

Effects of Low Temperature and Wind Treatment on Physiological Indexes, Rumen Microbiota, Immune Responses and Hormones in Sheep

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Research

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Abstract

Background: Low-temperature environments can strongly affect the normal growth and health of livestock. Previous studies have shown that cold exposure can alter the intestinal microbiota and thereby affect other traits. In winter, cold weather can be accompanied by strong winds that aggravate the effects of cold on livestock. In this study, an experiment was conducted to investigate the effect of low temperature and wind speed on physiological indexes, rumen microbiota, and immune responses in sheep.

Methods: The sheep were divided into control group and test group according to their ambient temperature. Sheep in the test group were divided into four groups according to wind-speed treatment: no wind (average wind velocity less than 0.5 m/s), low wind velocity (average wind velocity of 3 m/s), medium wind velocity (average wind velocity of 4 m/s) and high wind velocity (average wind velocity of 5 m/s).

Results: Average daily gain and the utilization of forage, especially soluble fiber, decreased with increasing wind velocity in cold temperature ($P<0.05$). In rumen, the enzyme activity of cellulose degradation was also lower with increasing wind velocity ($P<0.05$). The abundance of potentially beneficial bacteria showed differences among the wind treatments ($P<0.05$). The large fluctuations in the amount of bacteria provided a breeding opportunity for potentially harmful bacteria ($P<0.05$). In addition, there were significant decreases in the serum levels of IL-2 and IFN- γ ($P<0.05$) and a large increase in IL-4 level ($P<0.05$), which indicated that the sheep underwent immune suppression during the trial. The significant increase in the activities of the antioxidant enzymes SOD, GSH-PX, and CAT ($P<0.05$) indicated that the production of oxygen free radicals was increased.

Conclusions: The cold environment significantly reduced the growth of sheep and altered the composition of rumen microbiota, reducing the utilization of soluble fiber by the rumen flora. Furthermore, the sheep produced large amounts of enzymes to resist tissue damage and experienced immune suppression in the cold environment.

Background

Climate change can cause stress responses in animals[1], especially sharp decreases in temperature. In cold environments, the weight of livestock tends to be reduced. It has been found that cold exposure alters the composition of the intestinal microbiota by affecting food intake[2, 3], which could cause other changes in animal phenotype.

Studies in many monogastric animal models have investigated correlations between the intestinal microbiota and host phenotype[4]. In ruminants, the rumen is a large fermentation site containing microbes. Similar to the intestinal microbiota, the rumen microbiota could interact with the host[5]. Furthermore, the host could limit the abundance and community composition of rumen microbes to maintain homeostasis[6]. In turn, the rumen microbiota composition could affect inflammation and

oxidation, which can be measured by inflammatory-related markers, such as interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), and interferon- γ (IFN- γ), and markers of oxidative stress, such as malondialdehyde (MDA) and various antioxidant enzymes, respectively. The rumen epithelium could produce hormones that influence phenotype, such as peptide-1, peptide YY, ghrelin, and leptin[7, 8]. Changes in the rumen microbiota could affect metabolites and the energy supply to tissues by producing volatile fatty acids (VFAs) by fermenting carbohydrates and inflammatory reaction[7, 9, 10]. Cold exposure could lead to changes in the intestinal microbiota in monogastric animals and affect host oxidation and inflammation[11]. Many studies have shown that cold exposure could alter the intestinal microbiota. However, the effects of a cold environment on the rumen microbiota are unclear.

High-altitude areas in the Northern Hemisphere experience low temperatures in winter, which reduce the productivity and feed-utilization efficiency of livestock and severely constrain the economic benefits of animal husbandry[12]. In northern China, the cold winter temperatures are always accompanied by strong northwest winds. Wind and low temperature aggravate the convection and heat dissipation of the body, imposing cold stress on livestock. In this experiment, we varied wind speed to investigate how cold exposure affects rumen microbiota and metabolism in sheep. We hypothesized that a cold environment affects the rumen microbiota and that changes to their metabolites affect host phenotype in ruminants.

Material And Methods

Ethical approval

All the animal procedures were carried out in accordance with the guidelines of the China Council on Animal Care and the Ministry of Agriculture of the People's Republic of China. The use of animals and all experimental protocols (protocol number 100403) were authorized by the Institutional Animal Care and Use Committee of Northwest A&F University (Yangling, Shaanxi, China).

Experimental animals and design

In this experiment, the test site and the corresponding test materials were provided by Linze Experimental Station of Lanzhou University, China. Twelve healthy 6-month-old crossbred hybrid ewes (small-tailed Han sheep \times Hu sheep; mean body weight (BW) $30.36 \pm SE 1.68$ kg; not pregnant before the experiment) were randomly allocated into three groups: I, II and III (four sheep per group).

The experiment spanned 20 days, comprising 10 days for adaptation and 10 days for the trial period. The sheep were divided into a control group and treatment groups. And the experimental condition other than wind sheep were the same across the treatment groups (including feeding management, test time, time of use of metabolic cages, etc.). According to records of annual average wind speed in the Atlas of Natural Disaster Systems in China, the annual average wind speed in the study area is approximately 3~4 m/s; this range was used to set the wind-speed gradient. Increasing wind speed increases thermal convection, exacerbating the effect of cold stress on sheep. The sheep in the treatment groups were subjected to one of four wind-speed treatments: no wind (average wind speed or less than 0.5 m/s, low temperature (LT)),

low wind (average wind speed of 3 m/s, LW), medium wind (average wind speed of 4 m/s, moderate wind (MW)) and high wind (average wind speed of 5 m/s, high wind (HW)). To reduce the influence of diurnal temperature variation on the results, wind-speed treatment was performed from 8:00 p.m. to 8:00 a.m. of the second day, and the rest of the time was used as a rest and recovery period. During the test period, the outdoor night average temperature was -17°C and the average temperature in the sheep house was approximately 5°C.

The test was carried out in two stages. In the first stage, the test sheep of groups I, II and III were allocated as the control group(C), no wind under low temperature(LT), and low windunder low temperature (LW), respectively. In the second stage of the test, the sheep of groups II and III were treated with MW and HW, respectively, under LT. Stage 1 spanned days 10 to 15, and stage 2 spanned day 16 to 20. There was no interval between the two phases, which spanned 5 days each (See Additional file 1: Fig.S1). This procedure ensured that the test sheep gradually adapted to cold stress, which increased from low to high, avoiding injury or death due to direct exposure to high-intensity cold stress. In addition, compared with a sudden change, a gradual change in temperature more closely resembles temperature change in the field.

The wind treatments were carried out by industrial electric fan (fl65-1, Watson, China; maximum wind speed, 6.5 m/s). In each treatment, four fans were used to create wind in different directions to ensure a uniform wind speed. During the test period, a windproof barrier was built at the test site to avoid interference from external natural wind. The external wind speed was monitored throughout the experiment. Testo 405-v1 anemometer (testto405-v1,tmall, Germany) was used to measure the wind speed at different points in the sheepfold and calculate the average wind speed.

Feeding and management

To avoid excessive differences in wind speed among different places in the large sheepfold, two open sheep pens (2.5×2.5 m) were set up in the sheepfold before the experiment began and sterilized. The experimental sheep were fed twice daily (at 9:00 a.m. and 6:00 p.m.), and some residual feed remained after each feeding, indicating that the sheep had been adequately fed.

The sheep were fed complete formula pellet feed (582 Formula Feed, Yuansheng, 120 China). The feed composition and nutrient levels are shown in Table S1. Before the test period, due to the low ambient temperature, the water in the water tank provided for drinking completely froze for approximately 1 to 2 h each day. Therefore, the water tank was regularly monitored during the study and any ice removed to ensure sheep access to drinking water.

Sampling

In each treatment group, the sheep were fed in a metabolic cage from the third day to the fifth day of treatment. The sheep were weighed without feeding at 8:00 a.m. before entering the metabolic cage, and the average daily gain (ADG) was calculated. In the morning of the 4th, 5th, and 6th days, all feces and urine were collected and weighed, and the daily fecal and urine volumes were recorded. The collected

feces and urine were then stored at -20°C in a refrigerator. The collected fecal samples and feed samples were dried in an oven at 65°C for 24 h. The initial moisture was determined, and the dry fecal content and dry matter intake (DWI) were calculated. The dried fecal samples and feed samples were crushed into powder for testing. DWI, average daily weight gain, fecal volume, urine volume, and apparent digestibility of dry matter were measured.

On the last three days of every treatment, blood samples from all sheep were collected by jugular venipuncture into a serum separator tube and immediately centrifuged at 3,000 g for 20 min. The serum was then stored at -40°C for the analysis of biochemical indicators. The levels of antioxidant enzymes, including superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and glutathione peroxidase (GSH-PX); total antioxidant capacity (T-AOC); and the levels of immune factors IL-2, IL-4, IL-6, and IFN- γ were measured. The kits used to measure the indexes were purchased from Beijing Huaying Biotechnology Research Institute, Beijing, China. The timeline of the experiment was shown (See Additional file 1: Fig.S1). On the last day of each treatment, after fasting the sheep overnight, the rumen contents were carefully pumped out, separated and stored at -20°C for analysis.

Total DNA extraction from rumen fluid and quantification PCR

Total genomic DNA was extracted using the stool DNA kit (OMEGA Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The concentration and quality of DNA were measured using a K5800 microspectrophotometer (KAIAO, Beijing, China). Quantitative PCR was executed in triplicate using SYBR Premix Ex Taq II (RNaseH Plus) assay kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan). The reaction system and procedure followed a previous study[13]. The primer sequences were selected based on past research[14].

16S r RNA gene sequencing, data processing and functional prediction

The V3-V4 region of the total microbial 16S r RNA gene was amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') that were tailed with specific sequences and amplified genes. Amplification was performed with the following cycling conditions: 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min[14]. The products were separated on 2% agarose gel, and nucleotides were isolated via bead purification using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, USA). Each product was assembled in equimolar amounts and sequenced on the Illumina MiSeq platform (Illumina, San Diego, USA).

The sequence data from 16S rRNA MiSeq sequencing were analyzed, quality-filtered using Trimmomatic, and merged by FLASH[15, 16]. The RDP classifier can quickly and accurately classify sequences into high-order taxonomy, which can provide a range of classification structures from the domain to genus level and accurately evaluate each stage[15]. Reads of 97% similarity were clustered into operational taxonomic units (OTUs) with ≤1% incorrect bases using UPARSE[17]. Chimeric 16S rRNA sequences were removed after CS detection[18]. QIIME can be used to analyze a microbial community and graphically

display the results[19]. The functional prediction of rumen bacteria was performed based on previous work[14].

Volatile fatty acid (VFA) analysis

The rumen fluid was thawed on the ice and centrifuged to obtain the supernatant, which was stored at 4°C. For SCFA analysis, a solution was prepared by mixing the supernatant and the crotonic acid at the ratio of 10:1 and then filtered and analyzed using gas chromatography (Agilent Technologies 7820A GC system, Santa Clara, USA) according to previous studies[20].

Statistical analysis

For each 16S rRNA sample, the abundances of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were estimated by calculating the means and standard errors of the mean (SEM) using one-way ANOVA with SPSS version 17.0 (SPSS Inc.; Chicago, IL, USA). For the bacterial community, we performed alpha and beta diversity analyses; the alpha diversity indexes Simpson, Shannon, and Chao were calculated, and beta diversity was explored by PLS-DA graphs. Rarefaction curves were produced in R (version 3.5.1).

The data, including BW, antioxidant indexes, immune indexes, and SCFA concentrations, were analyzed using R (3.5.0). R was used to analyze the associations between microbial community composition and these factors[21]. Covariance analysis was used to determine the effect of cold stress (sheep number was included as a covariate). R was used to construct graphs. In addition, we performed correlation network analysis. All data are presented as the mean ± SE, and values of $P<0.05$ were considered statistically significant.

Results

Growth performance and nutrient digestibility

We measured and calculated some of the metrics to estimate the performance of the production quota. We found that the ADG of sheep significantly decreased ($P<0.05$) with increasing intensity of cold stress, and after initiating wind treatment, sheep weight began to decrease (Fig. 1a).

To study whether changes in energy intake or energy output were responsible for the change in BW, sheep were fed in the metabolic cage. We found that Dry matter intake (DMI) varied among the treatment groups. DMI was low in the C group and lowest in the LT group. DMI then increased with increasing wind velocity, being similar between the LW and MW groups and highest in the HW group ($P<0.05$) (Fig. 1b). The apparent digestibility of dry matter was much lower in LT and LW than C ($P<0.05$), whereas that in MW and HW was similar to that in C (Fig. 1c). As cold stimulation increased, the amount of crude fiber (CF) in feces increased significantly (Fig. 1d), possibly due to the degradation of primarily carbohydrate rather than cellulose by the rumen microbiota in the MW and HW groups. The levels of metabolic energy and digestibility energy were significantly lower in the LT and LW groups than the other groups (See

Additional file 1: Fig.S2). These data indicated that the cold environment led to weight loss in the sheep and reduced digestion of the fiber in their feed.

Rumen microbiota changes with environmental changes

We used 16S rRNA gene sequence technology to analyze the abundance of rumen microbiota. The coverage index indicated that cold temperatures influenced microbial diversity (See Additional file 2: Table S1, S2). Through partial least squares discriminant analysis (PLS-DA), we found that microbiota community structures differed among the treatments(Fig. 2a).

We investigated the α diversity of the microbiota. Eighteen taxa were identified at the phylum level. Among the phyla, *Bacteroidetes*($58.7962\pm2.0898\%$) had the highest diversity, followed by *Firmicutes* ($34.2305\pm1.6897\%$), *Proteobacteria*($2.7963\pm0.8040\%$), and *Fibrobacteres* ($1.0466\pm0.2894\%$). The remaining taxa had values less than 1%. The diversity of *Verrucomicrobia* ($0.3078\pm0.0486\%$) was significantly higher in the HW group than in the C and LT groups ($P = 0.043$).

At the genus level, 216 taxa were identified. Univariate ANOVA of the bacterial abundances revealed several significant differences in the rumen microbes among treatments(Fig. 2). The relative abundance of *Prevotellaceae_UCG-003*(*Bacteroidetes*, $P = 0.045$)and *Solobacterium*(*Firmicutes*, $P = 0.017$)were decreased in the LT, LW, MW and HW groups compared with the C group. Furthermore, the abundance of *Brachybacterium*(*Actinobacteria*, $P = 0.040$), *Devosia*(*Proteobacteria*, $P = 0.008$), *Sphingomonas*(*Proteobacteria*, $P = 0.000$) and *unclassified_f_Enterobacteriaceae*(*Proteobacteria*, $P = 0.050$) were higher in the LT group than in the other groups. The abundance of *Rhizobium*(*Proteobacteria*, $P = 0.007$)was higher in the LT and LW groups than the other groups. In contrast, the abundance of *Sphaerochaeta*(*Spirochaetae*, $P = 0.044$)was decreased in the LT and LW groups compared with the other groups. Furthermore, the abundance of *Pseudobutyrivibrio*(*Firmicutes*, $P = 0.039$) was higher in the MW group than in the other groups. The abundance of *Ruminiclostridium_1*(*Firmicutes*, $P = 0.020$), *Ruminococcaceae_UCG-005*(*Firmicutes*, $P = 0.044$), *norank_c_WCHB1-41*(*Verrucomicrobia*, $P = 0.043$)was increased in the HW group relative to the other groups. Interestingly, the abundance of *Lachnospiraceae_XPB1014*(*Firmicutes*, $P = 0.012$)exhibited highest levels in the LT group and the lowest levels in the LW group (Fig. 2).

We used qPCR to verify the changes in some bacterial groups (See Additional file 2: Table S3). The diversity of the dominant bacteriadid not differ significantly (See Additional file 2: Table S4).

Bacterial function prediction and molecular pathways in the rumen

We predicted the functions of the rumen bacteria and the associated molecular pathways in sheep to assess the impact of wind treatment. At KEGG level 1, there were 7 major categories, including Metabolism ($49.76\pm0.22\%$), Genetic Information Processing ($21.32\pm0.06\%$), Unclassified ($13.95\pm0.04\%$), Environmental Information Processing ($10.58\pm0.23\%$), Cellular Processes ($2.68\pm0.08\%$), Organism System ($0.77\pm0.01\%$), and Human Diseases ($0.75\pm0.01\%$). The gene abundance of Unclassified was

significantly lower in the HW group than the other groups ($P<0.05$); no other significant differences were observed (See Additional file 2: Table S5).

At KEGG level 2, a large percentage of 41 gene families were found to have correlations with Amino Acid Metabolism ($10.57 \pm 0.56\%$), Carbohydrate Metabolism ($10.12 \pm 0.05\%$), Replication and Repair ($9.88 \pm 0.04\%$), Membrane Transport ($9.13 \pm 0.21\%$), Translation ($6.00 \pm 0.02\%$), and Energy Metabolism ($6.03 \pm 0.03\%$) (See Additional file 2: Table S6). The gene abundance of Nervous System was significantly lower in the MW group than in the other groups ($P<0.05$). However, the gene abundance of Excretory System was lower in the LT, LW, and MW groups than in the C and HW groups, which had similar abundance ($P= 0.06$). At KEGG level 3, 328 KEGG orthology (KO) pathways were identified. The top 44 pathways with high expression are shown (See Additional file 2: Table S7). The gene abundance did not differ significantly among groups. These findings showed that LT and wind speed may affect the nervous system in the rumen.

Changes in the concentration of VFAs and cellulase activity in the rumen

Studies have shown that VFAs could provide energy to the host and participate in the host metabolism[4]. After discovering the changes in the rumen microbiota, we explored the levels of VFAs. We found that the concentration of total VFAs was significantly reduced in the MW and LW groups relative to the other groups ($P<0.05$) (Fig. 3a). Accordingly, the concentrations of acetic acid and propionic acid were decreased significantly in the MW and HW groups ($P<0.05$). However, the ratio of acetic acid to propionic acid did not markedly differ among the groups (See Additional file 2:Table S8). In addition, butyrate level was significantly reduced in the HW group relative to the other groups ($P<0.05$), whereas isobutyric acid and isovalerate levels were significantly increased in the MW and HW groups compared with the other groups ($P<0.05$). Cellulase activity in the rumen contents decreased with increasing wind speed, being significantly lower in the HW group than in the other groups($P<0.05$) (Fig. 3b). These findings suggested that in the cold environment, the rumen microbes reduced their digestion of CF.

Changes in inflammatory factors and antioxidant enzymes

As we expected, the contents of proinflammatory factors, such as IL-2, IL-6, and IFN- γ , in plasma were reduced in the wind-treatment groups relative to the C group ($P<0.05$). In contrast, the contents of anti-inflammatory factors, such as IL-4, were increased in the wind-treatment groups relative to the C group ($P<0.05$) (Fig. 4).

We found that MDA content was significantly decreased in the wind-treatment groups compared with the C group ($P<0.05$) (Fig. 5a). In contrast, the serum concentrations of SOD, CAT and GSH-PX showed similar trends as T-AOC (Fig. 5), being increased in the wind-treatment groups relative to the C group ($P<0.05$). The ratio of T-AOC to MDA reflects the relationship between the body's antioxidant capacity and oxidative damage. Low-temperature treatment significantly increased the ratio of T-AOC to MDA in serum ($P<0.05$), and this ratio increased significantly with increasing WV ($P<0.05$). However, the ratio of T-AOC to MDA was significantly lower in the LW group than in all of the other groups except the C group, for which

no significant difference was observed. These data showed that cold stimulation led the sheep to enter an immunosuppressive and antioxidant state.

Associations of rumen microbiota with host phenotype

We used correlation analysis to research the associations between microbiota and host phenotype (Fig. 6, Additional file 3: Table S9). ADG was negatively correlated with *Ruminiclostridium_1* ($r = -0.525, P < 0.05$), *Ruminococcaceae_UCG-005* ($r = -0.480, P < 0.05$), *Sphaerochaeta* ($r = -0.479, P < 0.05$), and *norank_c_WCHB1-41* ($r = -0.519, P < 0.05$) and the levels of isobutyric acid ($r = -0.500, P < 0.01$) and isovalerate ($r = -0.553, P < 0.05$). Positive relationships were detected between ADG and *Solobacterium* ($r = 0.583, P < 0.01$) and the levels of acetic acid ($r = 0.583, P < 0.01$), propionic acid ($r = 0.523, P < 0.05$), butyrate ($r = 0.638, P < 0.01$), valeric acid ($r = 0.521, P < 0.05$), and total VFA ($r = 0.499, P < 0.05$).

In addition, we found that some microbiota were associated with the levels of certain inflammatory factors. For example, the levels of the proinflammatory factors IL-2 and IFN- γ were positively associated with *Prevotellaceae_UCG-003* ($r = 0.618, P < 0.01$; $r = 0.708, P < 0.01$, respectively) and *Solobacterium* ($r = 0.541, P < 0.05$; $r = 0.675, P < 0.01$, respectively). Furthermore, a significant negative association was detected between IFN- γ level and *norank_c_WCHB1-41* ($r = -0.494, P < 0.05$), and a positive relationship was found between IL-4 level and *Lachnospiraceae_XPB1014* ($r = 0.553, P < 0.05$).

Furthermore, correlations between microbiota and oxidative stress markers were identified. *T Prevotellacee_UCG-003* had a significant positive correlation with MDA level ($r = 0.534, P < 0.05$) and negative correlations with T-AOC ($r = -0.451, P < 0.05$), SOD ($r = -0.646, P < 0.01$), CAT ($r = -0.664, P < 0.01$), and GSH-PX levels ($r = -0.532, P < 0.05$). *T Sphingomonas* had significant positive correlations with T-AOC ($r = 0.602, P < 0.01$), CAT ($r = 0.608, P < 0.01$), and GSH-PX levels ($r = 0.506, P < 0.05$).

Discussion

Winter in northwest China is not only cold but also subject to strong winds. We used the local temperature in this study and strictly controlled the wind speed. In the present study, we explored the whole-body and rumen responses of sheep to both cold and wind speed. When the sheep were exposed to cold temperature, DMI decreased sharply. In contrast, Bo reported that voles exposed to cold temperature (4°C) increased their food intake[22] to maintain a constant body temperature. However, in the present study, the range of temperature was very large. The sharp decrease in BMI with cold exposure observed in the present study corresponds to the first stage in the stress response, i.e., a panic reaction or mobilization phase, as proposed by Canadian pathologist Hans Selye. This reaction led to a decline in animal feed intake. In addition, the metabolic analyses revealed that the apparent digestibility of DM, DE, and ME decreased sharply upon cold exposure. Young et al concluded that each 10°C decrease in an environment below 20°C would cause 1.8 percentage points of DM digestibility change[23], reducing the feed-utilization efficiency of sheep. This conclusion is consistent with our results. Subsequently, wind treatment was applied to the sheep in the cold environment. Under wind exposure, the sheep increased the apparent digestibility of DM, DE, and ME. They then entered the second phase of the stress response:

the adaptation phase. They generated more heat from feeding and the body to maintain a constant body temperature. As has been described in previous studies, the ADG of sheep decreased significantly under wind treatment and became negative[22].

The rumen microbiota change when animals are exposed to cold conditions[2]. For example, when the sheep were subjected to cold treatment, the abundance of *Lachnospiraceae_XPB1014*, which is highly enriched in the gut of nonalcoholic fatty liver patients[24], increased, and the abundance of *Prevotellaceae_UCG003* decreased. These two bacterial taxa represent more than 1% of the bacterial community in the rumen and use dietary soluble fiber as substrate to produce short chain fatty acids[25]. Furthermore, several nitrogen-fixing microbial groups, such as *Devosia* and *Rhizobium*[26], were enriched in the wind-exposed sheep, which might increase the amount of ammonia and urea produced via the rumen nitrogen cycle. As a result, the abundance of *Brachybacterium* increased, which uses urea to breed and degrade harmful substances such as phenol[27]. In addition, the abundance of *Sphingomonas*, which is involved in redox reactions and has a reducing effect[28], was increased under cold temperature. Large fluctuations in the amount of bacteria in the rumen provide a breeding opportunity for pathogens such as *unclassified_f_Enterobacteriaceae*[29]. Treatments with different wind velocities were applied to sheep. As WV increased, the abundances of *Lachnospiraceae_XPB1014*, *Ruminiclostridium_1*, *Ruminococcaceae_UCG005* and *norank_f_WCHB1-41* increased.

Lachnospiraceae_XPB1014 digests soluble fiber; the other two groups had abundances between 0.1% and 1% and degrade cellulose[30-32]. Furthermore, the abundance of *Pseudomonas* increased, which might reduce oxidative stress[33]. We concluded that after cold stimulation, rumen bacteria that digest soluble fiber fluctuated in abundance, while the abundances of beneficial bacteria decreased and those of harmful bacteria increased.

PICRUSt1 functional prediction revealed that the rumen microbiota were regulated by the nervous system under cold temperature. Several studies have found that the gut microbiota in mice are regulated by neurons, such as VIP neurons[34]. We suspect that rumen microbes can similarly be regulated by neuronal factors. Changes in the rumen microorganisms caused changes in VFAs. Consistent with our findings, previous studies have found that cold conditions reduce the contents of total VFA, acetate, butyrate, and valerate[12]. These reductions occur due to the lower efficiency of rumen microorganisms in fermenting soluble fibers in cold conditions[35].

Some studies have shown that animals exposed to cold temperature can enter a severe inhibitory state of the immune response[36, 37]. This observation is consistent with our findings. IL-2, which could represent the level of cellular immunity[38], and IFN- γ , which is mainly involved in cellular immune-related immune responses, were decreased in the sheep exposed to cold temperature. Furthermore, the level of IL-4, which could represent the activation level of TH2 cells, was increased in these sheep. Following these changes, the content of TH2 likely increased sharply and that of TH1 likely decreased[39], which destroyed the normal dynamic equilibrium state of the two types of cells, causing the body to enter an immunosuppressive state. In addition, the serum content of the proinflammatory factor IL-6 was decreased in the cold-treated sheep in this study. Inconsistent with our results, Guo et al found that IL-6

levels increased under cold conditions[40].The difference may be due to study differences in the levels of cold stimulation in the experimental design and in the genetic backgrounds of the animals. The immune response is very complex, and proinflammatory factors are affected by many factors[34], which deserve further study.

When the sheep were exposed to cold temperature,they metabolized large amounts of nutrients to increase their bodies' heat production.During this process, the body could produce oxygen free radicals, resulting in oxidative damage[41]. However, in the present study,MDA content,which reflects the extent of cell damage[42], was decreased in the cold-exposed sheep. We speculate that the high levels of antioxidant enzymes, such as SOD[43], CAT[44], and GSH-PX[45], in plasma under cold temperature led to the degradation of the oxygen free radicals produced by sheep metabolism[46]. Studies have shown that low levels of cold stress enhance antioxidant capacity and reduce the body's exposure to the effects of oxidative stress[47]. Furthermore, studies have shown that elevated levels of antioxidant enzymes in sheep are indicative of oxidative stress, with high enzyme levels needed to avoid damage from oxygen free radicals[45]. These observations are consistent with our findings.

Accumulating studies have shown that microbes have significant associations with and can alter host phenotypes[48, 49]. VFA content was strongly correlated with ADG in this study. VFAs, especially butyrate[35], can provide large amounts of energy to fuel metabolism. However, the mechanisms underlying the associations between specific microorganisms and ADG need further study. Some studies have shown that the abundances of *Prevotellaceae_UCG003*[25] and *Lachnospiraceae_XPB1014*,which produce metabolites such as VFAs[50],influence the immune response. In addition, the abundance of *Sphingomonas*, which is involved in reduction reactions, has been shown to be increased in the rumen of animals subjected to cold stress[51]. The increase in *Sphingomonas* abundance resulted in a decrease in MDA content and a positive correlation with blood hormone content, consistent with our results. However, the associations between *Prevotellaceae_UCG003* and blood hormones indicative of oxidative damage deserve further study.

Conclusion

In the present study, when the sheep were exposed to the cold environment, animal growth and feed efficiency decreased significantly, and the fermentation of soluble fibers by rumen microorganisms decreased significantly. In addition, sheep increased the levels of antioxidant enzymes to resist damage; however, the sheep were in a state of immunosuppression.

Abbreviations

ADG: average daily gain; SOD: superoxide dismutase; GSH-PX: glutathione peroxidase; CAT: catalase; VFA: volatile fatty acid; BW: body weight; MDA: malondialdehyde; T-AOC: total antioxidant capacity; KEGG: Kyoto Encyclopedia of Genes and Genomes; SEM: standard error of the mean; DMI: dry matter intake; WV: wind velocity; DM: dry matter; CF: crude fiber; PLS-DA: partial least squares discriminant

analysis; PICRUSt2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States;qPCR: quantitative real-time polymerase chain reaction;SPSS: Statistical Package for the Social Sciences; ANOVA: one-way analysis of variance

Declarations

Ethics approval and consent to participate

All procedures in the present study involving animals were approved by the Animal Care and Use Committee of Lanzhou University (Lanzhou, China) and Northwest A&F University (Yanglin China).

Consent for publication

Not applicable

Availability of data and materials

Raw Illumina sequencing data have been deposited in Sequence Read Archive (SRA). BioProject's metadata are available at the following link:

<http://www.ncbi.nlm.nih.gov/bioproject/633534>

The other datasets analyzed in the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

YY and CY conceived and designed the experiments. ZG,ZS, TG and LY managed the sheep. ZG,CH,TG and LY collected samples. GH and DN performed the bacterial analysis. GH and ZG analyzed the other data.ZG and GH performed the statistical analyses, and GH and ZG wrote the manuscript. All authors read and approved the final version of the manuscript.

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Figures

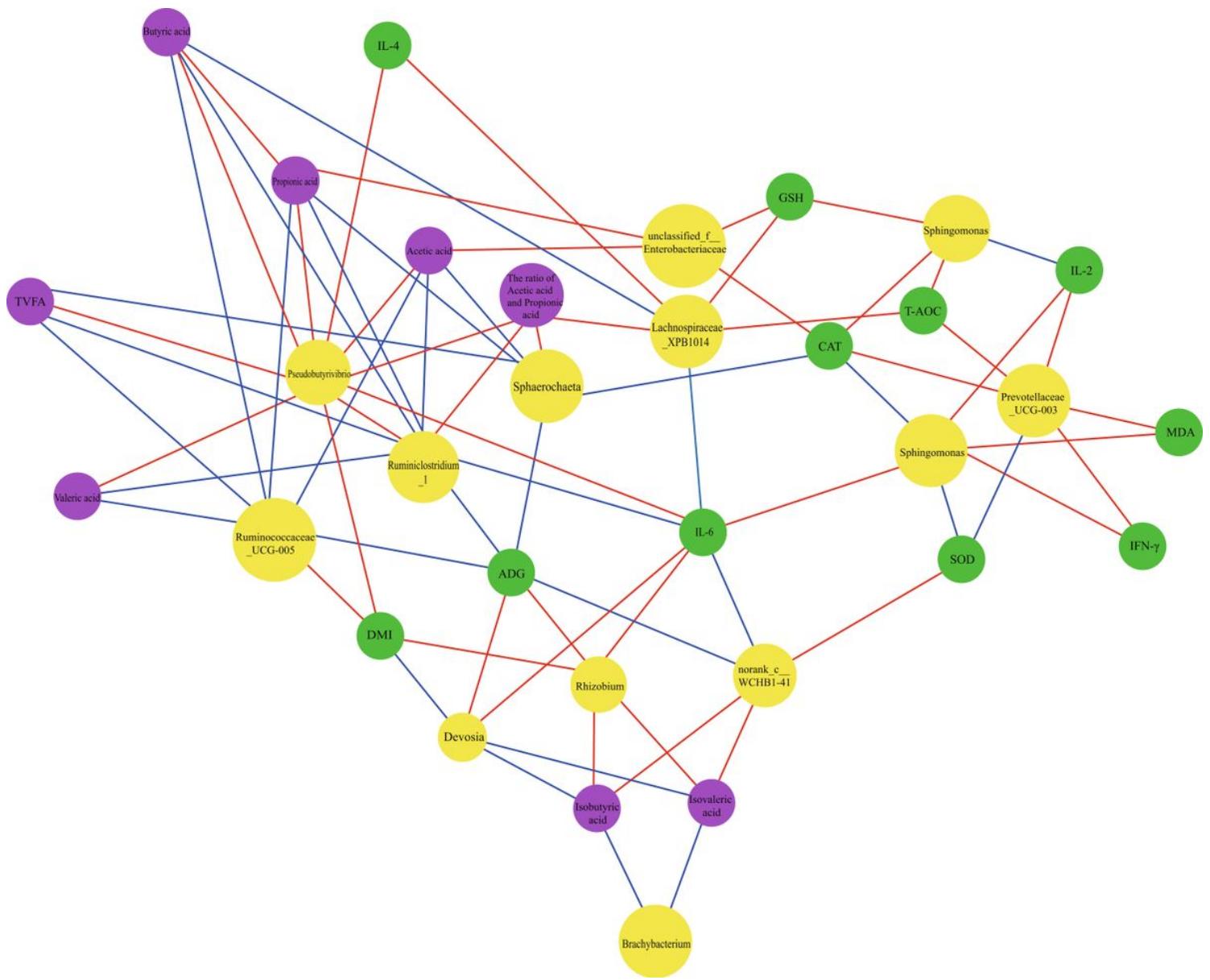


Figure 1

Correlation analysis of rumen microbiota and host phenotype.

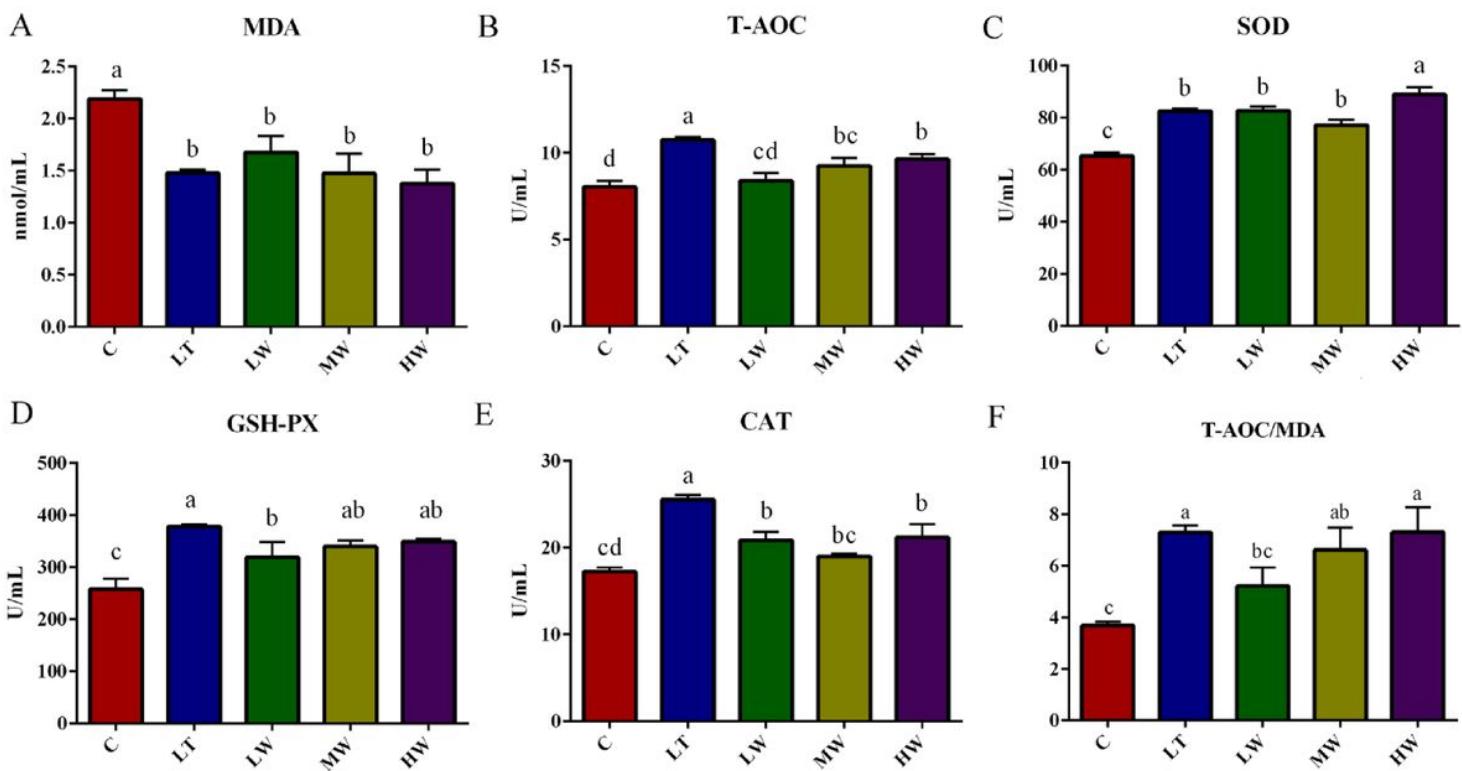


Figure 2

Effect of wind velocity on antioxidant indexes in sheep under low temperature. (A) Effect of wind velocity on MDA content in serum in sheep under low temperature. (B) Effect of wind velocity on T-AOC level in serum in sheep under low temperature. (C) SOD. (D) GSH-PX. (E) CAT. (F) T-AOC/MDA.

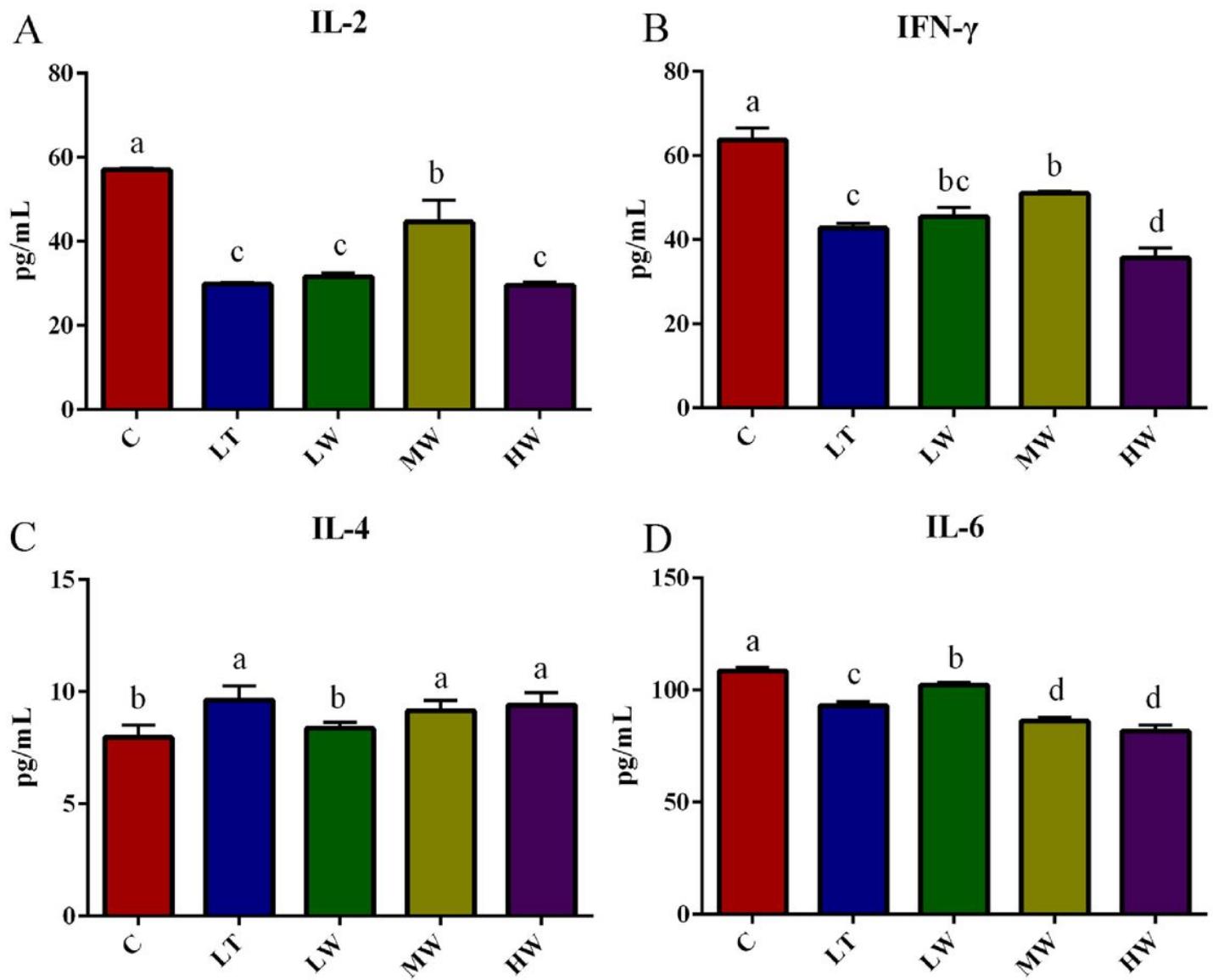


Figure 3

Effect of wind chill on serum levels of cellular inflammatory factors in sheep. (A) IL-2. (B) IL-4. (C) IFN- γ . (D) IL-6.

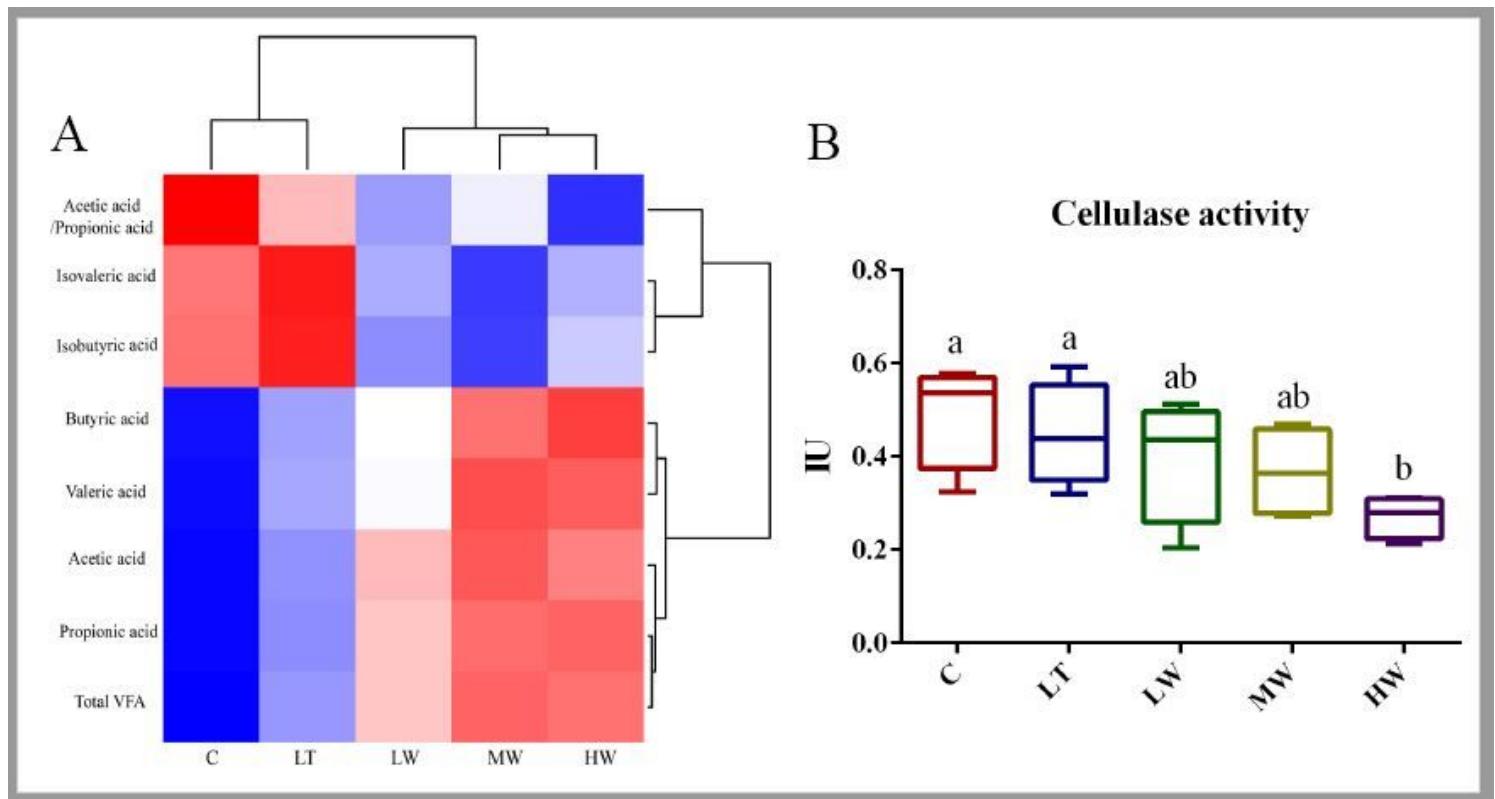


Figure 4

The effects of wind chill on the concentration of VFA and cellulase activity in the rumen. (A) VFA. (B) cellulase activity. Red indicates a high content, and blue indicates a low content.

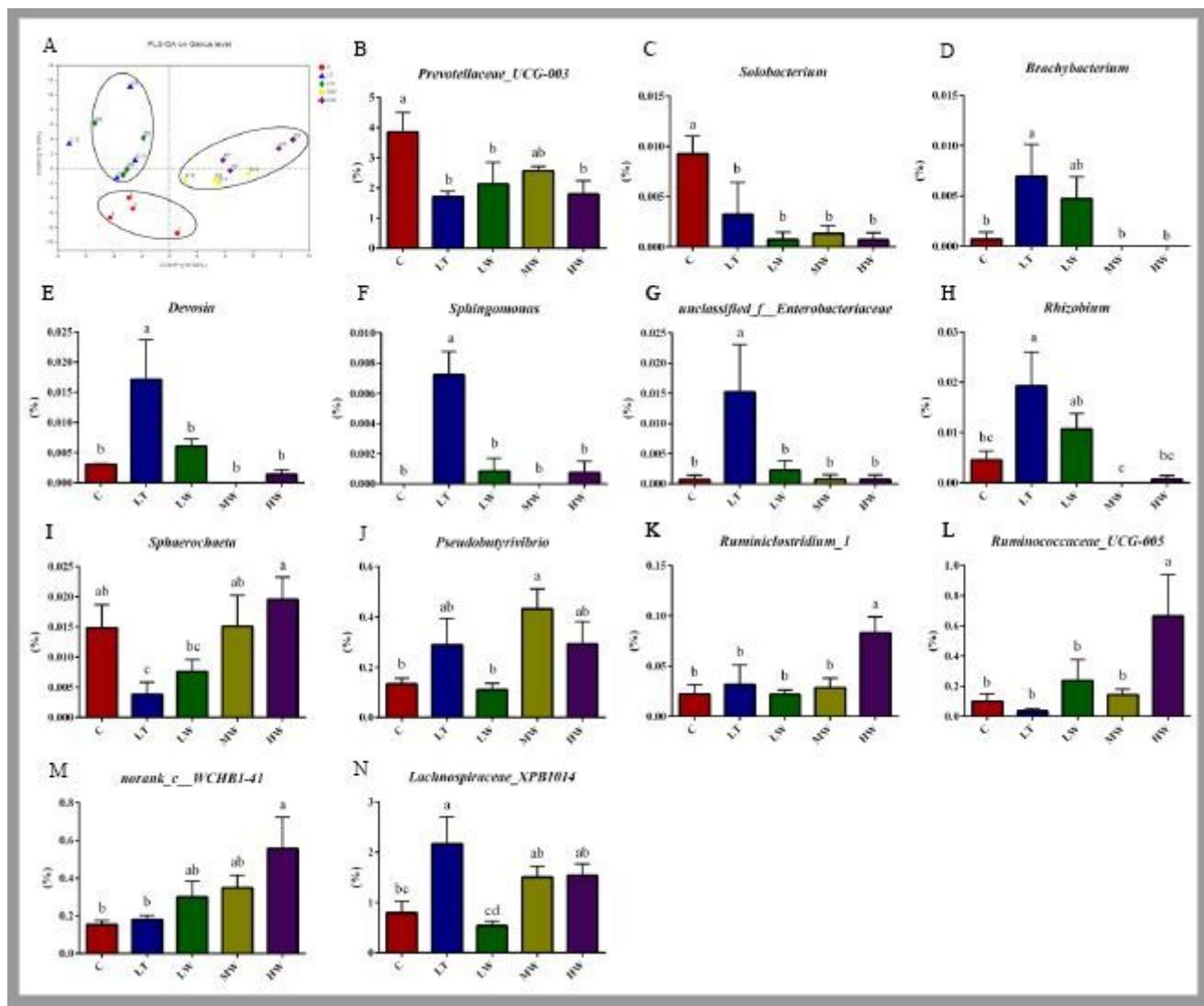


Figure 5

The effects of wind chill on the microbiota in the rumen. (A) Beta diversity as evaluated by PLS-DA. (B-N) Microbiota at the genus level were significantly changed. (B) *Prevotellaceae_UCG-003*, (C) *Solobacterium*, (D) *Brachybacterium*, (E) *Devosia*, (F) *Sphingomonas*, (G) unclassified_f_Enterobacteriaceae, (H) *Rhizobium*, (I) *Sphaerochaeta*, (J) *Pseudobutyryrivibrio*, (K) *Ruminiclostridium_1*, (L) *Ruminococcaceae_UCG-005*, (M) norank_c_WCHB1-41, (N) *Lachnospiraceae_XPB1014*.

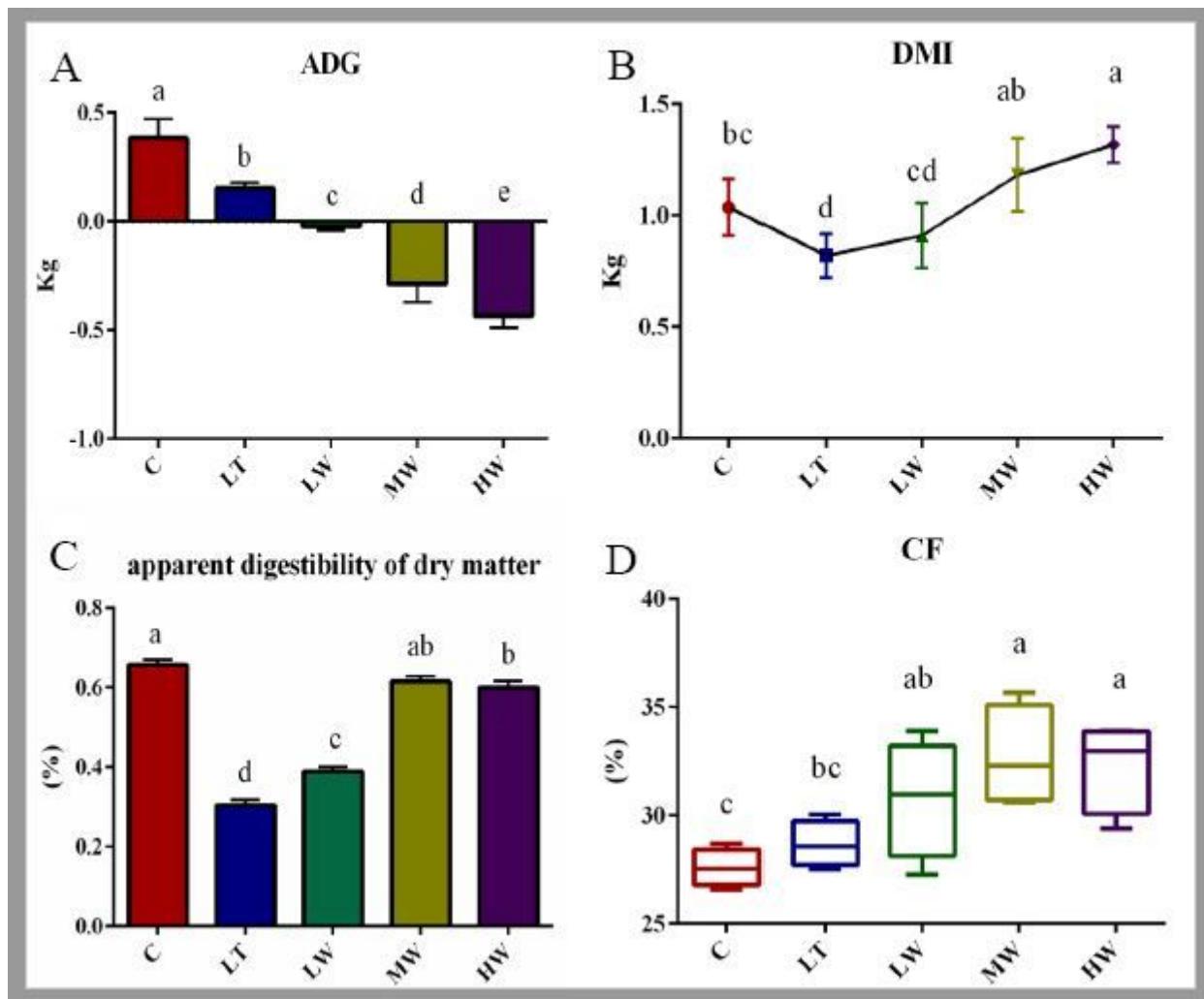


Figure 6

Effect of low temperature on the growth index of sheep.(A) Average daily gain (ADG) changes in sheep. (B) Dry matter intake (DMI) of each group of sheep. (C) Apparent digestibility of Dry matter (DM).d. Crude fiber (CF) in feces. C (sheep exposed to 5°C), LT (sheep exposed to -15°C and an average wind velocity less than 0.5 m/s), LW (sheep exposed to -15°C and an average wind velocity of 3 m/s), MW (sheep exposed to -15°C and an average wind velocity of 4 m/s), HW (sheep exposed to -15°C and an average wind velocity of 5 m/s).

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