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Age Differences in Ileum Microbiota Density: VFAs and Their Transport-Related Gene Interactions in Tibetan Sheep

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Abstract: Microbiota density plays an important role in maintaining host metabolism, immune function, and health, and age has a specific effect on the composition of intestinal microbiota. Therefore, the age-specific effects of age differences on the structure and function of the ileum microbiota in Tibetan sheep were investigated by determining the density of the ileum microbiota, the content of VFAs, and the expression levels of their transporter-related genes at different ages. The results showed that the contents of acetic acid and propionic acid in the ileum of Tibetan sheep in the 1.5-year-old group were significantly higher ($p < 0.05$) than those in other age groups, and that the contents of total VFAs were also significantly higher ($p < 0.05$) than those in other age groups. The relative densities of ileum *Rf*, *Ra*, and *Fs* were significantly higher in the 1.5-year-old group than in the other age groups ($p < 0.05$). The ileum epithelial VFAs transport-related genes *AE2*, *MCT-4*, and *NHE1* had the highest expression in the 1.5-year-old group, and the expression of *DRA* was significantly lower in the 1.5-year-old group than in the 6-year-old group ($p < 0.05$). Correlation analysis showed that *Cb*, *Sr*, and *Tb* were significantly positively correlated with butyric acid concentration ($p < 0.05$) and negatively correlated with acetic acid, but the difference was not significant ($p > 0.05$); *MCT-1*, *MCT-4*, and *AE2* were significantly positively correlated ($p < 0.05$) with acetic, propionic, and isobutyric acid concentrations; *NHE1*, *NHE2*, and *MCT-4* were highly significantly positively correlated ($p < 0.01$) with *Romboutsia* and *unclassified_Peptostreptococcaceae*, while acetic acid was significantly positively correlated ($p < 0.05$) with *NK4A214_group*; *Romboutsia*, and *unclassified_Peptostreptococcaceae* were significantly positively correlated ($p < 0.05$). Therefore, compared with other ages, the 1.5-year-old Tibetan sheep had a stronger fermentation and metabolic capacity in the ileum under traditional grazing conditions on the plateau, which could provide more energy for Tibetan sheep during plateau acclimatization.

Keywords: Tibetan sheep; ileum; VFAs; microbiota; related genes

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1. Introduction

The Tibetan sheep is a chordate of the Bovidae family of the mammalian order Artiodactyla and is native to Tibet, China; most of this primitive sheep breed lives in the Qinghai–Tibet Plateau region at 2500~5000 m above sea level under harsh habitat conditions (extreme cold, low oxygen, strong ultraviolet light, and nutrient stress in the cold season). The Tibetan sheep is also an indispensable economic animal for local herders [1], providing meat, wool, skin, fuel (animal dung as fuel), and other means of living. After a long period of natural selection, Tibetans and animals living on the Tibetan Plateau have adapted to the special plateau environment and have developed unique genetic predispositions, lifestyles, and eating habits [2]. The rumen microbiota of Tibetan sheep changed significantly during

adaptation to the alpine environment, and the host genome is significantly correlated with the rumen microbiota [3].

The digestive system of mammals is colonized by numerous complex microbiota, including bacteria, fungi, and protozoa. A close symbiotic relationship exists between the microbiota and the host, and the intestinal microbiota not only helps the host to digest and absorb food, thus providing energy and nutrients for the host, but also plays an important role in the process of the host's environmental adaptation [4]. Gut microorganisms help the host to degrade and metabolize cellulose, hemicellulose, and other indigestible substances so that these compounds can be utilized by the host. These microorganisms are classified into fiber-degrading bacteria (*Ruminococcus albus* (*Ra*), *Ruminococcus flavefaciens* (*Rf*), and *Fibrobacter succinogenes* (*Fs*)), acid-utilizing bacteria (*Clostridium butyricum* (*Cb*), *Selenomonas ruminantium* (*Sr*), and *Treponema bryantii* (*Tb*)) [5]. *Fs* is a dominant genus of cellulose-degrading bacteria that has a high capacity to break down pasture species and provide more energy in a short period of time, while also increasing the intestinal pH and acetic acid production, which in turn improves intestinal fermentation [6]. *Ra* is a highly dominant cellulose-degrading bacterium that can use cellulosomes to adhere to and break down cellulose [7]. *Fs* is an anaerobic bacterium that naturally colonizes the gastrointestinal tract of herbivores and is able to break down cellulose into cellulosic disaccharides and glucose, which are used as a carbon source for growth [8].

The composition of the gut microbiome is influenced by age, sex, genotype, diet, and health status [6]. Studies have shown that the diversity of gut microbiota changes as the host ages and stabilizes after reaching an adult microecology and digestive capacity. This trend is a result of a subsequent increase in microbial inoculation, colonization, and diversity in the host gut as the host ages, develops, and is exposed to different environments. Diversity improves the stability and function of the intestinal flora; in particular, intestinal flora diversity has been suggested to serve as a new biomarker of health and metabolic competence and to play an important role in host environmental adaptation [9,10]. An increasing gut microbial abundance with age has been demonstrated in humans [11], black howler monkeys [12], and rhesus monkeys [13]. Higher gut microbial diversity provides greater functional diversity to the host and contributes to a more stable gut microecosystem than that found in immature individuals. In addition, the composition and diversity of intestinal microbial metabolites (VFAs) play important roles in nutrient absorption, immunological resistance to disease, and the growth and metabolism of animals [14]. Studies have demonstrated that acetic acid and 3-hydroxybutyric acid produced by rumen fermentation are the main raw materials for fatty acid synthesis in the mammary epithelium [9]. In addition to being important energy substances in ruminants [15,16], VFAs are also involved in the regulation of leptin levels, insulin secretion, and immune responses; for example, VFAs regulate the immune response through *GPCR41* [17]. In addition, VFAs, as weak acids, are able to affect the pH in the gastrointestinal tract by dissociating hydrogen ions; lower pH values reduce intestinal enzyme activity, affecting the type of fermentation by the intestinal microbiota and an animal's productive performance [10]. After the removal of rumen anaerobic fungi from the rumen, the proportion of acetic acid and the concentration of total rumen VFAs in goats decreased significantly, but the proportion of propionic acid increased significantly [18]. The expression of intestinal VFAs transporter carriers is an important mode of nutrient transport in the intestine, and the main VFA transporter carriers are *Anion exchanger2* (*AE2*) [19], *Monocarboxylate transporter1* (*MCT-1*), *Monocarboxylate transporter4* (*MCT-4*) [20], and *Sodium hydrogen ion exchange protein* (*NHE*) [17]. *NHE*, as a cellular homeostatic regulator protein, maintains the normal physiological function of the cell by regulating the intracellular pH. In addition, *Down regulated in adenoma carrier* (*DRA*) is a Cl^-/HCO_3^- exchanger encoded by the *SLC26A3* gene, which is expressed mainly on intestinal epithelial cell membranes and is involved in physiological processes such as intestinal fluid absorption and cellular acid-base balance [21]. There is a synergistic interaction between *DRA* and *MCT-1*, and a functional coupling of *DRA* with *NHE2* and *NHE3* mediates the absorption of electrically neutral NaCl [22]. Therefore, the study of the

mechanism of absorption and transport of VFAs in the intestine is not only important for the regulation of the digestive and nutritional systems of animals, but also has a certain significance in guiding production practices.

Previous studies on the digestive flora of ruminants have focused mainly on the rumen, with less research conducted on the hindgut. The small intestine is an important organ involved in nutrient absorption, and changes in age cause rapid changes in microorganisms. As the end of the small intestine, the ileum has a high microbial content [23], and the roles played by the rumen and the ileum during the plateau acclimatization process of Tibetan sheep are different. The ileum microbiota also plays an important role [21], but the regulatory mechanism of its influence on the growth and development of the host and its metabolite absorption and metabolism is unclear. Studies on the ileum microbiota of Tibetan sheep at different ages and on their VFA absorption and metabolism are lacking. Therefore, the present study was conducted to comparatively analyze the fermentation function and flora structure of the ileum in Tibetan sheep of different ages to understand the changes in the uptake and metabolism of ileum VFAs and the expression of host genes, which will provide a new understanding of Tibetan sheep plateau acclimatization.

2. Materials and Methods

2.1. Test Animals and Sample Collection

In Haiyan County, Haibei Prefecture, Qinghai Province, China (altitude of 3500 m, plateau continental climate, annual precipitation of 400 mm), grazing Tibetan sheep (Eulerian type Tibetan sheep) were used as the research subject; four age groups—namely, 4-month-old (lambs, n = 6), 1.5-year-old (n = 6), 3.5-year-old (n = 6), and 6-year-old (adults, n = 6)—were randomly selected from the same pasture flock (approximately 200 sheep) to study their health statuses. There were six good Tibetan sheep ewes each, all of which grazed in the same pasture and were under local traditional natural grazing management (nomadic grazing: 'no winter grass in summer, no summer grass in winter') without any supplemental feeding, and 4-month-old lambs followed their mothers in grazing. The samples were collected in August 2020, and the pasture species and nutrient levels in the pastures are detailed in our team's previous research [21] (Table S1).

In accordance with ethical requirements, all the Tibetan sheep were euthanized—traditional jugular vein bloodletting was used to ensure rapid death—the gastrointestinal tract organs were removed immediately, and the ileum contents were collected. Approximately 200 mL of ileum contents were collected from each sheep, divided into freezer tubes, frozen in a liquid nitrogen tank, and returned to the laboratory for storage at −80 °C. This method was used to extract DNA from the ileum flora and to determine VFAs. In addition, a small part of the ileum tissue was cut immediately, the tissue contents were quickly washed with normal saline, and the tissue was cut into small pieces, divided into freezer tubes, stored in liquid nitrogen, and brought back to the laboratory for storage at −80 °C for subsequent RNA extraction.

2.2. Ileum VFA Determination

Ileum content samples were thawed at room temperature and centrifuged at 15,000 rpm for 15 min at 4 °C. The internal standard was 2-ethylbutyric acid (2 EB). One milliliter of the centrifuged supernatant was aspirated into a 1.5 mL centrifuge tube and 0.2 mL of 25% metaphosphoric acid solution (with internal standard 2 EB) was added. The solution was mixed well, placed in an ice bath for at least 30 min, and centrifuged at 15,000 rpm for 15 min. A quantity of 0.2 mL of 25% metaphosphoric acid solution (with internal standard 2 EB) was added, mixed well, and placed in an ice bath for 30 min or more. This was centrifuged at 15,000 rpm for 15 min. The supernatant was aspirated with a 2 mL syringe, placed in the organic phase of a 0.22 μm filter head, filtered put into 2 mL brown vials, and stored at −20 °C, pending online assay.

An Agilent 7890 B gas chromatograph was used for the determination of VFA content. The chromatographic column was an AT. The FFAP capillary column was 30 m × 0.32 mm × 0.50 μm and the temperature of the inlet port (SSL) was 250 °C; the temperature of the detector (FID) was

250 °C; the carrier gas was high-purity nitrogen (99.999%), the total pressure was 100 kPa, and the splitting ratio was 5:1. The flow rate of the gas was as follows: air at 400 mL/min, H_2 at 35 mL/min, and N_2 at 40 mL/min. The injection volume was 1 μL. The heating procedure was as follows: 120 °C for 3 min, 10 °C/min to 180 °C, and 180 °C for 1 min. The retention time for each sample was 10 min.

2.3. DNA Extraction and Detection

An E.Z.N.A.® Stool DNA Kit (Omega, Shanghai, China) was used to extract total ileum microbial DNA from the Tibetan sheep. For extraction, 19 mL and 80 mL of anhydrous ethanol were added to the VHB Buffer and DNA Wash Buffer reagents, respectively, for dilution. Samples of fresh intestinal fluid of less than 200 mg were transferred into a 2 mL inlet centrifuge tube. If the sample was a solution, 200 μL of solution was added to the centrifuge tube, whereas if the sample was not thawed, the sample was scraped into the centrifuge tube before thawing. SLX-Mlus Buffer was added before the sample thawed, and 200 mg Glass Beads X (on ice) were added. Then, 540 μL SLX-Mlus Buffer was added, and the mixture was swirled for 10 min at the maximum rotational speed (≥1000 r/min) to thoroughly homogenize the fecal samples. Then 60 μL of DS buffer and 20 μL of Proteinase K were added, and the mixture was swirled thoroughly. The mixture was incubated at 70 °C for 10 min (the sample was shaken up and down irregularly to help it crack) and the centrifuge tube was closed. After that, the extraction steps of the kit were strictly followed, and the DNA was stored in a −20 °C refrigerator for later use.

After extraction, a 3 μL DNA sample was taken and the concentration and purity were measured via a Therm Nano Drop-2000. Then, agarose gel electrophoresis was per-formed (Agient2100, LabChip GX) (Beijing Wuzhou Dongfang Technology Development Co) and EB staining was performed for 30 min. The DNA integrity was imaged under UV light from the gel imager (if there was obvious degradation, the DNA of the corresponding sample had to be extracted again) (Figure 1). The DNA samples were stored at −80 °C.

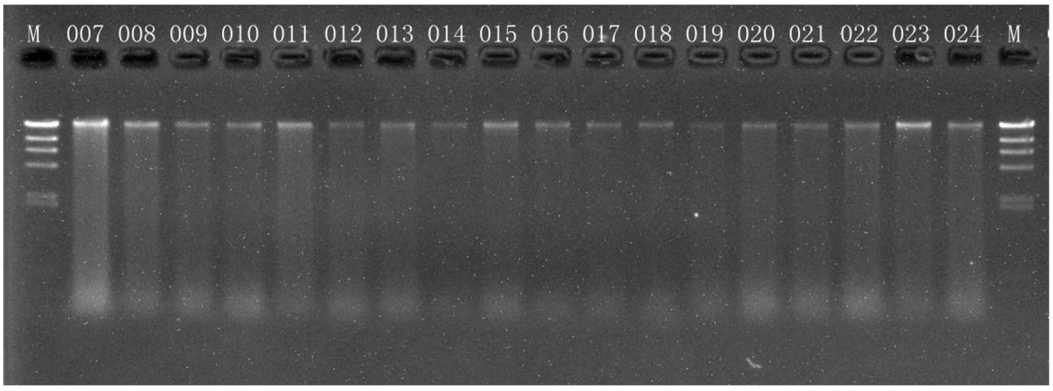

Figure 1. DNA concentration and purity gel electrophoresis assay plot.

2.4. RNA Extraction and Detection

Total RNA was extracted from the ileum tissue of Tibetan sheep using Trizol reagent. Before the extraction of total RNA, the extraction consumables were autoclaved and consumables, such as sterile enzyme-free gun tips and centrifuge tubes, were used. The frozen tissue samples were extracted from the frozen storage tube, and approximately 100 mg of each tissue sample was clipped and put into a mortar (adding new liquid nitrogen continuously), ground into a powder (showing no obvious particles), and transferred to a centrifuge tube. A total of 1000 μL of RNA separation solution was added, the mixture was allowed to stand for 5 min (the lysate was gently aspirated with a pipette to make it transparent), and the mixture was covered with a centrifuge tube. The traditional Trizol reagent method was subsequently used for extraction, and the extraction process was carried out on an ultraclean workbench. A small amount of sample was used for testing, and the rest was kept at −80 °C for later use. After extraction, 2 μL RNA samples were taken, the

concentration and purity of the RNA were detected via an ultramicrospectrophotometer (Therm Nano drop-2000), and the RNA concentration (ng/μL) and purity (260 nm/280 nm (about 1.8–2.1) were recorded, respectively. The integrity of the RNA was then detected via agarose gel electrophoresis. RNA concentration (ng/μL) = OD260 × dilution factor × 40.

cDNA was synthesized via the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) reverse transcription kit. The detailed operation was carried out in strict accordance with the kit instructions. The RNA stock solution was diluted before reverse transcription, and the amount of RNA used was controlled to be 1 pg^{-1} μg, with 20 μL of reaction mixture. After reverse transcription was completed, the cDNA stock solution was diluted 10 times and stored at −80 °C for subsequent fluorescence quantification.

2.5. Fluorescent Quantitative PCR Primer Design and Amplification

Using ileum microbial RNA as a template, VFA absorption-related genes (*AE2*, *DRA*, *MCT-1*, *MCT-4*, *NHE2*, and *NHE1*) were selected for a comparative quantitative study. Using ileum microbial DNA as a template (Table S2), a relative quantitative study was conducted on ileum microbe groups *Rf*, *Fs*, *Cb*, *Sr*, *Tb*, and *Ra* (Table S3); NCBI BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov) (accessed on: 1 June 2023.) was used to search for bacterial 16S rRNA sequences, and Primers 5.0 software was used to design specific primers. The primers were synthesized by the Shanghai Biological Corporation. Bacteria and β-actin were taken as internal parameters, and the sequences of the bacterial primers were referred to from Muyze et al. [21]. The relative contents of the *Cb*, *Sr*, *Tb*, *Rf*, *Fs*, *Ra*, and VFA absorbation-related genes *AE2*, *DRA*, *MCT-1*, *MCT-4*, *NHE2*, and *NHE1* in the ileum were determined via ABI QuantStudio-6. The PCR reaction system consisted of 20 μL, which included 2 × SuperReal PreMix Plus 10 μL, upstream and downstream primers 0.6 μL, template 1 μL, 50 × RO × Reference Dye 0.4 μL, and ddH_2O 7.4 μL. The reaction parameters were predenaturation at 95 °C for 15 min, denaturation at 95 °C for 10 s, and annealing at 60 °C for 32 s, and a total of 40 cycles was performed.

2.6. Ileum 16S rRNA Sequencing

This part of the data was already available and only interactions were analyzed in this study [21].

2.7. Data Statistics

Single factor variance in SPSS software (SPSS version 24.0, ANCOVA) was used to analyze the significance of differences in the expression of VFAs, VFA-related genes, and bacterial population density in the ileum of Tibetan sheep at different ages ($p < 0.05$). The difference of 0.05 was significant and had statistical significance. Spearman's correlation test was used to analyze the correlation between the expression of VFAs and related transporter genes and ileum colony density, as well as the correlation between the expression of VFAs, related transporter genes, and the ileum microbial species (relative abundance > 0.5%).

3. Results

3.1. Determination of VFA Concentrations and Proportions in the Ileum of Tibetan Sheep at Different Ages

As can be seen in Table 1, there were differences in ileum VFA concentrations among the different ages of Tibetan sheep, and the total ileum VFA content was higher in the 1.5-year-old group than in the other age groups ($p < 0.05$). Among them, acetic and propionic acid contents were significantly higher ($p < 0.05$) in the 1.5-year-old group than in other age groups; isobutyric acid content was significantly higher ($p < 0.05$) in the 3.5-year-old group than in the 1.5-year-old group; and butyric acid content was significantly higher ($p < 0.05$) in the 4-month-old group than in the 3.5-year-old group, and did not differ significantly ($p > 0.05$) from that of the 1.5-year-old group. Isovaleric acid content was higher in the 1.5-year-old group than in the 3.5-year-old and 4-month-old groups, with a non-significant difference ($p > 0.05$), but significantly lower than in

the 6-year-old group ($p < 0.05$); valeric acid content was significantly higher in the 6-year-old group than in the other age groups ($p < 0.05$).

Table 1. Concentrations of VFAs in the ileum of Tibetan sheep at different ages.

VFAs Concentration (mmol/L)	4 M	1.5 Y	3.5 Y	6 Y	*p*
Acetic acid	4.94 ± 1.990 ^{b}	9.70 ± 1.201 ^{a}	5.21 ± 0.691 ^{b}	4.53 ± 1.220 ^{b}	<0.01
Propionic acid	0.69 ± 0.431 ^{c}	1.76 ± 0.010 ^{a}	1.16 ± 0.140 ^{b}	1.26 ± 0.080 ^{b}	<0.01
Isobutyric acid	0.31 ± 0.082 ^{a}	0.13 ± 0.003 ^{b}	0.32 ± 0.030 ^{a}	0.28 ± 0.010 ^{a}	<0.01
Butyric acid	0.53 ± 0.171 ^{a}	0.43 ± 0.010 ^{ab}	0.28 ± 0.010 ^{b}	0.44 ± 0.040 ^{ab}	<0.01
Isovaleric acid	0.19 ± 0.060 ^{b}	0.26 ± 0.031 ^{b}	0.19 ± 0.051 ^{b}	0.96 ± 0.030 ^{a}	<0.01
Valeric acid	0.29 ± 0.061 ^{c}	0.26 ± 0.010 ^{c}	0.43 ± 0.001 ^{b}	0.61 ± 0.050 ^{a}	<0.01
Total VFAs	6.94 ± 1.670 ^{b}	12.53 ± 1.221 ^{a}	7.58 ± 0.481 ^{b}	8.08 ± 1.300 ^{b}	0.02

Note: Different lowercase letters in the same line indicate significant differences between different ages, $p < 0.05$ level. 4 M: 4-month age group; 1.5 Y: 1.5 years age group; 3.5 Y: 3.5 years age group; 6 Y: 6 years age group.

3.2. Determination of Ileum Flora Density of Tibetan Sheep at Different Ages

There were significant differences ($p < 0.05$) in ileum microbiota densities among different ages of Tibetan sheep (Figure 2); *Rf* had the highest relative densities in all the four age groups, and the relative densities of *Rf* were significantly higher in the 1.5-year-old group than in the other age groups ($p < 0.05$). The relative densities of *Cb* and *Tb* had significantly higher relative densities in the 3.5-year-old group than the other age groups ($p < 0.05$). *Fs* and *Ra* had significantly higher relative densities in the 1.5-year-old group than the other age groups ($p < 0.05$); the relative density of *Sr* was significantly higher ($p < 0.05$) than other age groups at 4 months of age, with the 1.5-year-old group being higher than the 3.5-year-old group and the 6-year-old group, which was not significant ($p > 0.05$).

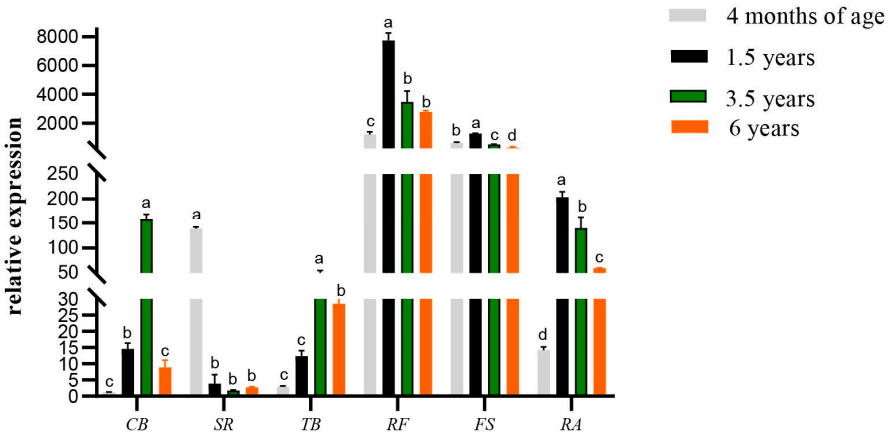

Figure 2. Study on ileum microbiota density of Tibetan sheep at different ages. Note: Lowercase letters on different colored bars indicate significant differences between ages, $p < 0.05$. *CB*: *Clostridium butyricum*; *SR*: *Selenomonas ruminantium*; *TB*: *Treponema bryantii*; *RF*: *Ruminococcus flavefaciens*; *FS*: *Fibrobacter succinogenes*; *RA*: *Ruminococcus albus*.

3.3. Determination of VFA-Related Gene Expression in the Ileum of Tibetan Sheep at Different Ages

Differences in the expression of genes related to the transport of VFAs in the ileum of Tibetan sheep of different ages are shown in Figure 3. Among them, *AE2*, *MCT-4*, and *NHE1* had the highest expression in the 1.5-year-old group, and the expression of *AE2* and *NHE1* was significantly higher in the 1.5-year-old group than in other age groups ($p < 0.05$). The expression of *DRA* in the 1.5-year-old group was significantly lower than that in the 6-year-old group ($p < 0.05$). The expression of *MCT-1* in the 6-year-old group was significantly higher than that in the 4-month-old and the 1.5-year-old groups ($p < 0.05$) The expression of *NHE2* was significantly higher in the 4-month-old group than in the other age groups ($p < 0.05$).

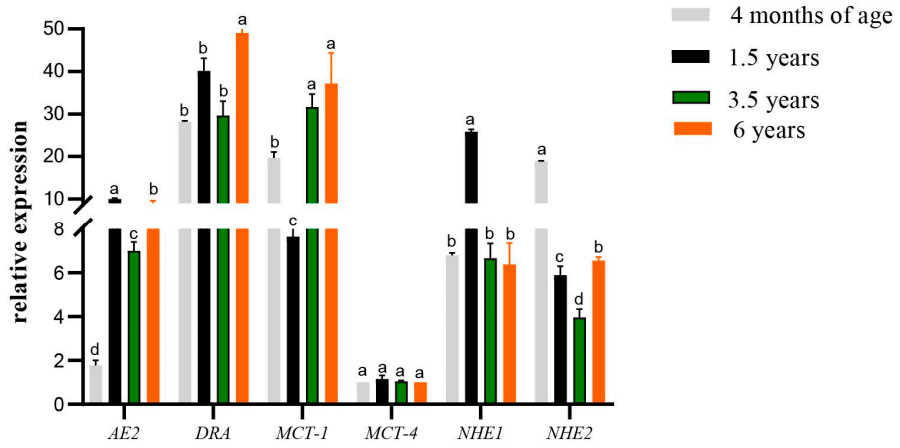

Figure 3. Expression of VFA-related genes in the ileum of Tibetan sheep at different ages. Note: Lowercase letters on different colored bars indicate significant differences between ages, $p < 0.05$.

3.4. Correlation Analysis of Ileum VFA Concentration and Bacterial Population Density of Tibetan Sheep at Different Ages

Through the correlation analysis of ileum VFAs and ileum flora density of Tibetan sheep at different ages, it was found that there was a correlation between ileum VFAs and ileum flora density of Tibetan sheep (Figure 4). Among them, *Cb*, *Sr*, and *Tb* were significantly positively correlated with butyric acid ($p < 0.05$), were negatively correlated with acetic acid operation, and had no significant difference. *Sr* and *Tb* were significantly positively correlated with propionic acid ($p < 0.05$). *Rf* showed significant positive correlation with acetic acid parameters, propionic acid, valeric acid, and isobutyric acid ($p < 0.05$); *Fs* showed a significant positive correlation with acetic acid parameters, propionic acid, valeric acid, isovaleric acid, and isobutyric acid ($p < 0.05$); and *Ra* was positively correlated with acetic acid operation, propionic acid, and isobutyric acid, with significant differences ($p < 0.05$). *Fs* and *Rf* were negatively correlated with butyric acid, with insignificant differences ($p > 0.05$).

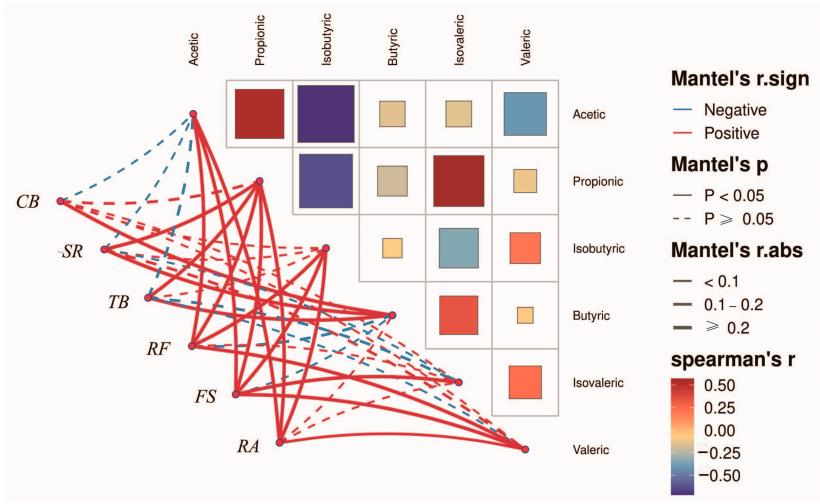

Figure 4. Study on the correlation between ileum microbiota density and VFA concentrations in Tibetan sheep at different ages. Note: The blue line indicates a negative correlation, the red line indicates a positive correlation, the solid line indicates $p < 0.05$, and the dotted line indicates $p \geq 0.05$. *CB*: *Clostridium butyricum*; *SR*: *Sele-nomonas ruminantium*; *TB*: *Treponema bryantii*; *RF*: *Ruminococcus flavefaciens*; *FS*: *Fibrobacter suc-cinogenes*; *RA*: *Ruminococcus albus*.

3.5. Correlation Analysis between VFA Concentration and Transport-Related Gene Expression in the Ileum of Tibetan Sheep at Different Ages

Through the correlation analysis of the ileum VFAs of Tibetan sheep at different ages and its transport-related genes, it was found that there was a correlation between the ileum VFAs of Tibetan sheep and their transport-related genes (Figure 5). The *AE2* gene showed a significant positive correlation with acetic acid, propionic acid, isobutyric acid, and butyric acid ($p < 0.05$); there was no significant negative correlation with isovaleric acid; the *DRA* gene was positively correlated with isovaleric acid and valeric acid ($p < 0.05$). *MCT-1* and *MCT-4* were positively correlated with and significantly different ($p < 0.05$) from acetic acid, propionic acid, and isobutyric acid, and negatively correlated with butyric acid, although the difference was not significant; *NHE1* showed significant positive correlation with acetic acid operation, propionic acid, and isobutyric acid ($p < 0.05$); *NHE2* was negatively correlated with acetic acid operation and isovaleric acid, but the differences were not significant ($p > 0.05$).

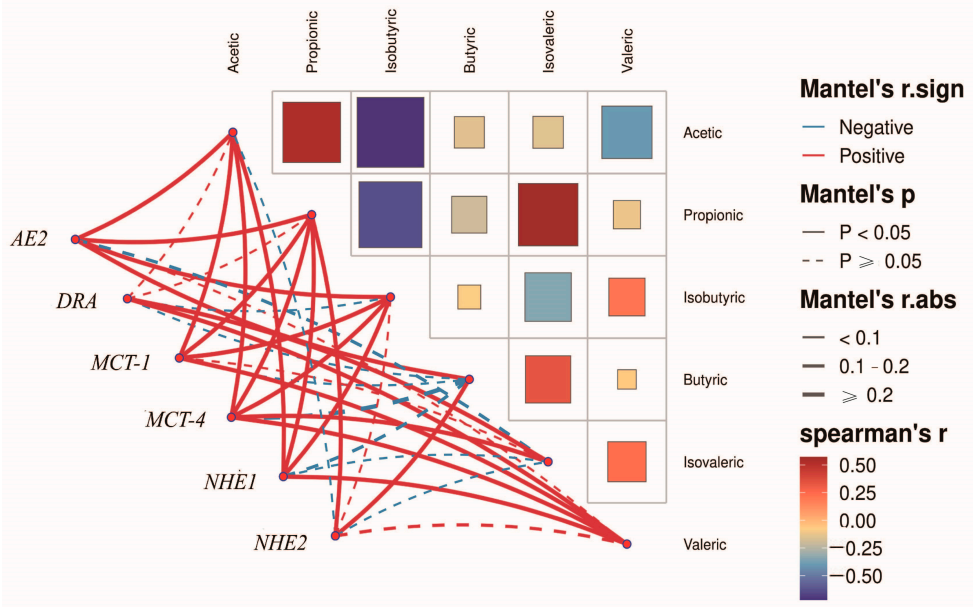

Figure 5. Correlation between VFA concentration and transport-related gene expression in the ileum of Tibetan sheep at different ages. Note: The blue line indicates a negative correlation, the red line indicates a positive correlation, the solid line indicates $p < 0.05$, and the dotted line indicates $p \geq 0.05$.

3.6. Association Analysis of Ileum Microbiota-VFAs and Their Transport-Related Genes in Tibetan Sheep of Different Ages

A heat map of the correlation between the ileum microbiota of Tibetan sheep (the top 20 genera in relative abundance) [21], the concentration of VFAs in the ileum, and the genes associated with VFA transport was generated (correlation threshold > 0.5) (Figure 6). It was found that *NHE1*, *NHE2*, and *MCT-4* were significantly positively correlated with *Romboutsia* and *unclassified_Peptostreptococcaceae* ($p < 0.05$), and that *AE2*, *MCT-4*, and *NHE1* were significantly positively correlated with *Candidatus_Saccharimonas* ($p < 0.05$); *AE2* showed a significant positive correlation with *uncultured_rumen_bacterium* ($p < 0.05$). *DRA* was significantly positively correlated with *Escherichia_Shigella* ($p < 0.05$), *MCT-1*, and the following: *unclassified_[Eubacterium]_coprostanoligenes_group*, *UCG_005*, and *unclassified_UCG_010*; *Christensen-ellaceae_R_7_group* was also significantly positively correlated ($p < 0.05$). *NHE2* was significantly positively correlated with *Clostridium_sensu_stricto_1* ($p < 0.05$). There was a significant negative correlation between *MCT-1* and *Romboutsia*; *unclassified_Peptostreptococcaceae* ($p < 0.05$), which showed significant negative correlation with *uncultured_rumen_bacterium* and *Candidatus_Saccharimonas* ($p < 0.05$), as well as *DRA* and *AE2* were significantly negatively correlated with *Brevinema* ($p < 0.01$). *NHE2* was negatively correlated with *Christensenellace-*

ae_R_7_group and *unclassified_[Eubacterium]_coprostanoligenes_group* ($p < 0.05$). Acetic acid was positively correlated with *NK4A214_group*, *Romboutsia*, and *unclassi-fied_Peptostreptococcaceae*. Valeric acid was positively correlated with *Romboutsia*; *unclassi-fied_Peptostreptococcaceae* ($p < 0.05$), propionic acid, and isovaleric acid were significantly positively correlated with *Brevinema* ($p < 0.05$), and propionic acid was significantly positively correlated with *unclassified_UCG_010* and *UCG_005* ($p < 0.05$). *Escherichia_Shigella* was significantly negatively correlated with isovaleric acid and butyric acid ($p < 0.05$), isobutyric acid was significantly negatively correlated with *UCG_005* and *Brevinema* ($p < 0.05$), and propionic acid was significantly negatively correlated with *uncultured_rumen_bacterium* and *Candidatus_Saccharimonas* ($p < 0.05$).

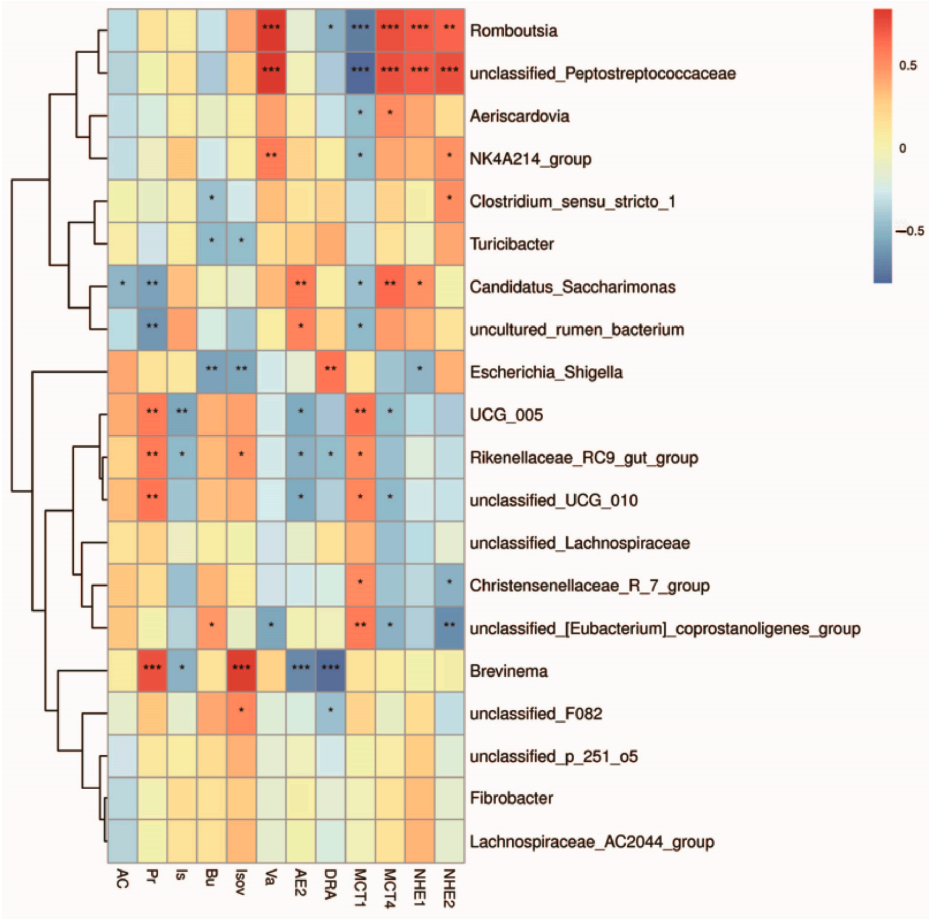

Figure 6. Correlation analysis of ileum microbiota-VFAs and their transport-related genes in Tibetan sheep of different ages. Note: AC: Acetic acid; Pr: Propionic acid; Is: Isobutyric acid; Bu: Butyric acid; Isov: Isovaleric acid; Va: Valeric acid, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The Tibetan sheep, a sheep breed endemic to the Tibetan Plateau, derives energy mainly through the gastrointestinal fermentation of natural pastures. The main components of these pastures are cellulose and hemicellulose, and cellulase, an important enzyme for the degradation and digestion of fibrous material, can be secreted by fibrolytic bacteria to improve this process [8]. *Ra* contains cellulosomes that can adhere to and digest cellulose; its genes encode cellulases and hemicellulases [24], and its final metabolites are various VFAs. *FS*, as a source of carbon for growth, is a potent biomass degrader, and its outer membrane proteins are involved in cellulose degradation [25]. In this study, the densities of *Ra* and *FS* were greater in the ileum of Tibetan sheep in the 1.5-year-old group, and *Ra* and *Fs* were significantly positively correlated with acetic, propionic, and isobutyric acids, indicating that the ileum of Tibetan sheep in the 1.5-year-old group had a strong capacity to degrade fibrous material and that the VFAs produced by its degradation entered

the bloodstream to provide energy for the organism. In practical production applications, part of the energy that enters the bloodstream is utilized by the body to promote growth, whereas the other part is deposited in the muscles. The diversity of the intestinal microbiota increases with age, and some studies have reported *Rf* and other fiber-degrading bacteria in the digestive tract of 5-week-old calves, although Streptococcus spp. and Chondrichthyes spp. were not observed [26]. The relative densities of *Rf* were the highest in all four age groups, and *Rf* was significantly positively correlated with acetic, propionic, valeric, and isobutyric acids. Thus, ruminants have been colonized by a large number of fiber-degrading bacteria in the gut since birth, and VFAs produced by fermentation of gut microorganisms provide a source of energy for their growth and development.

Microbial diversity is richer in the rumen than in the small intestine, but the rumen digestate passes through it more rapidly than it does the small intestine, and undigested nutrients in the small intestine encourage the growth of a large number of microorganisms to improve productivity. Intestinal VFAs are the products of carbohydrate degradation by intestinal microorganisms [27], which can regulate the intestinal pH, inhibit the proliferation of harmful pathogens [28], protect the intestinal mucosal barrier [29], and provide energy to the animal body and intestinal cells [30]. In this study, the total ileum VFA content of Tibetan sheep in the 1.5-year-old group was significantly greater than that in the other age groups, and the contents of acetic acid and propionic acid also significantly increased. At this age, the intestinal development of Tibetan sheep has matured, the amount and speed of feed intake have peaked, and the ileum fermentation capacity is strong. The VFAs produced are absorbed through the ileum epithelium and transported into the bloodstream to provide energy for the body via VFA transport carrier proteins, which ensures highly efficient production performance and better adaptation to the plateau environment. With age, the fermentation capacity of the ileum decreases, resulting in a decrease in the VFA content, so the total VFA content in the ileum of Tibetan sheep in the 6-year-old group was lower than that in the younger age groups. The content of butyric acid in the ileum was significantly greater in the 4-month-old group than in the 3.5-year-old group. Butyric acid, as a major microbial metabolite, not only improves the growth performance of the body, but also enhances the immune system [22], and a high concentration of butyric acid has an intestinal barrier function in 4-month-old lambs [12]. It has been reported that dietary fiber increases butyric acid-producing bacteria and improves the growth performance of weaned piglets [31]. Propionic acid is the main precursor for the synthesis of glucose required for metabolism by the gluconeogenesis pathway in liver tissues. In the present study, the ileum propionic acid content was significantly greater in the 1.5-year-old group than in the other age groups, and was able to provide more energy to the Tibetan sheep for growth and fat deposition. VFAs enter the bloodstream for body use through transporter carriers, and the relative expression of transporter carriers is an important indicator of the absorption capacity of the small intestine. In this study, the expression of *DRA* and *MCT-1* was significantly upregulated in the 6-year-old group; *MCT-1* plays a crucial role in the transport and absorption of VFAs [32], and there is a synergistic effect between the VFA^-/H^+ exchange carriers *DRA* and *MCT-1* [22]. It has been reported that the expression of *MCT-1* in the gastrointestinal tract of calves that have not started ruminating is lower than that of adult cattle, and that the concentration of VFAs is correlated with the expression of *MCT-1* in the gastrointestinal tract [33]. These findings suggest that, with age, Tibetan sheep need more VFAs to maintain energy requirements during adaptation to the plateau environment, which explains the lower concentration of VFAs in the ileum of Tibetan sheep in the 6-year-old group. Certain fragments in the transmembrane structural domain of *AE2* can play important roles in maintaining the cellular pH environment and intra- and extracellular ion homeostasis in intra- and extracellular pH-regulated ion transport [34]. Similarly, *NHE1* plays an important role in maintaining the cellular pH environment and its transport mode is an important mechanism for maintaining the pH of tumor cells [35]. In this study, the expression of *AE2*, *MCT-4*, and *NHE1* was significantly upregulated in the 1.5-year-old group, which may be related to the fact that the ileum microbes of Tibetan

sheep in the 1.5-year-old group produced more VFAs to prevent intestinal acidosis and regulate intestinal homeostasis. Correlation analysis revealed that *AE2*, *MCT-1*, and *MCT-4* were positively correlated with acetic, propionic, and isobutyric acid. Transporter carriers such as *MCT-1* transport VFAs, such as acetic acid and propionic acid, into the bloodstream, where they are absorbed and utilized by the body, suggesting that VFAs are transported by a variety of transporter proteins in the epithelium.

The interactions and relationships among VFA content, the intestinal microbiota, and the host are receiving increasing attention. In this study, by constructing a heatmap of the correlation between the ileum microbiota (genus level microorganisms in the top 20 in terms of relative abundance) [21], ileum VFA concentrations, and the genes related to the transport of VFAs in Tibetan sheep, a correlation was found. *Romboutsia* produces VFAs, which can improve metabolic endotoxemia and protect the intestinal barrier [36], as well as play an important role in gut health. Valeric acid modulates C3 signaling in the brain, including the modulation of immune and inflammatory responses and neuronal activity [37]. *Romboutsia* is highly significantly and positively correlated with valeric acid, and their combined effect protects the health of Tibetan sheep. Correlation analysis revealed that the *NK4A214_group* was significantly and positively correlated with ileum acetate, which in turn was significantly and positively correlated with glycolysis [38]. During exercise training in mice, acetic acid significantly increased muscle expression of key enzymes involved in fatty acid oxidation and sugar degradation, leading to oxidative fiber-type conversion [39]. Acetyl coenzyme A produced by acetic acid can be preferentially used for citric acid synthesis [39]. These findings suggest that the *NK4A214_group* may have some effect on acetic acid by affecting the glycolytic pathway, further leading to differences in the ileum acetic acid content at different ages. The intestinal flora plays an extremely important role in nonspecific prevention of colonization by enteric pathogens. Resistance to *Escherichia_Shigella* proliferation in mixed cultures was found to be due to the production of acetic acid and other VFAs by *E. coli*, which has a bactericidal effect on *Escherichia_Shigella* [38]. Therefore, VFAs are considered to be important factors in the exclusion of pathogens from the intestinal tract. *Escherichia_Shigella* was significantly negatively correlated with isovaleric and butyric acids in this study, and it has been reported that certain low levels of VFAs and the low pH of the intestinal contents are key factors in preventing the growth of enteropathogenic bacteria [40]. Therefore, Tibetan sheep can effectively inhibit *Escherichia_Shigella* proliferation via VFAs produced by the organism. Propionic acid was significantly negatively correlated with *uncultured_rumen_bacterium* and *Candidatus_ Saccharimonas*, which are associated with inflammatory diseases and obesity [41], suggesting that the VFAs produced by the intestinal flora can protect against various diseases caused by microorganisms in addition to aiding their growth and development. The ileum microbiota of Tibetan sheep of different ages under natural grazing conditions on the plateau differed to some extent, and with growth and development the diversity of the intestinal flora increased and the abundance of microorganisms related to nutrient digestion increased, which ultimately led to differences in their fermentation functions. Microbiota produce energy substances, VFAs, through fermentation, which are further transported to the bloodstream via ileum epithelial transit carrier proteins to provide energy. This interaction mechanism plays an important role in maintaining the nutrient balance between tissue cells and the gut environment and regulating the homeostasis of the gut environment (Figure 7). At present, we only studied the ileum of Tibetan sheep of different ages, and found that there are differences in the fermentation ability of the ileum of Tibetan sheep based on age. We need to analyze the remaining intestinal segments in detail, which will provide new research ideas for the plateau adaptation of Tibetan sheep and a certain experimental basis for the daily feeding management of Tibetan sheep.

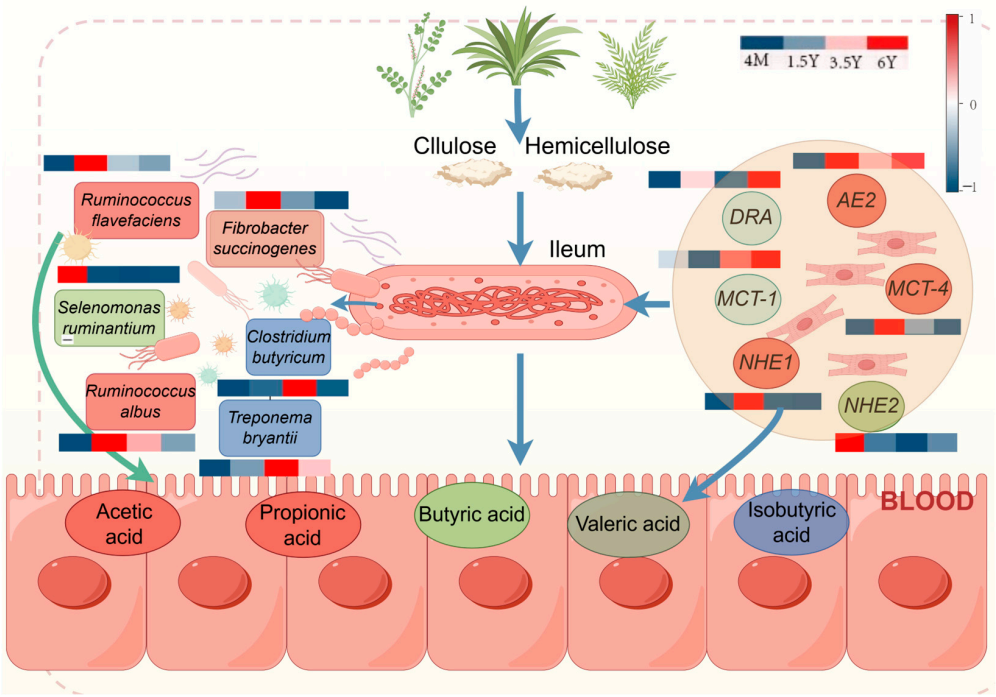

Figure 7. The expression interaction between ileum microbiota and VFAs and their transport-related genes in Tibetan sheep of different ages. Note: 4 M: 4-month age group; 1.5 Y: 1.5 years age group; 3.5 Y: 3.5 years age group; 6 Y: 6 years age group. The heatmaps in the pathway represent the concentrations of flora, genes, and VFAs in the four age groups: the redder the color, the higher the gene expression.

5. Conclusions

The total VFA, acetic acid, and propionic acid contents in the ileum of 1.5-year-old Tibetan sheep were significantly greater than those in the other age groups under the same feeding management method; moreover, the VFA transporter-related genes were highly expressed in the ileum of 1.5-year-old Tibetan sheep, which presented better energy-material transporter efficiency and transported more VFAs into the bloodstream to provide energy. A large number of fibrinolytic bacteria were found in the ileum of Tibetan sheep in all four age groups, with the highest relative densities of *Rf*, *Fs*, and *Ra* found in the ileum of 1.5-year-old Tibetan sheep. The degradation and fermentation of pasture were thereby promoted. The microbial and fermentation metabolic capacities of the ileum of Tibetan sheep differ, and the relative density of fibrinolytic bacteria in the ileum of 1.5-year-old Tibetan sheep is significantly greater than that of sheep of other ages. This enables 1.5-year-old Tibetan sheep to decompose and ferment more cellulose to produce the energy substances, VFAs, and the VFA transporter proteins are able to transport more VFAs to be absorbed and utilized by the ileum epithelium. Therefore, the ileum of 1.5-year-old Tibetan sheep has a greater fermentation and metabolic capacity under traditional grazing conditions on the plateau than that of sheep of other ages. In practical production applications, 1.5-year-old Tibetan sheep have strong carcass production performance and fat deposition capacity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10100509/s1, Table S1: Forage species and nutrient levels; Table S2: Short chain fatty acid related gene primer sequence; Table S3: Flora primer design.

Author Contributions: Conceptualization, Y.S.; validation, X.L.; writing—original draft preparation, F.W.; writing—review and editing, X.L. and Y.S.; statistical analysis, F.W.; experimental operations, X.C., W.Y., Q.C., M.G. and W.H.; supervision, Y.H., Z.H., and J.W.; project management, X.L.; experi-

mental design and sample collection, L.W. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The Ethical Institutional Review Board of the Animal Husbandry Committee of Gansu Agricultural University approved the conduct of the study (Approval No. GAU-LC-2020-27), and the selection and treatment of experimental animals were conducted in strict accordance with the ethical requirements, and the informed consent of the animal owner was obtained.

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. The microbial sequence data are available in the NCBI database under accession PRJNA1063531.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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