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A Study on the Mechanism Regulating Acetate to Propionate Ratio in Rumen Fermentation by Dietary Carbohydrate Type

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Abstract

The research direction of our team is nutrition and physiology of ruminants, including dietary nutrition metabolism and rumen microorganisms. Previous research has shown that ruminal acetate-to-propionate ratio is related to diet utilization efficiency. At present, it is believed that the main factors affecting the ruminal acetate-to-propionate ratio are the degradation rate of the diet and the rumen microbial structure, but the main mechanism is unclear. This study found that the effect of ruminal acetate-to-propionate ratio was not affected by the concentration of the fermentation substrate, but was affected by the structure of the rumen microbiota. We believe that changes in the rumen microflora structure are the main mechanism for regulating the ruminal acetate-to-propionate ratio. This will help people to further understand the rumen physiology, thereby gradually improving feed conversion efficiency and reducing production costs. **Abstract:** In order to explore the mechanism by which diet regulates the acetate-to-propionate molar ratio (A: P ratio), we compared the effect on rumen fermentation parameters and the microbiome by altering the ratio of dietary concentrates to roughage ratio and calcium pyruvate infusion. The test animals were Laoshan dairy goats, and were fed continuously through an automatic feeder. The test groups were fed a base diet of low concentrates, and intraruminally infused with calcium pyruvate at two concentrations. The infusion concentrations were derived from the difference in the rate of carbohydrate degradation of the high and low concentrate diets, and they were artificially set such that the high concentration infusion group was infused with twice the concentration as the low concentration infusion group. The control groups were fed high concentrate (6:4) and low concentrate (3:7) diets, respectively. The following results were obtained by measuring rumen fermentation parameters and microbial composition: the rumen A: P ratio was significantly lower in the high-concentrate

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diet group than in the low concentrate diet group ($P < 0.05$). Infusion of low concentration calcium pyruvate had no significant effect on rumen A: P ratio ($P > 0.05$), while infusion of high concentration calcium pyruvate significantly increased the rumen A: P ratio ($P < 0.05$). Relative to goats fed the low concentrate diet, those fed the high concentrate diet had a greater abundance of microbes related to propionate production and a reduced abundance of microbes related to fiber degradation. Infusion of pyruvate had no significant effect on rumen microbial structure. The above results indicate that increasing the concentration of the fermentation substrate without affecting the composition of the microflora does not reduce the A: P ratio. Microbiological results showed that the A: P ratio was more closely related to the rumen microflora structure. Therefore, it is believed that rumen microflora structure is the main mechanism regulating A: P ratio in rumen fermentation.

Keywords

Rumen Acetic Acid Propionate Ratio, Calcium Pyruvate, Rumen Microbiome, Volatile Fatty Acid

1. Introduction

Reducing the ruminal acetate-to-propionate ratio (A: P ratio) can improve the efficiency of dietary energy utilization. Early studies have found that ruminal A: P ratio is related to dietary structure [1] [2]. However, a large number of studies have also shown that under the same diet structure, the ruminal A: P ratio can be changed by the rate of dietary degradation [3] [4]. If changes in the rate of dietary degradation also affect the ruminal microbial composition, what is the main mechanism of the dietary regulation of rumen-fermented A: P ratio?

Before a carbohydrate becomes a volatile fatty acid (VFA), it must be converted to pyruvic acid [5]. Pyruvate molecules are small and can easily pass through the cell membrane and are readily utilized by most microorganisms in the rumen. Compared to other hydrolytic products of carbohydrates, pyruvate is more evenly distributed among microorganisms. In this study, different carbohydrate degradation rates were simulated by ruminal infusion of high and low concentrations of calcium pyruvate, and the pyruvate level was adjusted such that animals had similar pyruvate level regardless of their dietary concentrate-to-roughage ratio. By comparing the effects of diets with different concentrate-to-roughage ratios and different concentrations of calcium pyruvate on ruminal VFA and microflora structure, the main mechanism of dietary regulation of rumen A: P ratio was explored.

2. Materials and Methods

2.1. Animals

Eight Laoshan dairy goats were selected that were in good health, had similar

milk yield (2.00 ± 0.4 kg), similar body weights (45 ± 2 kg), and multiparous non-pregnant. Four of them were fitted with permanent rumen fistula.

2.2. Trial Diets

Trial diets were prepared according to UK AFRC (1993). The high concentrate diet contained 6 parts of concentrate, and 4 parts of roughage, while the low concentrate diet contained 3 parts of concentrate and 7 parts of roughage. The diets were pelletized with a granulator (Table 1).

2.3. Feeding Management

Goats were kept individually in single-animal cages, fed 350 g through an automatic feeder every 4 hours, and had free access to drinking water. Goats were

Table 1. Ingredients and chemical composition of the diets.

	Treatment	
	H	L
Ingredients (% DM)		
Concentrate	60	30
Roughage	40	70
Corn	27.5	10
Soybean meal	15	15
Bran	15	5
peanut vine	10	42.5
Alfalfa Hay	30	25
$CaHPO_4$	0.1	1
limestone	0.9	0
NaCl	0.5	0.5
Premix[1]	1	1
Nutrient level (%)		
NEL/(Mcal/kg)	1.49	1.28
DM	91.36	92.22
OM	81.15	80.99
CP	16.58	15.21
NDF	30.60	35.18
ADF	20.25	27.26
NFC[2]	31.04	29.09
EE	2.93	1.51
ADL	3.22	6.48

[1]Premix contained: VA 1000 KIU/kg; VD 3250 KIU/kg, VE 2400 mg/kg, niacin 2000 mg/kg, Fe 2000 mg/kg; Mn 3000 mg/kg, Cu 3000 mg/kg, Zn 14,000 mg/kg, Se 100 mg/kg, I 180 mg/kg, and Co 40 mg/kg. [2]NFC = 100 − (% NDF + % CP + % EE + % Ash). NFC and NEL were calculated values and others were determined experimentally. H is the high concentrate diet. L is the low concentrate diet.

dewormed and hoofed according to routine management procedures before entering their respective cages. Fistulated animals were given 30 days to recover from the rumen surgery. During the first week of recovery, high quality hay was provided. The amount of feeding increased subsequently and a small amount of drinking water was given. In the later stage of recovery, goats were given free access to sweet potato vine.

2.4. Experimental Design

The effective ruminal degradation rates of the two diets (as measured with the nylon bag method) were 33.48% and 33.42% in the high and low concentrate groups, respectively (Table 2 and Table 3). The difference in fermentable carbohydrate degradation between the two diets in the rumen was calculated to be 5.27 g/d, which corresponds to 6.47 g/d crude pyruvate, as calculated by molecular formula and purity. Therefore, the low concentration group was infused with 6.47 g/d calcium pyruvate through a constant flow pump. Referring to the dietary components, the high-concentrate diet group was infused with twice as much calcium pyruvate as the low-concentrate diet group.

The calculated fermentable carbohydrate difference between high and low concentrate diets was equivalent to 6.46 g/d pyruvate.

Table 2. Rumen real-time degradation rate.

Time	Degradation rate %		SEM	P
	H	L		
1 h	18.84	20.52	1.586	1.000
2 h	21.84	21.75	1.656	1.000
4 h	20.67	24.59	1.584	0.495
8 h	26.81	26.35	1.584	1.000
16 h	28.86	30.56	1.742	1.000
24 h	35.84	38.72	1.463	0.837
36 h	34.35	37.89	1.625	0.709
48 h	40.09	41.23	1.573	1.000

H is the high concentrate diet. L is the low concentrate diet.

Table 3. Effective degradation rate parameters.

	H	L
a	19.5350	19.1614
b	24.7006	24.1493
c	0.0441	0.0490
k	0.0340	0.0340
ED	33.4841	33.4230

H is the high concentrate diet. L is the low concentrate diet.

A 2 × 2 interactive design was used for the rumen calcium pyruvate infusion test. The test had two treatment groups. Each group contained two fistulated goats, which were fed with only low-concentration diets. Different concentrations of calcium pyruvate solution were infused at a constant rate throughout the day through a constant flow pump via the rumen fistula. The other 4 non-fistulated goats were randomly divided into two control groups, and the high- and low-concentrate diets were fed according to the 2 × 2 interactive design. Each trial period lasted 20 days, with a pre-feeding period of 16 days and a sampling period of 4 days. At the end of each trial, animals were transferred to the next trial.

2.5. Sample Collection

During each sampling period, rumen chyme was collected through the rumen fistula after the morning feeding. A rumen fluid sample was obtained by pressurized filtration through 2 layers of gauze. Immediately after the rumen fluid was collected, the pH was measured with a pH meter. The collected rumen fluid samples were then stored as 4 mL aliquots in 5 mL cryotubes. Two of them were used at −20˚C for the determination of volatile fatty acids. One aliquot was frozen in liquid nitrogen and then stored at −80˚C for microbiological determination, and the remainder was frozen at −80˚C for backup.

2.6. Sample Measurement

The dry matter (DM) of the diet was determined according to GB 6435-86. The crude protein (CP) was determined by following GB/T 6432-94. The determination of lignin (ADL) and neutral detergent fiber (NDF) was based on GB/T 20806-2006. The acid detergent fiber (ADF) was determined as described in NY/T 1459-2007. The coarse ash (Ash) was determined according to GB/T 6438-86. The crude fat (EE) was determined according to GB/T 6433-2006. Rumen fluid VFA was determined by gas chromatography. The rumen fluid microflora composition was determined using MiSeq high-throughput sequencing technology.

The work of microbial sequencing and otu map was entrusted to Shandong Kaiyuan Gene Technology Co., Ltd. The raw data were filtered to eliminate the adapter pollution and low-quality reads to obtain clean sequences. Sequence reads with an average quality of under 20 over a 30-bp sliding window as per the phred algorithm were truncated, and trimmed reads having less than 75% of their original length, as well as its paired read, were removed. Then, paired-end reads with overlap were merged into tags using FLASH Tags were clustered to OTU at 97% sequence similarity by scripts of software USEARCH. Venn Diagram and package "ade4" of software R (v3.0.3) were used separately in Venn diagram and OTU PCA analysis. The tag numbers of each taxonomic rank (phylum, class, order, family, genus, and species) or OTU in different samples were summarized in a profiling table. The species with abundances of less than 0.5% were classified into "others" in other ranks for all samples.

2.7. Data Analysis

Rumen pH, VFA and other data were analyzed by SAS 9.1 software MIXED process for analysis of variance. The statistical model is:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk}$$

where μ = population mean; α_i = fixed effect of the ith experimental period; β_j = continuous effect of calcium pyruvate dose; γ_k = random effect of the kth animal; e_{ijk} = random error. The linear and secondary effects of the calcium pyruvate dose were initially tested. When the secondary effects were found to be not significant, they were removed from the model. The Person's correlation test procedure was performed. The effect was considered significant at $P < 0.05$, and an influential trend at $0.05 < P < 0.1$.

3. Results

3.1. Effects of the Dietary Concentrate Ratio and Calcium Pyruvate Infusion on VFA Content of Rumen Fermentation

Table 4 shows the effects of different concentrate ratios of diets and different calcium pyruvate infusion concentrations on rumen pH and rumen volatile fatty acid (VFA) concentrations. As shown in the table, marked differences were seen in average rumen pH values between animals fed diets of different concentrate levels. The average ruminal pH was significantly lower in the high concentrate diet group than in the low concentrate diet group ($P < 0.01$). Infusion of calcium pyruvate increased the average ruminal pH, though this was not significant. The differences in total VFA among treatments were not significant ($P > 0.05$), and

Table 4. Rumen fermentation parameters.

	Treatment[1]				P
	H	L	LL	LH	
Mean Ph	6.27^{a} ± 0.21	6.72^{b} ± 0.07	6.96^{b} ± 0.07	6.87^{b} ± 0.04	<0.01
TVFA mmol/L	81.65 ± 10.48	77.73 ± 7.52	87.96 ± 6.35	98.96 ± 8.97	0.35
Acetate mmol/L	38.56 ± 2.23	44.37 ± 3.01	41.09 ± 1.86	42.97 ± 1.98	0.37
Propionate mmol/L	25.17^{a} ± 3.66	21.00^{ab} ± 2.69	17.49^{b} ± 1.14	16.14^{b} ± 0.72	0.02
Butyrate mmol/L	23.25 ± 2.07	22.58 ± 4.30	24.05 ± 1.33	24.73 ± 1.18	0.94
Isobutyric acid mmol/L	1.27 ± 0.45	1.33 ± 0.31	2.22 ± 0.28	2.03 ± 0.13	0.12
Valerate mmol/L	3.66 ± 0.17	3.73 ± 0.28	3.97 ± 0.20	3.65 ± 0.21	0.76
Isovaleric acid mmol/L	2.13 ± 0.75	2.10 ± 0.49	3.32 ± 0.64	3.04 ± 0.19	0.28
Caproic acid mmol/L	1.29^{a} ± 0.16	1.19^{a} ± 0.05	0.79^{b} ± 0.17	0.61^{b} ± 0.05	<0.01
A: P ratio	1.47^{a} ± 0.29	1.90^{ab} ± 0.15	2.08^{ab} ± 0.13	2.36^{b} ± 0.17	0.047

H indicates high-concentration diet group; L indicates low-concentration diet group; LL indicates low-concentration diet infusion low-concentration calcium pyruvate group; and LH indicates low-concentration diet infusion high concentration calcium pyruvate group. Values in the same row with the same superscript or without a superscript were not significantly different ($P > 0.05$), and those with different superscript were significantly different ($P < 0.05$).

there were no significant differences in rumen acetic acid concentrations ($P > 0.05$). Numerically, the concentration of acetic acid in the high concentrate diet group was lower than that in the low concentrate diet group. The infusion of calcium pyruvate had little effect on the concentration of rumen acetic acid. The propionate was significantly lower in the low concentrate diet group than in the high concentrate diet group ($P < 0.05$). After infusion of calcium pyruvate into the rumen, the concentration of ruminal propionate further decreased; thus, a high concentration of calcium pyruvate significantly reduced the concentration of propionate in the rumen. The ruminal butyric acid concentration increased after infusion of calcium pyruvate, but the difference of butyric acid concentration in each treatment group was not significant ($P > 0.05$). The differences in isobutyric acid concentration were not significant among treatments ($P > 0.05$), nor were the differences in valeric acid concentration ($P > 0.05$). Infusion of calcium pyruvate increased the concentration of isovaleric acid but the difference was not significant ($P > 0.05$). The effect of dietary concentrate ratio on rumen hexanoic acid concentration was not significant. However, the concentration of hexanoic acid decreased significantly with increased calcium pyruvate infusion concentrations ($P < 0.01$). The rumen A: P ratio was significantly lower ($P < 0.05$) in the high concentrate diet group relative to the low concentrate diet group, but the ratio increased after infusion of calcium pyruvate. However, there was no significant difference in ruminal A: P ratio between the low concentration pyruvate infusion group and the low concentrate diet group, while it was significantly increased in the group with low concentrate diet and high-concentration calcium pyruvate infusion (Table 4).

3.2. Effects of Dietary Concentrate Ratio and Calcium Pyruvate Infusion on Rumen Microflora Structure

3.2.1. OTU Clustering and Abundance

The abundance of each operational taxonomic unit (OTU) in each sample was obtained by clustering analysis. The OTU abundance preliminarily illustrates the species richness of the sample. In order to obtain species classification information corresponding to each OTU, the RDP classifier Bayesian algorithm was used to classify the OTU representative sequence at a 97% similarity level, and the community composition for each sample was calculated at each taxonomic level: domain, kingdom, phylum, class, order, family, genus, and species. The high concentrate *diet group had an average of 3*309 OTUs, and the low concentrate diet group had an average of 3032 OTUs. The low concentration pyruvate infusion group had an average of 3412 OTUs, while in the high pyruvate infusion group had an average of 3479 OTUs. It can be seen from the heat map of the 100 most abundant OTUs (Figure 1) that the OTU enrichment profiles were different among different treatments and different individuals. It can also be seen that higher abundance OTUs of the high concentrate diet group were mainly concentrated in the top, while the higher abundance OTUs in the high calcium pyruvate infusion group were mainly concentrated at the bottom.

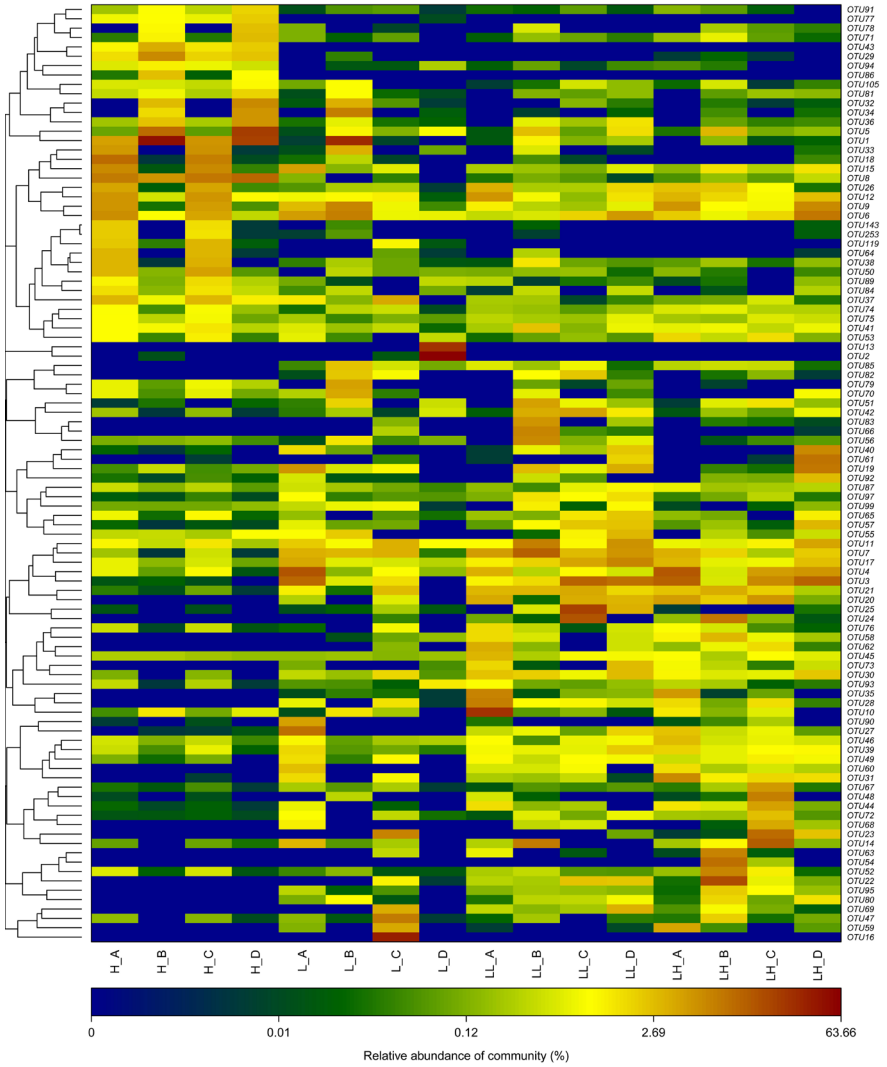

Figure 1. Heat map of top 100 abundant OTUs. H indicates high concentrate diet group. L indicates low concentrate diet group. LL indicates low concentrate diet infusion low concentration pyruvate group. LH indicates low concentrate diet infusion high concentration calcium pyruvate group.

Overall, the rumen microbial composition of high-concentrate diets was significantly different those of the low-concentrate diet and calcium pyruvate-infused groups.

3.2.2. Community Analysis

The sequencing results showed that the rumen fluid samples covered a total of 28 phyla, 62 classes, 114 orders, 185 families, 414 genera, and 752 species of bacteria. Among the genera that contributed greater than 1% of the microbiome included Prevotella (Prevotella_1, Prevotellaceae _unclassified, Prevotellaceae_UCG−001, Prevotellaceae_UCG−003, Prevotellaceae_Ga6A1_group, Prevotellaceae_YAB2003 _group), norank, Vibrio (Succinivibrionaceae_UCG−002, Succiniclasticum, Succinivibrio), Rikenella (Rikenellaceae _RC9_gut_group), Veillonella (Veillonellaceae_unclassified, Veillonellaceae_UCG−001), Streptococcus, Methanobrevibac-

ter, Selenomonas (Selenomonas_1, Selenomonas_3), Treponema (Treponema_2), Ruminococcus (Rumincoccaceae_NK4A214_group, Ruminococcaceae_UCG−014, Ruminococcus_1, Ruminococcaceae_UCG−002, Ruminococcaceae _V9D2013_ group, Ruminococcus_2), Megasphaera, Christensenella (Christensenellaceae_ R−7_group), Roseburia, Lachnospira (Laxnospiraceae_NK3A20_group, Lachnospiraceae_unclassifi Ed, Lachnospiraceae_ND3007_group), Fibrobacter, Butyrivibrio (Butyrivibrio_2), Quinella, Bacteroides (Bacteroidales_unclassified, Bacteroidetes_unclassified), uncultured, Anaerovibrio (Anaerovibrio, Anaeroplasma), Moryella, Erysipelas (Erysipelotrichaceae_UCG−004), Phocaeicola, Tyzzerella_3, Ruminiclostridium_6, [Eubacterium]_coprostanoligenes_group, and Methanimicrococcus. The distributions of rumen flora in each sample are shown in Figure 2.

3.2.3. Differences in Species Diversity

The rumen microbial diversity indices of all groups are shown in Table 5. There were no significant differences in rumen microbial diversity indices among groups ($P > 0.05$). Therefore, these differences were not marked in the table. However, the total reads of each group increased after the infusion of calcium pyruvate ($P = 0.076$), indicating that the infusion of calcium pyruvate had a tendency to increase total bacterial numbers; however, these differences were not significant. According to the Shannon and Simpson indices, it can be seen that the species diversity of the low concentrate diet group was higher than that of the high concentrate diet group. The rumen microbial diversity was further increased after rumen infusion of calcium pyruvate. However, the differences between groups were not significant.

3.2.4. Significant Analysis of Differences between Treatments

Table 6 shows the results of Wilcoxon rank sum test at the genus level between treatment groups. It focuses on species with abundances greater than 1% and significant differences. Overall, the difference was significantly concentrated in

Table 5. Rumen microbial diversity index.

Treatment	Reads	0.97					
		OTU	ace	chao	coverage	shannon	simpson
H	493,204	13,237	13,237 (13,237, 13,237)	13,237 (13,237, 13,237)	1.000000	5.22 (5.21, 5.23)	0.026 (0.0258, 0.0262)
L	417,465	12,126	12,142 (12,136,12,152)	12,126 (12,126, 12,128)	0.999907	5.54 (5.53, 5.55)	0.0194 (0.0192, 0.0196)
LL	507,798	13,646	13,646 (13,646, 13,646)	13,646 (13,646, 13,646)	1.000000	5.66 (5.64, 5.67)	0.0144 (0.0143, 0.0145)
LH	541,018	13,919	13,919 (13,919, 13,919)	13,919 (13,919, 13,919)	1.000000	5.56 (5.55, 5.57)	0.0135 (0.0134, 0.0136)

H indicates high concentrate diet group. L indicates low concentrate diet group. LL indicates low concentrate diet low concentration pyruvate infusion group, and LH indicates low concentrate diet high concentration calcium pyruvate infusion group.

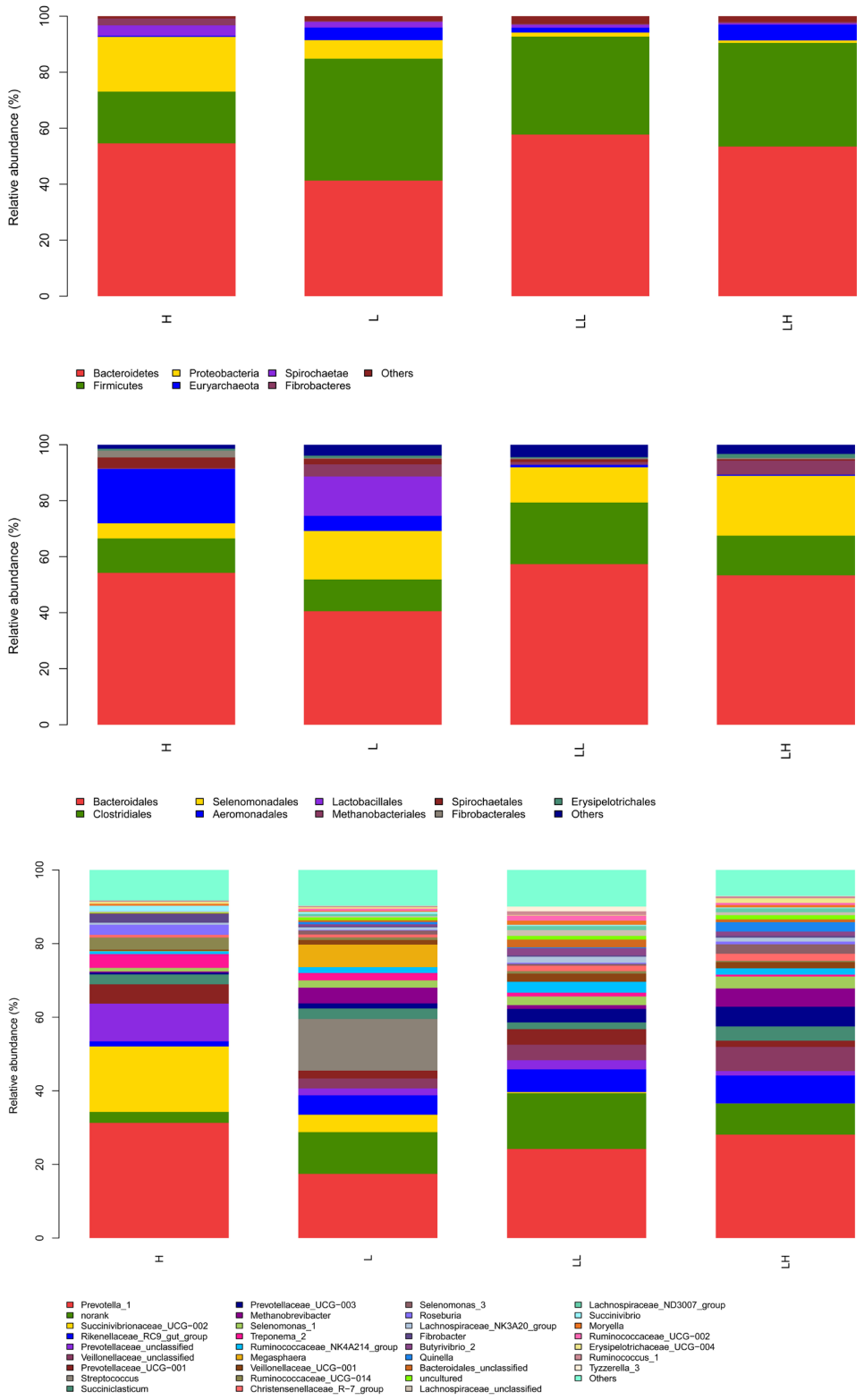

Figure 2. Classification and species composition in each treatment group. H indicates high concentrate diet group. L indicates low concentrate diet group. LL indicates low concentrate diet low concentration pyruvate infusion group, and LH indicates low concentrate diet high concentration calcium pyruvate infusion group. The upper, middle and lower panels are phylum level, order level and genus level, respectively. Only the taxa with abundances of more than 1% are displayed.

Table 6. Wilcoxon test result.

Genus[1]	Mean[2] (H)	Sd[3] (H)	mean (L)	sd (L)	p-value[4]
[Eubacterium]_coprostanoligenes_group*	0.0003	0.0002	0.0046	0.0068	0.0304
Fibrobacter*	0.0276	0.0115	0.0046	0.0063	0.0304
Prevotella_1	0.3732	0.1072	0.2352	0.1359	0.1939
Prevotellaceae_Ga6A1_group	0.0114	0.0152	0.0008	0.0007	0.665
Prevotellaceae_UCG-001	0.062	0.0658	0.03	0.0217	1
Prevotellaceae_YAB2003_group*	0.0093	0.0092	0.0005	0.0004	0.0304
Rikenellaceae_RC9_gut_group	0.0162	0.0113	0.075	0.0798	0.1124
Roseburia*	0.0308	0.0206	0.0026	0.0041	0.0304
Ruminococcaceae_UCG-002*	0.0001	0.0001	0.0082	0.0075	0.0304
Ruminococcaceae_UCG-014	0.0384	0.0278	0.0113	0.0135	0.1939
Ruminococcaceae_V9D2013_group*	0	0	0.0074	0.0096	0.0211
Selenomonas_1	0.0108	0.006	0.0233	0.0284	1
Succiniclasticum	0.031	0.027	0.0363	0.0316	0.8852
Succinivibrio	0.0179	0.0183	0.0079	0.0108	0.4705
Succinivibrionaceae_UCG-002	0.1966	0.2011	0.0693	0.1385	0.1939
Treponema_2	0.0453	0.0406	0.0281	0.0431	0.1939
	mean (H)	sd (H)	mean (LL)	sd (LL)	p-value
[Eubacterium]_coprostanoligenes_group*	0.0003	0.0002	0.0016	0.0008	0.0304
Anaeroplasma*	0.0025	0.0014	0.0111	0.0046	0.0304
Butyrivibrio_2*	0.0012	0.0013	0.0255	0.0096	0.0304
Fibrobacter*	0.0276	0.0115	0.0045	0.0045	0.0304
Lachnospiraceae_ND3007_group*	0.0006	0.0006	0.015	0.0032	0.0304
Megasphaera*	0.001	0.0005	0	0	0.0265
Prevotella_1	0.3732	0.1072	0.3142	0.1266	0.8852
Prevotellaceae_Ga6A1_group	0.0114	0.0152	0.0008	0.0007	0.4705
Prevotellaceae_UCG-001	0.062	0.0658	0.0703	0.0962	0.665
Prevotellaceae_UCG-003*	0.0057	0.0017	0.0502	0.0254	0.0304
Prevotellaceae_YAB2003_group*	0.0093	0.0092	0.0003	0.0003	0.0304
Rikenellaceae_RC9_gut_group*	0.0162	0.0113	0.082	0.0237	0.0304
Roseburia*	0.0308	0.0206	0.0044	0.0034	0.0304
Ruminococcaceae_UCG-002	0.0001	0.0001	0.0173	0.0213	0.0304
Ruminococcaceae_UCG-014	0.0384	0.0278	0.009	0.0065	0.0606
Ruminococcaceae_V9D2013_group*	0	0	0.008	0.0111	0.0211
Ruminococcus_2*	0.0017	0.0002	0.0006	0.0004	0.0304
Selenomonas_1	0.0108	0.006	0.0325	0.0312	0.4705
Selenomonas_3*	0	0	0.0066	0.0051	0.0265
Succiniclasticum	0	0	0	0	0.4533
Succinivibrio	0.0179	0.0183	0.0048	0.0039	0.4705

Continued

Succinivibrionaceae_UCG-002*	0.1966	0.2011	0.0035	0.0048	0.0304
Treponema_2	0.0453	0.0406	0.016	0.0178	0.3123
Tyzzerella_3*	0	0	0.0151	0.0283	0.0211
Veillonellaceae_UCG-001*	0.0039	0.0027	0.0331	0.0211	0.0304
	mean (H)	sd (H)	mean (LH)	sd (LH)	p-value
[Eubacterium]_coprostanoligenes_group*	0.0003	0.0002	0.0021	0.0007	0.0304
Butyrivibrio_2*	0.0012	0.0013	0.0136	0.0088	0.0304
Fibrobacter*	0.0276	0.0115	0.0062	0.0044	0.0304
Lachnospiraceae_ND3007_group*	0.0006	0.0006	0.0123	0.0048	0.0304
Megasphaera*	0.001	0.0005	0	0	0.0265
Prevotella_1	0.3732	0.1072	0.3461	0.1071	0.8852
Prevotellaceae_Ga6A1_group	0.0114	0.0152	0.0022	0.0024	0.8852
Prevotellaceae_UCG-001	0.062	0.0658	0.0214	0.0237	0.4705
Prevotellaceae_UCG-003*	0.0057	0.0017	0.0687	0.0497	0.0304
Prevotellaceae_YAB2003_group*	0.0093	0.0092	0.0005	0.0007	0.0304
Rikenellaceae_RC9_gut_group*	0.0162	0.0113	0.0905	0.0607	0.0304
Roseburia	0.0308	0.0206	0.0094	0.0083	0.1124
Ruminococcaceae_UCG-002*	0.0001	0.0001	0.0069	0.0073	0.0304
Ruminococcaceae_UCG-014*	0.0384	0.0278	0.0041	0.003	0.0304
Ruminococcaceae_V9D2013_group*	0	0	0.0004	0.0006	0.0211
Ruminococcus_2*	0.0017	0.0002	0.0003	0.0003	0.0304
Selenomonas_3*	0	0	0.0323	0.0321	0.0265
Succiniclasticum	0.031	0.027	0.0462	0.0323	0.4705
Succinivibrio	0.0179	0.0183	0.0032	0.002	0.4705
Succinivibrionaceae_UCG-002*	0.1966	0.2011	0.0004	0.0006	0.0304
Treponema_2*	0.0453	0.0406	0.0064	0.0052	0.0304
Veillonellaceae_UCG-001*	0.0039	0.0027	0.0209	0.0159	0.0304
	mean (L)	sd (L)	mean (LL)	sd (LL)	p-value
Christensenellaceae_R−7_group*	0.0093	0.0038	0.0203	0.0094	0.0304
Methanimicrococcus	0.0475	0.0859	0.0129	0.0148	0.8852
Prevotella_1	0.2352	0.1359	0.3142	0.1266	1
Prevotellaceae_UCG−001	0.03	0.0217	0.0703	0.0962	0.665
Prevotellaceae_UCG−003	0.0179	0.0125	0.0502	0.0254	0.1124
Rikenellaceae_RC9_gut_group	0.075	0.0798	0.082	0.0237	0.3123
Ruminococcaceae_NK4A214_group	0.0216	0.0174	0.0389	0.0301	0.3123
Ruminococcaceae_UCG−014	0.0113	0.0135	0.009	0.0065	0.8852
Selenomonas_1	0.0233	0.0284	0.0325	0.0312	0.8852
Selenomonas_3	0.0123	0.0194	0.0066	0.0051	0.8845
Succiniclasticum	0.1265	0.253	0	0	0.1859
Succinivibrionaceae_UCG−002	0.0693	0.1385	0.0035	0.0048	0.3123

Continued

Treponema_2	0.0281	0.0431	0.016	0.0178	0.8852
Veillonellaceae_UCG−001	0.0148	0.016	0.0331	0.0211	0.1124
	mean (L)	sd (L)	mean (LH)	sd (LH)	p-value
Methanimicrococcus	0.0475	0.0859	0.0619	0.0641	0.4705
Prevotella_1	0.2352	0.1359	0.3461	0.1071	0.4705
Prevotellaceae_UCG−001	0.03	0.0217	0.0214	0.0237	0.665
Prevotellaceae_UCG−003	0.0179	0.0125	0.0687	0.0497	0.1939
Rikenellaceae_RC9_gut_group	0.075	0.0798	0.0905	0.0607	0.4705
Ruminococcaceae_NK4A214_group	0.0216	0.0174	0.0199	0.019	1
Ruminococcaceae_UCG−014	0.0113	0.0135	0.0041	0.003	1
Selenomonas_1	0.0233	0.0284	0.0363	0.0694	0.3123
Selenomonas_3	0.0123	0.0194	0.0323	0.0321	0.1913
Succiniclasticum	0.0363	0.0316	0.0462	0.0323	0.4705
Succinivibrionaceae_UCG−002	0.0693	0.1385	0.0004	0.0006	0.8852
Treponema_2	0.0281	0.0431	0.0064	0.0052	0.665
Veillonellaceae_UCG−001	0.0148	0.016	0.0209	0.0159	0.4705
	mean (LH)	sd (LH)	mean (LL)	sd (LL)	p-value
Butyrivibrio_2	0.0136	0.0088	0.0255	0.0096	0.1124
Christensenellaceae_R−7_group	0.023	0.0144	0.0203	0.0094	0.665
Erysipelotrichaceae_UCG−004	0.0141	0.0253	0.002	0.0004	1
Lachnospiraceae_ND3007_group	0.0123	0.0048	0.015	0.0032	0.4705
Lachnospiraceae_NK3A20_group	0.0125	0.0175	0.024	0.013	0.3123
Methanimicrococcus	0.0619	0.0641	0.0129	0.0148	0.1939
Prevotella_1	0.3461	0.1071	0.3142	0.1266	0.665
Prevotellaceae_UCG−001	0.0214	0.0237	0.0703	0.0962	0.4705
Prevotellaceae_UCG−003	0.0687	0.0497	0.0502	0.0254	0.665
Quinella	0.0328	0.0381	0.0029	0.0032	0.2454
Rikenellaceae_RC9_gut_group	0.0905	0.0607	0.082	0.0237	0.3123
Ruminococcaceae_NK4A214_group	0.0199	0.019	0.0389	0.0301	0.3123
Selenomonas_1	0.0363	0.0694	0.0325	0.0312	0.8852
Selenomonas_3	0.0323	0.0321	0.0066	0.0051	0.0606
Succiniclasticum	0.0462	0.0323	0.0273	0.0223	0.4705
Veillonellaceae_UCG−001	0.0209	0.0159	0.0331	0.0211	0.3123

H indicates high concentrate diet group. L indicates low concentrate diet group. LL indicates low concentrate diet low concentration pyruvate infusion group, and LH indicates low concentrate diet high concentration calcium pyruvate infusion group. [1]Genus: genus level; [2]Mean: Average relative abundances of species in the group of samples; [3]Sd: Standard deviation of relative abundances of species in the group of samples; [4]p-value: the probability that the hypothesis is true in the test for the two groups. Differences were considered significant at $p < 0.05$, indicated by *, and considered extremely significant at $p < 0.01$, indicated by **.

the comparison of the high concentrate diet group with the other three groups. The vast majority had no significant differences between the low concentrate diet group and the calcium pyruvate infusion group. In the four groups of samples, those with abundances always greater than 1% were Prevotella (Prevotella_1 and Prevotellaceae_UCG−001), Rikenellaceae_RC9_gut_group, Selenomonas_1 and Succiniclasticum, and there were no significant difference among groups in these genera except Rikenonella. The abundance of Prevotella_1 was not significantly different among the four treatment groups and it was always the most abundant in the rumen. The abundance of Prevotella_1 was consistently above 23% in the four groups of samples.

As shown in Table 6, the high concentrate diet group had significantly lower contents of Eubacterium (coprostanoligenes_group) and Ruminococcus (Ruminococcaceae_UCG-002 and Ruminococcaceae_ V9D2013_group) than the low concentrate diet group, and it had significantly higher contents of Fibrobacte, Prevotellaceae_YAB2003_group and Roseburia. Compared with the low concentration pyruvate infusion group, the high concentrate diet group had significantly lower contents of coprostanoligenes_group, Anaeroplasma, Butyrivibrio_2, Lachnospiraceae_ND3007_group, Prevotellaceae_UCG−003, Rikenellaceae_RC9_gut_group, Ruminococcaceae_UCG−002, Ruminococcaceae_V9D2013_group, Selenomonas_3, Veillonellaceae_UCG−001, and Tyzzerella_3, and it had significantly higher contents of Fibrobacter, Megasphaera, Prevotellaceae_YAB2003_group, Ruminococcus_2, Roseburia and Succinivibrionaceae_UCG−002. Compared with the high concentration pyruvate infusion group, the high concentrate diet group had significantly lower contents of coprostanoligenes_group, Butyrivibrio_2, Lachnospiraceae_ND3007_group, Prevotellaceae_UCG−003, Rikenellaceae_RC9_gut_group, Ruminococcaceae_UCG −002, Ruminococcaceae_V9D2013_group, Selenomonas_3 and Veillonellaceae_UCG−001, and it had higher contents of Fibrobacter, Megasphaera, Prevotellaceae_YAB2003_group, Ruminococcaceae_UCG−014, Ruminococcus_2, Succinivibrionaceae_UCG−002, and Treponema_2.

There were no significant differences between the two calcium pyruvate infusion groups and the low concentrate diet group, and only the low concentrate diet group had significantly lower Christensenellaceae_R−7_group than the low concentration pyruvate infusion group. Ruminococcaceae_UCG-002 and Ruminococcaceae _V9D2013_group) and the eubacteria coprostanoligenes_group were significantly higher in the low concentrate diet group and the calcium pyruvate infusion group than in the high concentrate diet group, and the contents of Prevotellaceae genus Prevotellaceae_ YAB2003_group and Fibrobacter were significantly lower in the low concentrate diet group and the calcium pyruvate infusion group than in the high concentrate diet group.

4. Discussion

4.1. Effect of Pyruvate Infusion on Ruminal A: P Ratio

An increase in the dietary NDF level will reduce DMI. The main reason for this

is that excessive intake of NDF increases the volume of rumen chyme and increases satiety, which reduces DMI. Arelovich *et al.* summarized a number of studies which demonstrated a significant inverse correlation between DMI and NDF content if the NDF content of dairy cows changed between 22.5% and 45% [6]. In addition to affecting feed intake, dietary NDF levels also affect feeding behavior and nutrient digestibility [7]. Increased feed intake may introduce other factors into our research, such as feeding time, chewing time, salivation, and so forth. The way in which feed is processed can also be an influencing factor. Although Jiang's research found that the roughage particle size has little effect on salivation [8], we know that changing the size of roughage grains will affect the degradation rate. Therefore, in order to eliminate the interference of factors which may affect the test, such as feed intake, picky feeding behavior and dietary processing methods, this experiment used continuous feeding to keep the rumen environment as stable as possible; each test animal was fed the same amount of feed, which was processed in the same way. The results showed that there was no significant difference in the effective degradation rate of fermentable carbohydrates between the two diets. This may be related to the way the diets were processed. Pulverization and granulation increase the contact area between dietary fiber and rumen microorganisms and promotes the degradation of dietary fiber. Studies have shown a linear decrease in the degradation rate occurs with increases in the average particle sizes of barley, wheat, rye, and pea flour [9]. Therefore, the effective degradation rate in the rumen of low concentrate diet was not much worse than that of the high concentrate diet. Although the difference in the effective degradation rates between the two diets was not significant, the difference in fermentable carbohydrate content was significantly higher in the high concentrate diet than that in the low concentrate diet. Therefore, the concentration of the fermented substrate produced per unit time from the high concentrate diet was significantly higher than that from the low concentrate diet. Our rumen fermentation results were identical to those of most studies. The increased in dietary concentrate proportion was accompanied by a decreased rumen A: P ratio. For the low concentrate diet, rumen infusion of low concentration pyruvate did not significantly change the rumen A: P ratio, while a significant increase in A: P ratio did occur with infusion of high concentration pyruvate. Chen *et al.* also found that the addition of pyruvate increased the ruminal A: P ratio [10].

After ruminal infusion of calcium pyruvate in the low concentrate diet group, the proportion of ruminal propionate was significantly reduced, the ruminal acetate-to-propylene ratio was significantly increased, and the proportion of hexanoic acid was significantly reduced. Meanwhile, the differences in ruminal pH were not significant, although the total volatile fatty acid concentration increased. In the process of producing VFAs, carbohydrate is first degraded into pyruvic acid by microbial cells. Infusion of calcium pyruvate is equivalent to increasing the substrate concentration of the fermentation. After infusion of calcium pyruvate in this experiment, the substrate level in the low concentrate diet

group reached a level as high as that in the high concentrate diet group. According to the results of previous studies, increased dietary NFC level is accompanied by a rapid reduction in ruminal A: P ratio [11] [12] [13] [14]. However, the changes in A: P ratio from the high-concentrate diet group followed the opposite trend compared to results of previous studies after the calcium pyruvate infusion. Ruminal infusion of calcium pyruvate may not mimic the true pyruvate distribution, and this may result in the differences. As pyruvic acid is mainly present in microbial cells, the concentration of pyruvate that can be detected in the rumen fluid is very low. In the high concentrate diet, the NFC level was high. Oligosaccharides and monosaccharides produced by NFC degradation can only be utilized by some microorganisms. For example, rumen Ruminococcus flavefaciens cannot utilize glucose, while rumen Ruminococcus albus preferentially utilize cellobiose. As a result, the fermentation substrates are distributed differently in the rumen, which affects the composition of the microflora. In the high-concentrate diet group, microorganisms associated with NFC degradation are multiplied, and monosaccharides or oligosaccharides produced by NFC degradation are rapidly captured and utilized by such microorganisms. Such microorganisms are often closely related to the formation of propionate, such as starch-degrading bacteria and lactic acid-producing bacteria. In the weakly acidic rumen environment, pyruvate becomes pyruvic acid. As a substrate of fermentation, pyruvic acid may be evenly or slightly unevenly distributed, absorbed and utilized by a wide range of microorganisms. For a low-concentrate diet, rumen fermentation occurs mainly as the acetic acid type. Therefore, after pyruvate infusion, calcium pyruvate is used by a large number of microorganisms related to acetic acid production, which promotes the growth of this group of microorganisms. Subsequently, the ruminal A: P ratio does not decrease, but increases. The results of rumen VFAs do not support one of the previous assumptions. The rate of carbohydrate fermentation may not be a major factor in regulating the A: P ratio. Therefore, there is skepticism about this hypothesis, which is because pyruvate infusion does not truthfully simulate the true distribution of pyruvate among rumen microorganisms under differential rates of degradation.

4.2. Effect of Rumen Microorganisms on Rumen Fermentation

4.2.1. Differences in Microbial Flora between Rumen Fluid of High Concentrate and Low Concentrate Diets

The three most abundant phyla in the rumen fluid samples were Bacteroidetes, Firmicutes and Proteobacteria, and this result is consistent with those of previously studies [15]. Firmicutes were significantly more abundant in the low-concentrate diet group than in the high-concentrate diet group. Conversely, Proteobacteria and Bacteroides were significantly lower in the low-concentrate diet group than in the high-concentration diet group. At the genus level, Eubacteria (coprostanoligenes_group) and Ruminococcus (Ruminococcaceae _UCG-002 and Ruminococcaceae_V9D2013_group) were significantly lower in the high-concentrate diet group than in the low concentrate diet group, while Fi-

brobacter, Prevotella (Prevotellaceae _YAB2003_group) and Roseburia were significantly higher in the high concentrate diet group than in the low-concentrate diet group. Rumenococci were one of the most abundant among Clostridiales in Firmicutes, and are the main fiber-degrading bacteria in the rumen. They can produce a large amount of cellulose and hemicellulose, and generally do not produce propionic acid. The genus Fibrobacter is the only genus of the Fibrobacteres which are fiber-degrading bacteria. It is also known as Fibrobacter succinogenes, and the fermentation product is usually succinic acid. Acid-utilizing bacteria use succinic acid to produces propionic acid via the succinic acid pathway, which increases the proportion of propionate in the rumen. The genus Roseburia is an actinomycete that can ferment glucose and maltose. Upon fermentation of glucose, Roseburia produces mainly lactic acid and a small amount of acetic acid, formic acid and succinic acid. Prevotella is the most abundant bacteria in the rumen. Although it is a starch-degrading bacterium, it is potent and can degrade plus utilize starch and plant cell wall polysaccharides such as xylan and pectin. It can also degrade protein, but not cellulose. Its fermentation products are mainly acetic acid, succinic acid and propionic acid. By comparison, we found that the low-concentration diet group had more abundant fiber-degradation-related flora than the high-concentration diet group. The high-concentration diet group was associated with high propionic acid-producing bacteria compared to the low-concentration diet. This may be the reason why diets of different concentrates differ in A: P ratio.

4.2.2. Changes in Rumen Flora after Infusion of Different Concentrations of Calcium Pyruvate

After rumen infusion of different concentrations of calcium pyruvate, the content of Proteobacteria was significantly lower than that of both the high concentrate and low concentrate groups. In the two calcium pyruvate infusion groups, Bacteroidetes were most common, followed by Firmicutes. The content of Euryarcheaota was also significantly higher in these two groups than in the high concentrate diet group. Euryarcheaota are a major category otherwise known as archaea, and include many methanogens in the rumen. At the genus level, the Wilcoxon rank sum test showed that the ruminal microbes in the pyruvate infusion group were not significantly different from the low concentrate diet group and significantly different from the high concentrate diet group. It is likely that compared to different types of dietary carbohydrates, pyruvic acid enters easily the cell membrane and is more easily utilized by most microorganisms, which results in smaller differences in distribution between microorganisms.

The high-concentrate diet group had significantly higher contents of Megasphaera, Succinivibrio (Succinivibrionaceae_UCG-002), Rumenococcus (Ruminococcus_2), Prevotella (Prevotellaceae_YAB2003_group), and Fibrobacter than did the two pyruvate infusion groups. Megasphaera elsdenii is the common Megasphaera species found in the rumen of juvenile and adult animals fed high-grain diets. As a lactic acid-utilizing bacteria, its main role is the fermenta-

tion of D-type and L-type lactic acid, and the fermentation products are mainly propionic acid, butyric acid and the like. A previous study found that increases in dietary NFC/NDF significantly increased the number of M. eldenii [16]. Succinivibrionaceae_UCG-002 is a hemicellulose-degrading bacterium. The fermentation products of Succinivibrio are mainly acetic acid and succinic acid. A study by R. John Wallace *et al.* found that ruminal contents of Succinivibrio were four times higher in low-methane-producing cattle than in high methane-producing cattle [17]. Based on this observation, it is a plausible assumption that Succinivibrio may help reduce inter-species hydrogen transfer and reduce methane production. At the phylum level, we found that Euryarcheaota was indeed less abundant in the high-concentrate diet group than in the low-concentrate diet group. This means that the high concentrate diet group will produce less methane than the pyruvate infusion group. This also provides an explanation for the lower A: P ratio in the high-concentrate diet group than in the pyruvate-infused group.

The two pyruvate infusion groups had significantly higher contents of Selenomonas_3, Butyrivibrio_2, Russococcus (Ruminococcaceae_UCG-002, Ruminococcaceae_V9D2013_group), Lacnospiraceae_ND3007_group, Prevotellaceae_UCG-003, Rikenellaceae_RC9_gut_group and Veillonellaceae_UCG-001 relative to the high concentrate diet group, but there were not significantly different from the low concentrate group. Selenomonas are starch-degrading bacteria; All Selenomonas strains can ferment starch and produce acetic acid and propionic acid, and cannot ferment structural carbohydrates; they can effectively utilize degradation products of structural carbohydrates, such as cellobiose. In addition, Selenomonas can also utilize lactic acid. However, unlike *M. elsdenii*, their ability to ferment lactic acid is inhibited by increased soluble sugar. Lachnospira are hemicellulose degrading bacteria, and their fermentation products are mainly formic acid, acetic acid, lactic acid and the like. Other studies have shown that Lachnospira can produce butyric acid [18]. It is currently believed that bacteria of genus Rikenella are widely distributed in the digestive tracts of animals. They also produce short chain fatty acids, which mainly include acetic acid and propionic acid. Zhang Ke *et al.* found that the proportion of Rikenellaceae_RC9_gut_group in the rumen of Shanbei White Cashmere Goats increased from 28 days of age, and this was thought to be related to the digestion of cellulose [19]. Veillonella are lactic acid-utilizing bacteria that cannot ferment sugars, but can ferment lactic acid, pyruvic acid, fumaric acid, L-malic acid, and the like. These bacteria were less abundant in the rumen and do not act like M. elsdenii. In this test, the difference in Veillonella is probably due to the fact that the infusion of calcium pyruvate increases the fermentation substrate and benefits the reproduction of these bacteria.

4.3. Comparison of Effects of Rumen Microbes and Pyruvate Infusion Concentration on A: P Ratio

By comparison, it can be seen that calcium pyruvate infusion does not signifi-

cantly affect rumen microorganisms. However, the flora associated with propionic acid production was significantly higher in the high concentrate diet group than in the two pyruvate infusion groups, which might result in the significantly higher A: P ratio in the high concentrate diet group than was seen in the two pyruvate infusion groups. The high concentrate diet group had a lower concentration of fermentation substrate than the other groups, and in particular, the high-concentration calcium pyruvate infusion group. However, greater numbers of microorganisms associated with propionic acid production are associated with a lower rumen A: P ratio. In contrast, when comparing the two calcium pyruvate infusion groups with the low concentrate group, calcium pyruvate infusion did not result in the expected decrease in the A: P ratio, and the A: P ratio was significantly increased in the high-concentration group. This result does not support the notion that the rate of carbohydrate fermentation is the main mechanism regulating A: P ratio, but the microbial composition results support the hypothesis that A: P ratio is regulated by microorganisms.

This is different from the conclusions of previous studies, which have mostly shown that elevated dietary NFC levels or promotion of carbohydrate fermentation can reduce A: P ratio [8] [20]. This may be due to an alteration of rumen flora structure through increases in NFC levels. However, some studies have found that a reduction in dietary NFC levels resulted in no change to A: P ratio, or even an elevated rumen A: P ratio [21] [22]. The reason may be that the magnitude of NFC change was insufficient to cause a change in the structure of the relevant microorganisms due to the redundancy of rumen microbial function and the strong elasticity and resilience of rumen microorganisms [23]. The rate of carbohydrate fermentation is likely to have greater effects on rumen microbes, rather than the insignificant effects from calcium pyruvate in this experiment. This may be the reason that the results of this test differed from results from other studies, where the A: P ratio was reduced by changing feed source and processing. It can be seen that changes in the rumen A: P ratio are more closely related to changes of the rumen microflora structure.

5. Conclusion

The results showed that the main regulatory mechanism of rumen A: P ratio is more closely related to the structure of rumen microflora, and does not support the hypothesis that the rate of carbohydrate degradation regulates rumen A: P ratio.

Author Contributions

Conceptualization, Xueyan Lin and Zhonghua Wang; methodology, Xueyan Lin and Guanwen Cheng; software, Zhiyong Hu and Yun Wang; validation, Qiuling Hou and Zhengui Yan; formal analysis, Guanwen Cheng and Kerong Shi; investigation, Guanwen Cheng; resources, Zhiyong Hu; data curation, Guanwen Cheng and Shizhe Zhang; writing—original draft preparation, Guanwen Cheng;

writing—review and editing, Xueyan Lin and Shizhe Zhang; visualization, Zhonghua Wang; supervision, Zhonghua Wang; project administration, Zhonghua Wang and Xueyan Lin; funding acquisition, Zhonghua Wang and Xueyan Lin.

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Conflicts of Interest

The authors declare no conflict of interest.

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