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Calm *Hu* Sheep Have a Different Microbiome Profile and Higher Energy Utilization Efficiency Than Nervous *Hu* Sheep

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Abstract: In sheep, temperament is known to affect animal welfare and the quality of animal products. While the composition of the gut microbiota is different between depressed patients and healthy human patients, in sheep, the influence of temperament on ruminal microbial species and abundance remains unknown. This study investigated the effects of temperament on parameters of rumen fermentation and microbial composition of rumen contents of *Hu* ram lambs. Using the pen score test, 6 lambs that scored 2 points or below (calm) and 6 lambs that scored 4 points or more (nervous) were selected from 100 ram lambs. The sheep were fed a standard diet for 60 days and rumen samples were collected at slaughter. The concentrations of propionic acid, isovaleric acid, valeric acid, and the ammonia nitrogen concentration were different between the calm and the nervous groups ($p < 0.05$). At the phylum level, there were significant differences in *Bacteroidetes*, *Tenericutes*, and *Spirochetes* ($p < 0.05$); and at the genus level, there were significant differences in the *Christensenellaceae R-7* group, *Treponema 2*, *Fibrobacter*, and *Ruminococcaceae UCG-003* ($p < 0.05$). The present study suggests that differences in the rumen microbiota between the calm group and the nervous group could have an impact on the metabolism of carbohydrates and polysaccharides and explain why Calm *Hu* sheep have a higher energy utilization efficiency than nervous *Hu* sheep. More studies are needed to further understand the effect of temperament on specific pathways of the rumen microbiota.

Keywords: temperament; *Hu* sheep; rumen; microorganism; calm; nervous

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1. Introduction

In animals, temperament commonly refers to social or aggressive behavioral and physiological differences that are observed in individual animals in response to stressors or environmental challenges [1–3]. Temperament can be assessed by quantifying the response of animals to standardized tests [4–6]. For example, reactions caused by human operation or environmental stimulation include escape responses to human and environmental conditions [7], aggressive behaviors between individuals [8], emotional responses to stress [9], cognitive responses to new stimuli [10], and the calf-protecting behavior of cows [11]. Overall, animals with a calm temperament show less fear and anxiety, while nervous animals show higher levels of fear and anxiety [12]. Thus, temperament is considered as an objective measure of the degree of fear and anxiety that an animal experiences in the face of threats [13,14].

In farm animals, temperament can also affect animal welfare and productivity [15]. Cattle with a quiet temperament have higher feed efficiency than those with a nervous temperament [16]. Several studies have shown that production animals that are calm and docile grow faster, are easier to transport and feed, and have better meat quality than

do individuals that are nervous and aggressive [4,17–21]. Calmer cows had better ADG, higher feed efficiency, and pregnancy rates than nervous cows [19], while irritable cattle had compromised performance in a feedlot, and poor carcass and meat quality traits than did calmer cattle [22]. In sheep, selection for temperament affects the sexual and maternal behavior of the female and the survival of newborn lambs [23].

Animal temperament can be influenced by many factors such as genetics and aspects of the environment. The temperament of farm animals such as sheep, cattle, and quail have a strong genetic basis. In sheep, the temperament of young lambs born to calm or nervous mothers is not affected by the genotype of the nurturing mother, as demonstrated by cross-breeding experiments [24]. Temperament, at least in part, determines how an individual responds to stressful situations and can vary considerably across species and sexes. Some studies have shown that temperament is related to the experience of stress during rearing [25]. In response to environmental stress, calm animals show a sluggish response while nervous animals are overreactive [23]. In association with the different behavioral responses, animals with calm or nervous temperaments have different cortisol responses to stressors [26].

In recent years, research on the gastrointestinal microbiota has become a new research hotspot. The gastrointestinal tract harbors a complex microbial network and its composition reflects the constant co-evolution of these microorganisms with the environment of the host [27]. In humans, the composition of the gut microbiota is altered in people with depression [28,29] and the abundance of specific genera is correlated with behavioral characteristics that are linked to personality [30]. Similarly, in animals, the gut microbiome can influence the stress response, anxiety, and depressive-like behaviors, as well as social behavior and communication [31,32]. There is also evidence that the relationship operates both ways, because as well as the gastrointestinal microbiome being a probable cause of temperament, physiological and behavioral changes that are associated with temperament can affect the gut microbiome [33]. In mice, the intestinal microbiota may be involved in depression-like behavior by altering glycerophospholipid metabolism in the gut–brain axis [34]. The composition of intestinal microbiota is different between mice with depression behavior and healthy mice [35]. In addition, the loss of intestinal microbiota can induce depression-like behavior in mice [35].

All the above studies show that, at least in monogastric animals, there is an interaction between temperament and the gastrointestinal microbiota. In ruminant animals, rumen function has a critical impact on the production and health of the host ruminant. Bacteria, which are the most abundant, diverse, and metabolically active ruminal microbes, enable the ruminant to ferment plant proteins and polysaccharides to generate the nutrients that are necessary for maintenance and growth [36,37]. However, it is not known if, such as in monogastrics, the gastrointestinal microbiota of ruminants is affected by temperament. Using *Hu* sheep, a breed of sheep that are fed all year round in Taihu Lake basin in China, we explored the relationship between temperament and the rumen microbiome. The rumen microbial composition was quantified using high-throughput sequencing technology and associated with the nutritional and physiological function of the *Hu* sheep with different temperament.

2. Materials and Methods

2.1. Selection for Temperament

In 2017, one hundred 5-month-old *Hu* ram lambs with similar body weights (30 ± 2 kg) were selected from a commercial farm (Huai'an, China).

The temperament of the individual lambs was scored on a 1 to 5 scale using the pen score test described by Kunkle et al. (1986) as cited by Hammond et al. (1996) [38,39]. Briefly, the sheep were kept in pen of 50 sheep and the individual response to the approach of an evaluator was made using the scoring scale: 1 = nonaggressive (docile): walks slowly, can approach closely, not excited by humans or facilities; 2 = slightly aggressive: runs along fences, will stand in corner if humans stay away, may pace fence; 3 = moderately aggressive:

runs along fences, head up, and will run if humans move closer, stops before hitting gates and fences, avoids humans; 4 = aggressive: runs, stays in back of group, head high, and very aware of humans, may run into fences and gates even with some distance, will likely run into fences if alone in pen; and 5 = very aggressive: excited, runs into fences, runs over humans, and anything else in path, "crazy". The scoring was done by two evaluators, and each evaluator repeated the scoring on each individual animal twice. Sheep with an average of 2 trials score of 2 points or lower were classified as calm and those sheep with an average of 2 trials score of 4 points or more were classified as nervous. Six ram lambs with the lowest score for calm temperament and six ram lambs with the highest score for nervous temperament were moved to individual pens for the duration of the experiment.

2.2. Animal Feeding Management

Each sheep was kept in an individual pen (1.25 m × 1 m) for the total duration of the feeding trial (7 + 60 days, see below). The feeding and management conditions were the same for all twelve animals in the two groups.

The basic feed was formulated according to the NRC Sheep Feeding Standard of the United States (2007). During the feeding period, the diet was fed in the form of TMR with a concentrate to forage ratio of 60:40. The feed samples were fried in an oven at 135 °C for 3 h to obtain the DM (AOAC, 1990 [40]; Method No. 930.15). The total N was detected using the Kjeldahl method; the crude protein content was calculated as 6.25 × N (Method No. 984.13); the ether extract (EE) was measured using the Soxhlet system (Method No. 954.02); the acid detergent fiber (ADF); and neutral detergent fiber (NDF) of diet were analysed using method described by Soest, Robertson, and Lewis (1991) [41]. Digestible energy, calcium, and total phosphorus were calculated from the composition of ingredients (Table 1).

Table 1. Composition and nutrient levels of the basal diet (DM basis).

Items	Content (%)
Silage corn	24.80
Leymus chinensis	15.20
Corn	18.00
Wheat bran	9.78
Soybean meal	16.20
Brown rice	8.52
Beer yeast	4.02
Corn gluten meal	1.20
Calcium dihydrogen phosphate	0.72
Stone powder	0.96
Salt	0.30
Premix [(1)]	0.30
Total	100.00
Nutrient [(2)]	
Digestible Energy (MJ/kg)	13.12
Crude protein (%)	20.72
Neutral detergent fiber (%)	31.80
Acid detergent fiber (%)	19.62
Ether extract (%)	2.10
Calcium (%)	0.74
Total phosphorus (%)	0.51

[(1)]: The premix provided per kg of diet: $CuSO_4$ 25 mg, $FeSO_4 \cdot H_2O$ 75 mg, $ZnSO_4 \cdot H_2O$ 105 mg, $CoCL_2$ 0.0024 mg, Na_2SeO_3 0.016 mg, VA 12,000 IU, VD_3 10,000 IU, VE 25 mg, Nicotinic acid 36 mg, Choline 1000 mg. [(2)]: Digestible energy, calcium, and total phosphorus were calculated from the composition of ingredients.

The feed refusals were measured daily before the morning feeding. Each daily feed was about 3% of the body weight with the amount offered being adjusted daily to provide

a slight surplus each day. The sheep were fed twice a day, at 6:00 am and 6:00 pm. The sheep were free to eat and drink at all times.

The sheep were acclimated to the diet for a pre-trial period of seven days and then the experimental feeding trial lasted for 60 days. During the experiment, the feed remaining each day was collected and weighed, and the dry matter intake of each experimental animal was recorded. After the 60-day feeding trial, the ram lambs were transported approximately 50 km to an abattoir where they were kept in a lairage and supplied only with water for 24 h before slaughter, as per industry practice. The animals were slaughtered and processed by professional butchers.

2.3. Sample Collection and Processing

Fresh rumen content was collected from each sheep within 15 min after slaughter using an aseptic technique as previously validated [42,43]. The pH of the rumen fluid was measured using a portable pH meter (PB-21, Beijing Sartorius Scientific Instruments, Beijing, China). Samples of rumen content were filtered through four layers of sterilized gauze to collect filtrate, and distributed into 10 mL centrifuge tubes and stored at −20 °C. In addition, samples of unfiltered rumen content were stored in liquid nitrogen for the determination of rumen microbial diversity.

2.4. Measurement Indexes and Methods

2.4.1. Determination of Rumen Fermentation Parameters

Concentrations of volatile fatty acid (VFA) in the rumen fluid were determined by gas chromatography using a Shimadzu GC-14B (Kyoto, Japan) fitted with a capillary column (CP-WAX52 CB, 30 m × 0.53 m × 1 μm). The rumen contents were thawed at 4 °C and centrifuged at 12,000× *g* for 5 min. One milliliter of supernatant was taken and added to 0.2 mL of 20% metaphosphoric acid containing 60 mM Crotunic acid (internal standard method), filtered through a 0.22 μm needle filter, and analyzed by gas chromatography. The temperature of the injector and detector was 200 °C. The initial column temperature was 100 °C, and the temperature was raised to 150 °C at 3 °C/min. The sensitivity was set to 10^1, and the attenuation was 2^5 [44]. The concentration of ammonia nitrogen was determined by phenol-sodium hypochlorite colorimetry [45].

2.4.2. DNA Extraction and High-Throughput Sequencing

The total microbial DNA of the rumen fluid from each animal was extracted using a fecal genome extraction kit (Tiangen Biotechnology Co., Ltd., Beijing, China). The concentration and purity of total DNA (OD 260/280 and OD 260/230) was determined by an ultramicro spectrophotometer (NanoDrop-1000, Thermiel), and DNA quality was determined by agarose-gel electrophoresis.

The DNA was stored in a −20 °C refrigerator until high-throughput sequencing. A Novaseq-PE 250 sequencing platform was used in the sequencing system (SanDiego, CA, USA), and the sequencing company was Nanjing Jisi Huiyuan Biotechnology Co., Ltd. (Nanjing, China). Specific primers of the 16S v4–v5 region were designed to amplify specific regions, and fragments of about 420 bp were amplified. The fragments were sequenced using a Hiseq 2500 platform.

The raw data obtained from the sequencer were spliced and filtered. Then, OTUs (operational taxonomic units), clustering, and species classification analysis were performed. The sequence of each OTU was annotated to obtain the corresponding species information and the species-based abundance distribution. Multi-sequence alignment of OTUs was performed to construct the phylogenetic relationship.

2.5. Statistical and Abundance Analyses

The effect of temperament on pH, fermentation parameters, phylum strains, and genus present in the rumen fluid were analyzed using t-tests performed in SPSS 13.0 software for Windows. The community structure was compared between temperaments using a

principal component analysis. The bar graph of KEGG function was made by GraphPad Prism 6.0 software. The Software package R (V3.1.1.) was used to build the Venn diagram, PCA, and the graphs of abundance of microbiome composition. The results are presented as the mean ± SEM. Significance was considered when $p \leq 0.05$.

3. Results

3.1. The Effect of Temperament on Growth and Feed Utilization

There was no difference in the body weight of the two groups of lambs at the start of the experiment and after 67 days of dietary treatment (Table 2). There was no effect of temperament on the average daily weight gain or the daily feed intake of dry matter (Table 2).

Table 2. Bodyweight, average weight gain, and dry matter intake in *Hu* sheep with different temperaments.

Items	Calm Group	Nervous Group	*p*-Value
Bodyweight (kg)			
Start on the experiment	29.7 ± 2.2	30.0 ± 1.8	0.30
End of the experiment	47.7 ± 0.23	49.3± 1.63	0.20
Average daily weight gain (kg/d)	0.26 ± 0.22	0.36 ± 0.05	0.49
Daily dry matter intake (kg/d)	1.48 ± 0.17	1.51 ± 0.23	0.30

3.2. Rumen Fermentation Parameters

The total volatile fatty acids, as well as the proportions of individual acids making up the total, pH, and ammonia nitrogen concentrations are presented in Table 3. The concentrations of propionic acid, isovaleric acid, and valeric acid were significantly higher in the nervous group than in the calm group (Table 3). There were no significant differences in the proportion of acetic acid, isobutyric acid, isobutyric acid, butyrate acid, total VFA concentration, acetic acid, or pH between the calm group and the nervous group ($p > 0.05$). The concentration of ammonia nitrogen was significantly higher in the calm group than it was in the nervous group ($p < 0.05$).

Table 3. Rumen fermentation parameters in *Hu* sheep with different temperaments.

Items	Calm Group	Nervous Group	*p*-Value
pH	6.70 ± 0.10	6.57 ± 0.06	0.116
Acetic acid (mmol/L)	15.86 ± 1.71	16.52 ± 1.06	0.754
Propionic acid (mmol/L)	2.71 ± 0.23	3.55 ± 0.28	0.045
Isobutyric acid (mmol/L)	0.28 ± 0.03	0.36 ± 0.05	0.194
Butyrate acid (mmol/L)	1.13 ± 0.18	1.41 ± 0.23	0.372
Isovaleric acid (mmol/L)	0.30 ± 0.03	0.45 ± 0.04	0.018
Valeric acid (mmol/L)	0.13 ± 0.01	0.21 ± 0.03	0.022
Total VFA concentration (mmol/L)	20.43 ± 1.83	22.50 ± 1.68	0.424
Acetic acid/acid ratio (%)	5.00 ± 0.80	4.67 ± 0.08	0.134
Ammonia nitrogen concentration (mg/100 mL)	3.68 ± 0.43	1.94 ± 0.09	0.007

3.3. The Effect of Temperament on Rumen Microbial Structure

3.3.1. Rumen Bacterial Sequencing OTUs and Alpha Diversities

Of the 1414 OTUs detected overall, only 11 OTUs were unique to the calm group, and 20 to the nervous group. The other 1383 were shared between the groups (Figure 1). The detected OTU coverage index was greater than 97.67% for the treatment. There was no significant difference in Chao index between the two groups ($p > 0.05$). Shannon's index and Simpson's index were significantly lower in the calm group than in the nervous group ($p < 0.05$; Table 4). A principal component analysis of β diversity showed that there

were significant differences in the bacterial composition between the two groups ($p < 0.05$; Figure 2).

Figure 1. The distribution of OTUs in *Hu* sheep with different temperaments.

Table 4. Effect of temperament on the number of species (Chao value) and indices of biodiversity (Shannon and Simpson indices) in the microbial population in the rumen fluid of *Hu* sheep.

Alpha Diversity Index	Calm Group	Nervous Group	*p*-Value
Chao value	1288.51 ± 12.08	1279.92 ± 13.77	0.649
Shannon index	7.78 ± 0.06	8.15 ± 0.03	< 0.01
Simpson index	0.9850 ± 0.001	0.9907 ± 0.0002	< 0.01

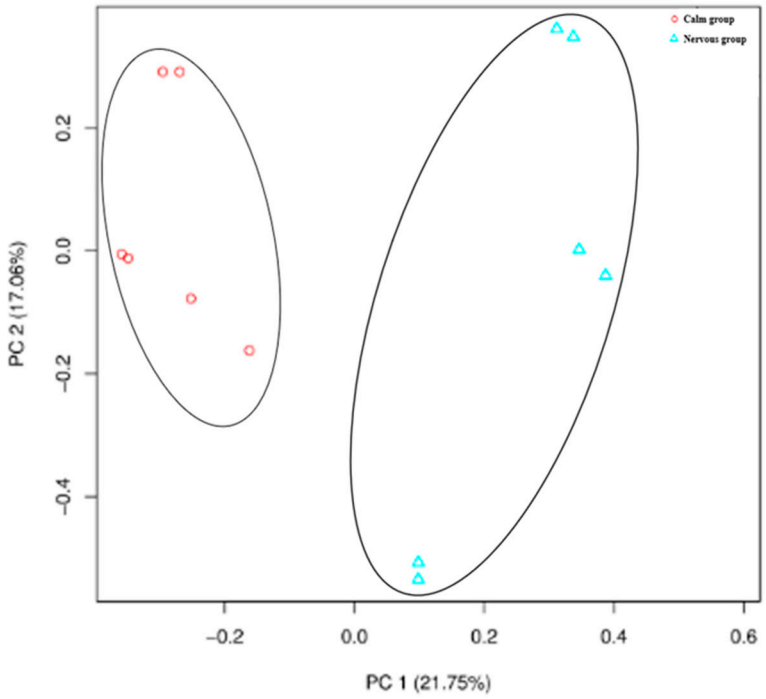

Figure 2. Principal component analysis of the β Diversity of the microbiome in *Hu* sheep with different temperaments.

3.3.2. The Effect of Temperament on the Relative Abundance of the Rumen Bacterial Community at the Phylum and Genus Level

Our taxonomic analysis showed that at the phylum level the bacterial community was predominantly *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, and *Tenericutes* (Figure 3). At the genus level, the dominant groups were *Prevotella* and *Prevotellaceae UCG-001* of phylum Bacteroidetes; *Christensenellaceae R-7 group*, *Ruminococcaceae NK4A214 group*, *Ruminococcaceae UCG-010*, *Ruminococcaceae UCG-003* and *Fibrobacter* of phylum *Firmicutes*; and *Treponema 2* of the phylum *Spirochaetes* (Figure 4).

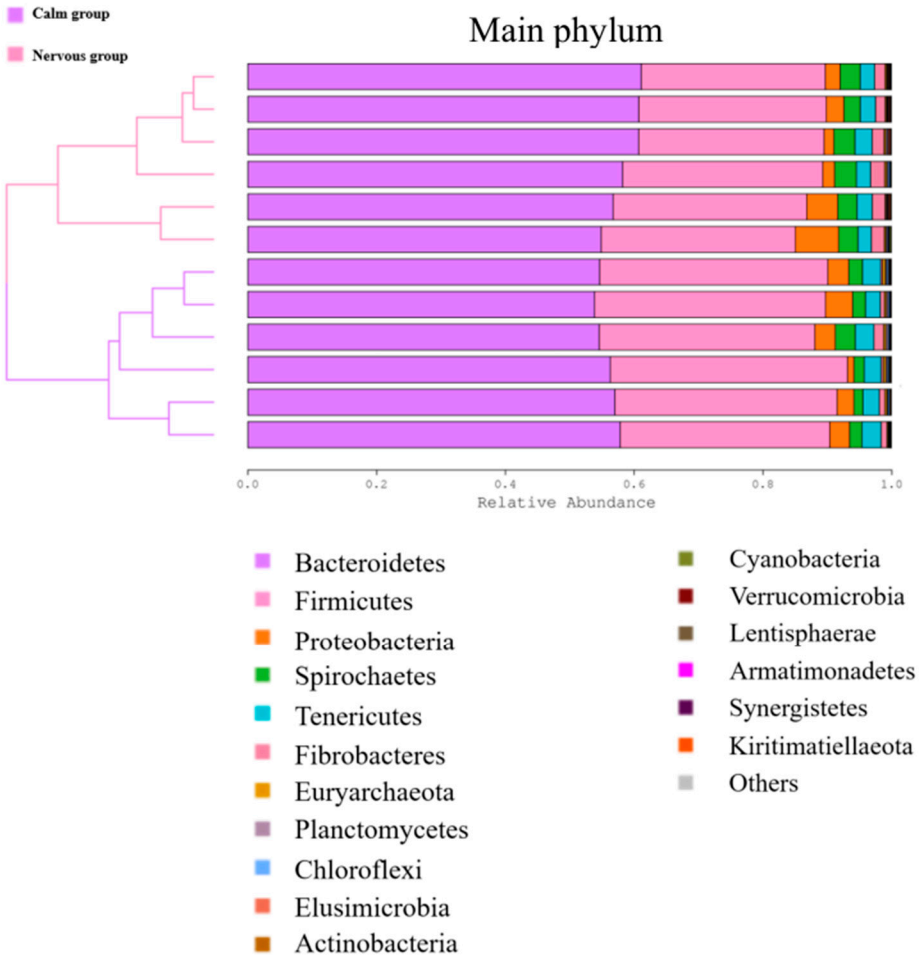

Figure 3. Composition of rumen bacteria at the phylum level in *Hu* sheep with different temperaments.

At the phylum level, the relative abundance of *Bacteroidetes* and *Spirochetes* was significantly higher in the nervous group than that in the calm group ($p < 0.05$; Table 5). The relative abundance of *Firmicutes* was significantly higher in the calm group than in the nervous group ($p < 0.05$; Table 5). There was no significant difference in the relative abundance of Proteobacteria between the two groups ($p > 0.05$; Table 5).

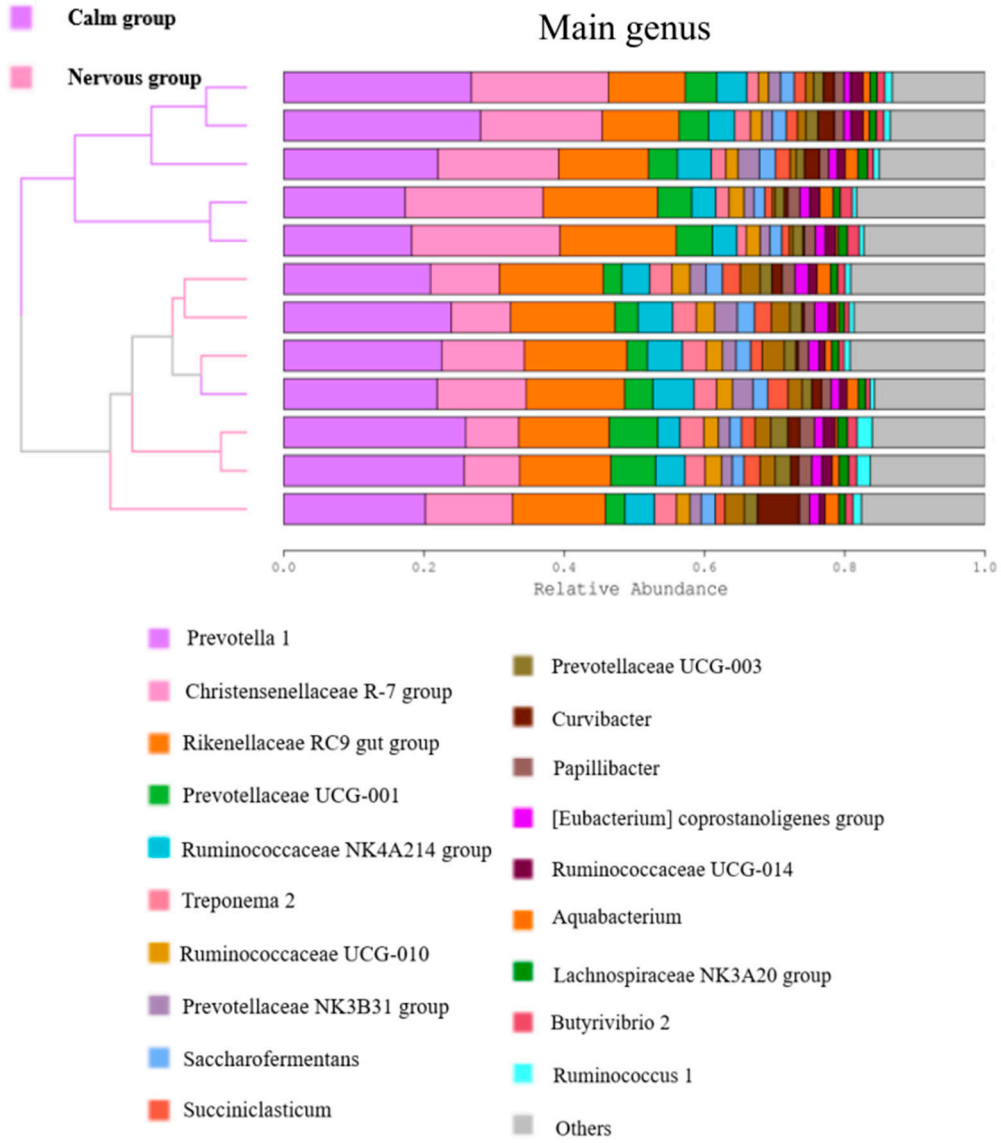

Figure 4. Composition of rumen bacteria at the genus level in *Hu* sheep with different temperaments.

Table 5. Relative abundance (%) of phylum strains of *Hu* sheep with different temperaments.

Phylum	Calm Group	Nervous Group	*p*-Value
Bacteroidetes	55.72 ± 0.64	58.74 ± 1.04	0.033
Firmicutes	34.78 ± 0.65	29.63 ± 0.39	< 0.01
Proteobacteria	2.87 ± 0.44	3.30 ± 0.83	0.650
Spirochaetes	2.04 ± 0.24	3.06 ± 0.12	0.004
Tenericutes	2.69 ± 0.10	2.32 ± 0.08	0.020

At the genus level, there were no significant differences in the relative abundance of *Prevotella*, *Prevotellaceae UCG-001*, *Ruminococcaceae UCG-010*, and *Ruminococcaceae NK4A214* between the two groups ($p > 0.05$; Table 6). The relative abundance of *Christensenellaceae R-7 group* in the calm group was significantly higher than in the nervous group ($p < 0.05$; Table 6). The relative abundance of *Treponema 2*, *Fibrobacter*, and *Ruminococcaceae UCG-003* in the nervous group was significantly higher than in the calm group ($p < 0.05$; Table 6).

Table 6. Relative abundance (%) of genus of *Hu* sheep with different temperaments.

Genus	Calm Group	Nervous Group	*p*-Value
Prevotella 1	16.77 ± 1.42	16.60 ± 0.70	0.917
Christensenellaceae R-7 group	13.37 ± 0.82	6.91 ± 0.61	< 0.01
Prevotellaceae UCG-001	3.35 ± 0.10	2.98 ± 0.58	0.546
Ruminococcaceae NK4A214 group	3.20 ± 0.32	3.03 ± 0.19	0.649
Treponema 2	1.56 ± 0.21	2.31 ± 0.60	0.006
Ruminococcaceae UCG-010	1.40 ± 0.96	1.65 ± 0.75	0.067
Ruminococcaceae UCG-003	1.05 ± 0.05	1.38 ± 0.10	0.014
Fibrobacter	0.76 ± 0.19	1.87 ± 0.11	< 0.01

3.4. Prediction of Bacterial Functions via KEGG

The predictions of bacterial function from KEGG pathways are shown in Figure 5A,B. The genes that were identified are related to metabolism, genetic information processing, and environmental information processing. Based on metabolic pathways, most of the predicted genes are involved in carbohydrate metabolism and amino acid metabolism.

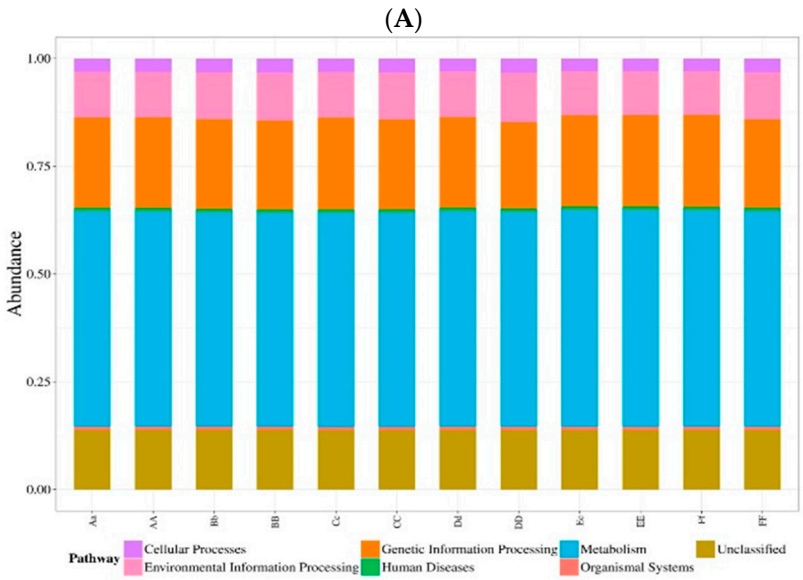

Figure 5. *Cont.*

Figure 5. KEGG functional annotation cluster heat map by pathways (**A**) and by functional group (**B**).

A comparative analysis of KEGG function prediction (Figure 6) showed that most of the functional genes that were different between the two temperament groups are related to carbohydrate metabolism and polysaccharide synthesis and metabolism. The genes related to carbohydrate metabolism were genes involved in N-glycan biosynthesis, mutual transformation of pentose and glucuronic acid, starch, and sucrose metabolism, glycosaminoglycan degradation, isoquinoline biogenic base biosynthesis, galactose metabolism, and glycosaminoglycan degradation. In addition, genes involved in lipoic acid metabolism, lipopolysaccharide biosynthesis, and lipopolysaccharide biosynthesis were also different between the two temperament groups.

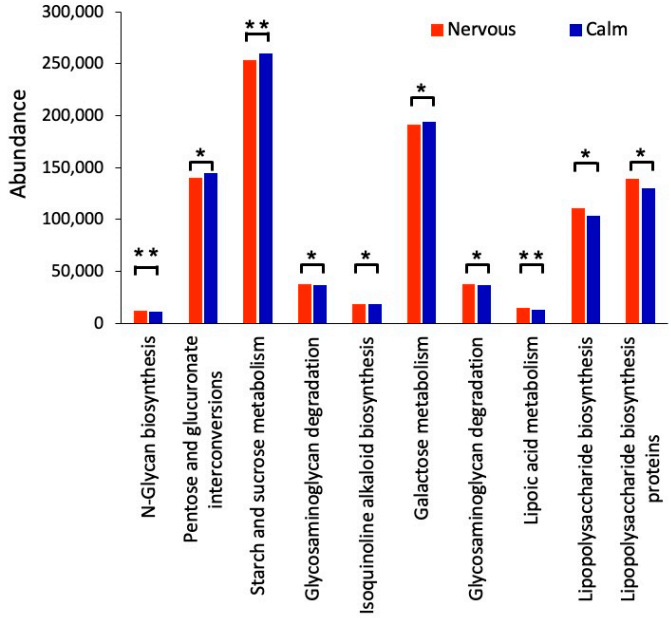

Figure 6. Comparison of the of KEGG function prediction between *Hu* sheep with different temperaments. *: $p < 0.05$, **: $p < 0.01$.

4. Discussion

The aim of this study was to assess the effect of temperament on the rumen microbiome and associated fermentation parameters in *Hu* Sheep when they were fed a standard ration. Our results suggest that there is a strong interaction between temperament and the rumen bacterial population. Differences in the bacterial population at both the phylum and genus levels between the two temperaments could explain the differences in metabolite profiles that we observed in our study. In addition, the differences in bacterial abundance suggest that some specific metabolic pathways, such as pathways in carbohydrate metabolism, could be different between calm and nervous sheep. Being a correlational study, it is not possible for us to conclude whether the temperament of sheep affects their rumen microbiome or whether the bacterial population affects the temperament. To our knowledge, the present study is the first demonstration of an interaction between temperament and the microbiome in sheep.

Rumen function was different between the groups as shown by the specific differences in fermentation parameters between the two temperament groups. While the pH of the rumen fluid was not different between the two temperament groups, the rumen ammonia nitrogen was higher in the calm group than it was in the nervous group. The ruminal pH value of *Hu* sheep was maintained between 6.51 and 6.71 in both groups, which would benefit the decomposition of fiber and protein synthesis by bacteria [46,47]. Interestingly, the differences in the ruminal concentration of ammonia nitrogen between the two temperament groups suggest that bacterial activity was lower in the calm sheep because most bacterial species are known to be able to utilize ammonia for the synthesis of nitrogenous compounds [48]. The differences in bacterial activity could have affected the balance states of protein degradation and microbial protein synthesis and ultimately the concentration of ammonia nitrogen [49]. It has to be noted that the concentration of ammonia nitrogen measured in the present study (2 to 4 mg/100 mL) was low compared to published values for the optimal concentration of ruminal ammonia nitrogen to synthesize microbial protein (8 to 30 mg/100 mL) [50]. It is possible that the time of sampling or the duration of storage of the samples prior to analysis might have led to a loss of rumen ammonia from the samples. However, the difference in ammonia nitrogen that we identified between the groups is probably reliable because the rumen samples were all collected within one hour and stored and processed together.

The concentrations of propionic acid in the rumen fluid of calm *Hu* sheep were significantly lower than they were in the nervous *Hu* sheep, while the concentration of acetic acid was similar between the two temperament groups. Therefore, the greater ratio of acetic acid to propionic acid (Calm: ~6:1, Nervous: ~4.5:1) indicates that there was a change in rumen fermentation mode towards propionic acid fermentation in the calm *Hu* sheep. Overall, the specific differences in fermentation parameters that we observed in the present study concurs with our finding that the microbiome was different between the two temperament groups.

The bacterial population was different between the two temperament groups and the differences were observed at the phylum level and at the genus level suggested that there are differences in metabolic pathways between the calm and nervous sheep. The bacterial population was less diverse (Shannon index) and less rich (Simpson index) in the calm sheep than in the nervous sheep, but the number of species (Chao value) was not different between the two temperament groups.

The difference in the relative abundance of the two most dominant phyla, *Bacteroidetes* and *Firmicutes*, suggests that the temperament of *Hu* sheep could be linked to these two phyla in the rumen. The relative ratio of *Bacteroidetes* and *Firmicutes* was different between the calm and the nervous sheep, suggesting differences in metabolic pathways between the two temperaments. In cattle, both of those phyla are involved in carbohydrate and protein metabolism [51,52]. In the present study, the relative abundance of *Bacteroidetes* was significantly higher in the nervous group than it was in the calm group. The different abundance of *Bacteroidetes* should result in a better efficiency of degradation of carbohydrate and protein and non-fiber plant polysaccharides [53] in the nervous sheep. The nervous *Hu* sheep could have had a higher carbohydrate metabolism and decomposition rate than the calm *Hu* sheep. The abundance of *Bacteroidetes* can be affected by the type of plant fiber that is ingested [54]. It could be possible that the nervous *Hu* sheep ate less fiber substances and more non-fiber substances during feeding, which would have induced an increase in the *Bacteroidetes* population. Interestingly, since the pH of the rumen fluid was similar between the two temperament groups, the decreased abundance of *Bacteroidetes* in the calm sheep was not due to a decrease of rumen pH value which is known to negatively affect the size of the *Bacteroidetes* population [52]. By contrast, the relative abundance of *Firmicutes* was significantly higher in the calm group than it was in the nervous group. *Firmicutes* are involved mainly in carbohydrate metabolism and are well suited to a wide range of environmental conditions [55]. So, the greater abundance of *Firmicutes* suggests that the calm sheep might be better adapted to a wider range of environmental conditions.

Amongst the least abundant phyla, the relative abundance of *Spirochetes* was significantly higher in the nervous group than it was in the calm group. The phylum *Spirochetes* is known to ferment plant polymers such as pectin, xylan, and arabinogalactan [56]. *Spirochetes* can also interact with other cellulolytic bacteria [57]. We suggest that the nervous *Hu* sheep had a better absorption and possibly utilization of carbohydrates from the feedstuff than did the calm *Hu* sheep. Although there was no difference in the relative abundance of *Proteobacteria* between the calm and nervous groups, *Proteobacteria* was the third most abundant phylum, and plays an important role in rumen metabolism [58–60].

Our analysis of the microbiome at the genus level supports the notion that temperament affected the capacity of sheep to process plant materials by affecting the interactions between rumen microorganisms. The relative abundance of *Fibrobacter, Treponema 2*, and *Ruminococcaceae UCG-003* was significantly higher in the nervous group than in the calm group. *Fibrobacter* are known to facilitate the degradation of cellulose and hemicellulose in the rumen [61,62]. In the rumen, *Treponema* are associated with the degradation of hemicellulose but do not utilize cellulose [63]. *Treponema* can affect the number of cellulolytic bacteria and then use soluble sugars that are released from cellulose by cellulolytic bacteria to produce metabolites such as succinate, acetate, and formate [63]. Altogether, *Treponema* enhances cellulose decomposition and, therefore, the rate of degradation of cellulose is higher in the nervous than the calm *Hu* sheep. Similarly, studies have provided

evidence supporting the involvement of *Ruminococcaceae* in fiber degradation and ruminal biohydrogenation [64–66]. Overall, it is highly possible that the nervous *Hu* sheep are better at decomposing cellulose than the calm sheep. Lastly, the relative abundance of *Christensenellaceae R-7 group* in the calm group was significantly higher than that of the nervous group. *Christensenellaceae R-7 group* belongs to the *Firmicutes* phylum which are often the dominant bacteria promoting the decomposition of cellulose by gastrointestinal microorganisms [54,67]. Interestingly, the relative abundance of *Christensenellaceae*, and the gut microbiome can be affected by several factors including host genetics [68]. Therefore, we can speculate that the calm *Hu* sheep have more *Christensenellaceae R-7 group* due to an impact of their temperament genetics. Further studies are required to identify the mechanisms that connect temperament genetics to the abundance of *Christensenellaceae*. In depth studies are needed to better understand the specific pathways that underly the interactions between the rumen microbiota and temperament in sheep.

5. Conclusions

The differences between temperaments at the phylum and genus levels suggest that sheep with different temperaments could have a different carbohydrate and polysaccharide metabolism. The nervous sheep could be more likely to metabolize carbohydrates and more inclined to metabolize polysaccharides into glycosaminoglycan and lipopolysaccharides.

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