.murinus; ABX, antibiotic cocktail.

Data Sharing Statement

The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

Ethics Approval and Consent to Participate

All animal experiments in this study received approval from the Institutional Ethics Committee of Qinghai University (No;2022-43).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors assert that they possess no recognizable conflicting monetary concerns or individual connections that may have seemed to impact the research detailed in this paper.

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