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Rumen Bacteria Abundance and Fermentation Profile during Subacute Ruminal Acidosis and Its Modulation by *Aspergillus oryzae* Culture in RUSITEC System

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Abstract: This study aimed at characterizing changes in rumen bacteria abundance and fermentation profiles by artificial saliva (AS) pH, and at evaluating the potential modulatory role of *Aspergillus oryzae* culture (AOC) in a rumen simulation technique (RUSITEC) system. The treatment included high AS pH (pH 6.8) or low AS pH (pH 5.5) according to the McDougall's method, and low AS pH was sustained by changing the composition of the AS ($NaHCO_3$ from 9.8 to 1.96 g/L, Na_2HPO_4 from 9.3 to 1.86 g/L). In low AS pH condition, the diets contained either 0% AOC, 1.25% AOC, or 2.5% AOC. Therefore, there are four treatments: (1) high AS pH, 0% AOC (HASP); (2) low AS pH, 0% AOC (AOC0); (3) low AS pH, 1.25% AOC (AOC1); (4) low AS pH, 2.5% AOC (AOC2), respectively. The experimental diets were supplemented with 16 g basic diets with the forage to concentrate ratio of 40:60. The experiments were conducted two independent 13 days, with 9 days adaption periods and 4 days sample collection. The results showed that low AS pH decreased the degradabilites of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) ($p < 0.05$), which occurred due to a decreased abundance of fibrolytic *Ruminococcus albus* ($p < 0.001$). The total concentration of volatile fatty acid (VFA) and proportion of propionate were decreased in the low AS pH ($p = 0.026$) and tended to increase the molar proportion of butyrate ($p = 0.086$) and the ratio of acetate to propionate ($p = 0.088$). The abundances of phylum *Firmicutes* ($p = 0.065$) and *Proteobacteria* ($p = 0.063$) tended to be greater in low AS pH group than high AS pH group. Low AS pH increased the abundance of phylum *Actinobacteria* ($p = 0.002$) compared to the high AS pH and decreased the abundances of phylum *Spirochaetes* ($p = 0.032$). Compared with the high AS pH, low AS pH increased the abundances of *Prevotella* ($p = 0.003$), *Pseudoscardovia* ($p = 0.001$), *Mitsuokella* ($p = 0.005$), and *Dialister* ($p = 0.047$), and decreased the abundances of *Olivibacter* ($p = 0.026$), *Ruminobacter* ($p = 0.025$), *Treponema* ($p = 0.037$), and *Sphaerochaeta* ($p = 0.027$) at genus level. Under a severe SARA in RUSITEC, supplementation of 2.5% AOC increased OM degradability, the copy numbers of *Selenomonas ruminantium* and *Fibrobacter succinogenes*. These findings indicate that the reduction AS pH at 5.5 caused a strong shift in bacterial composition in rumen. In addition, the addition of AOC in diets increased the growth rate of certain rumen bacteria that digest fiber or utilize lactate under SARA condition in RUSITEC system.

Keywords: fermentation; degradability; artificial saliva; in vitro; *Aspergillus oryzae* culture

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1. Introduction

Subacute rumen acidosis (SARA) has a negative impact in the dairy industry by decreasing day matter intake (DMI), milk production, profitability, and increasing culling rate and death loss [1]. An overall reduction in rumen pH below 5.6 for more than 3 h per day had been defined as SARA for dairy cows [2]. In recent years, major efforts in

characterizing the rumen microbial composition and function have been made, along with studying the factors that affect rumen microbiota. Studies have shown that the SARA decreased the richness, diversity, and functionality of microbiota in rumen, but the results vary greatly among studies [3–5]. Studies suggested SARA effects are caused by rumen pH and dietary type [6,7]. However, the effect of diet types on rumen bacteria community was different from that of rumen pH. Li et al. demonstrated that there are three groups of bacteria communities in the rumen, including pH-sensitive but substrate insensitive bacteria, pH-insensitive but substrate sensitive, and bacteria that are both pH- and substrate-sensitive [5]. It is difficult to test this hypothesis in vivo because of the differences in DMI and the passage rate of rumen content for ruminants varied intake and forage to concentrate diet. The induced-SARA, by reducing the buffer captivity in vitro, could avoid these differences.

Rumen pH is the most known abiotic factor that influences rumen bacteria community, particularly through inhibition pH-sensitive cellulolytic bacteria [5]. Studies on dairy goats, dairy cows, and beef steers indicated that the count of cellulolytic bacteria decreased when animals experienced SARA [4,8]. Nevertheless, some essential factors (e.g., vitamins, additive) can also facilitate and enhance functionality of specific rumen bacteria [9]. The AOC is one of several fungal products and has been widely used as a feed additive in ruminant production [10,11]. The AOC has richer multiple fiber enzymes rather than the single enzymes secreted by the other additive (e.g., xylanase, cellulase). Studies demonstrated that AOC could regulate the rumen bacteria [12] and increase fiber digestion [13]. Earlier studies have investigated the effect of AOC on the degradation of common feeds through rumen fermentation [14,15]. Supplementation of AOC also influences the metabolism of rumen bacteria. For example, adding AOC in diets increased the number of *Selenomonas ruminantium* and *Fibrobacter succinogenes* in rumen bacteria in vitro [16]. However, there is little information about the effects of supplementation of AOC on fermentation profiles and the rumen bacteria community under a severe SARA.

Considering that such severe models of SARA were difficult to establish in vivo conditions, due to ethical and health issues, the RUSITEC system was deemed appropriate for such long-term studies [17,18]. Therefore, this study aimed at characterizing changes in rumen bacteria abundance and fermentation profiles by AS pH, and to evaluate a potential modulatory role of AOC in RUSITEC system.

2. Materials and Methods

2.1. Animals and Procedures

The study was carried out using RUSITEC fermenters (Sanshin, Tokyo, Japan) as described by Kajikawa et al. and Zhao et al. [19,20]. The RUSITEC system included eight fermenters with an effective volume of 800 mL. The inoculum was obtained from four rumen fistulated Hu lambs that received two meals in the form of totally mixed ration (TMR) pellets at 0700 and 1900 h daily. Rumen contents were obtained from the rumen fistula before feeding in the morning, strained through 4 layers of surgical gauze, and separated into liquid and solid fractions. On the first day, each vessel was inoculated with 400 mL of liquid inoculum under CO_2 flux and mixed with 400 mL of AS. Then, two bags (100 µm pore) were deposited in the fermenters, one with feed and the other with solid inoculum (70 g wet weight). After 24 h, solid inoculum bag was substituted with a new feed bag. Subsequently, the feed bag incubated 48 h was substituted with a new feed bag. The fermenters were deposited at 39 °C waters and infused AS at a rate of 600 mL/day.

2.2. Experimental Diets and Sampling

The treatment contained high AS pH (pH 6.8) or low AS pH (pH 5.5) following the McDougall's method (Table 1) [21]. In the low AS pH condition, the diets contained either 0% AOC, 1.25% AOC, and 2.5% AOC. Therefore, there are four treatments: (1) high AS pH, 0% AOC (HASP); (2) low AS pH, 0% AOC (AOC0); (3) low AS pH, 1.25% AOC (AOC1); (4) low AS pH, 2.5% AOC (AOC2), respectively. The treatments were randomly assigned

to one of eight fermenters and two batches were fermented with an identical procedure. Each treatment had four replications. The experiment was conducted in two independent 13 days, with 9 d for adaption period and 4 d for sample collection.

Table 1. The composition of the buffer.

	High AS pH	Low AS pH
$NaHCO_3$	9.8 g/L	1.96 g/L
Na_2HPO_4	9.3 g/L	1.86 g/L
KCl	0.57 g/L	0.57 g/L
NaCl	0.47 g/L	0.47 g/L
$MgSO_4 \cdot 7H_2O$	0.12 g/L	0.12 g/L
$CaCl_2 \cdot 2H_2O$	0.045 g/L	0.045 g/L

The diets were ground to pass through a 2 mm sieve and supplemented with 16 g of the experimental diets with forage to concentrate ratio of 40:60. The ingredients and chemicals of the experimental diets are presented in Table 2. The pH of all the fermenters was determined at 0800 h, 1400 h, and 2000 h daily throughout the experiment periods.

Table 2. Composition and nutritional level of diets (%).

Ingredients	Contents
Corn straw	10
Alfalfa hay	30
Corn	35
Corn gluten feed	5
Soybean meal	10
Cottonseed meal	5
Wheat bran	5
Total	100
Nutritional level, % DM	
DM	91.00
OM	84.60
CP	17.36
NDF	28.73
ADF	15.18
Starch	25.35
DE[1], MJ/kg	11.52

DE[1] = digestible energy. The DE was calculated according to the Chinese Feed Database (http://www.chinafeeddata.org.cn/admin/Login/index.html (accessed on 22 June 2022)).

2.3. Sample Collection

To stop fermentation, the ice was placed around the over-flow bottle. 10 mL of rumen fluids were collected before feeding, and the pH in the fermenter was immediately measured with a mobile meter (PHB-4, Shanghai Hongyi instrument limited, Shanghai, China). Five milliliters of rumen fluid were mixed with 1 mL of metaphosphoric acid (25% wt/vol) and preserved at −20 °C to determine the VFA. On day 12 and 13, 10 mL of rumen fluid in the fermenter was collected and preserved at −80 °C for bacteria DNA extraction. On days 10, 11, and 12, the bag with residue from each fermenter was collected and washed with 100 mL of AS. Then, the bag was washed with cold water and preserved to analyze DM, OM, NDF, ADF, and CP.

2.4. Analytical Procedures

The DM, ash, and CP were measured according to the Association of Official Analytical Chemists (AOAC) [22]. The DM content was measured at 105 °C in a forced-air oven for 4 h. The ash content was determined in a muffle furnace (PrepASH-340, Precisa, Switzerland) at 550 °C for 6 h. The CP content was determined using a protein analyzer (K9840, Hanon Advanced Technology Group Co., Ltd., Jinan, China) following the method of Kjeldahl, and

CP was calculated as N × 6.25. The NDF and ADF analysis were carried out as described by Van Soest et al. [23]. The starch content was determined by a commercial assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

To determine the concentration of individual VFA, thawed rumen fluids were centrifuged at 2500× *g* for 5 min following the procedure of Liang et al. [24]. The concentrations of VFA were determined using the gas chromatography (TRACE 1300, Thermo Scientific, Milan, Italy) with a silica capillary column (DB-FFAP, 30 m × 0.32 mm × 0.25 µm, Agilent Technologies Co., Ltd., Santa Clara, CA, USA) by the method of Li et al. [25]. The 1% (wt/vol) crotonic acid was used as the internal standard. The temperatures of injector and detector were set at 240 °C. The column temperature was raised from 50 °C to 190 °C at a rate of 25 °C/min, and programmed to increase 200 °C at 10 °C/min and held for 5 min. Finally, the temperature was raised to 220 °C at 10 °C/min and held for 5 min.

The DNA of rumen bacteria was extracted according to the method of Murray et al. and Zhou et al [26,27]. The DNA concentration and purity were determined by an ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of DNA was determined by running aliquot on 1% agarose gel. Primers for all the rumen bacteria were selected from the published literature (Table 3). The qPCR assays were determined using a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA), and qPCR program was as described by Liang et al. [24].

Table 3. The sequence of primers used to determine the relative abundance of bacteria.

Primer Name	Primer Sequences (5′–3′)	Reference
Fibrobacter succinogenes	F: 5′– GGTATGGGATGAGCTTGC-3′R: 5′-GCCTGCCCCTGAACTATC-3′	[28]
Butyrivibrio fibrisolvens	F: 5′-GCCTCAGCGTCAGTAATCG-3′R: 5′-GGAGCGTAGGCGGTTTTAC-3′	[29]
Ruminococcus flavefaciens	F: 5′-CGAACGGAGATAATTTGAGTTTACTTAGG-3′R: 5′-CGGTCTCTGTATGTTATGAGGTATTACC-3′	[29]
Prevotella brevis	F: 5′-GGTTCTGAGAGGAAGGTCCCC-3′R: 5′-TCCTGCACGCTACTTGGCTG-3′	[30]
Selenomonas ruminantium	F: 5′-CAATAAGCATTCCGCCTGGG-3′R: 5′-TTCACTCAATGTCAAGCCCTGG-3′	[30]

The variable regions V3–V4 of bacterial 16S rRNA gene were amplified using specific primers (341F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′). The primers were tagged with Illumina adapter, pad, and linker sequences. PCR enrichment was carried out in a 50 µL reaction including 30 ng template, fusion PCR primer, and PCR master mix. The PCR program was as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were purified using AmpureXP beads and eluted by elution buffer. Libraries were assessed using an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). The sequence was performed on the platform of lllumina MiSeq (BGI, Shenzhen, China) and generating 2 × 300 bp paired-end reads. Overlapping paired-end sequence of the DNA fragments (minimum overlap of 15 bp, maximum mismatch rate set 0.1) was combined by FLASH (Fast Length Adjustment of Short reads, v1.2.11) to get tags of hypervariable regions [31]. Then, the tags were clustered using the USEARCH software to get operational taxonomic units (OTU) (version v7.0.1090) [32]. The chimeras generated by PCR amplification were filtered by UCHIME (v4.2.40) [33]. Then, The Ribosomal Database Project (RDP) Classifier v.2.2 was used to classify the OTU representative sequences with a minimum confidence threshold of 0.6, and QIIME v1.8.0 was used to train on the Greengenes database v201305 [34]. The OTU abundance statistics table of each sample was obtained by USEARCH_global. Alpha and beta diversity were performed by MOTHUR (v1.31.2) [34] and QIIME software (v1.8.0) at the OTU level [35], respectively. Sample cluster was carried out by QIIME software (v1.8.0) based on UPGMA [34]. The LEfSe software (http://huttenhower.sph.harvard.edu/lefse/ (accessed on 22 June 2022)) was

used to determine the difference of rumen bacteria among the AS pH and supplementation AOC. The species was considered as a biomarker when the LDA value was ≥4.

2.5. Statistical Analysis

The number of rumen bacteria was expressed as a proportion of total rumen bacterial 16S rRNA according to Chen et al. and Liang et al. [24,36]. Statistical analysis was performed by SPSS version 17.0 (IBM, Armonk, NY, USA). The differences between the high AS pH and low AS pH group were calculated using independent sample T-test. The effect of AOC on nutrients degradability, fermentation variables, and rumen bacteria community were used to the linear and quadratic responses of dietary inclusion levels of AOC. The multiple comparisons of treatment means were performed using Duncan's test. Significance was set at $p < 0.05$, and a tendency for treatment effect was observed when $0.05 < p \leq 0.10$. Pearson correlation and significance tests was used to analyze the correlation between rumen bacteria at the genus level and VFAs.

3. Results

3.1. Nutrient Degradability and Fermentation Profiles

The effect of AS pH and supplementation of AOC on nutrient degradability and fermentation variables is shown in Table 4. Compared with the high AS pH, low AS pH decreased degradabilities of DM (66.6 vs. 57.6%), OM (66.0 vs. 56.2%), NDF (32.0 vs. 21.1%), ADF (25.5 vs. 15.2%), and CP (64.0 vs. 41.8%). Supplementation of AOC had no effect on the degradabilities of DM, NDF, ADF, and CP under a severe SARA in RUSITEC ($p > 0.05$), and the OM degradability was greater in AOC2 than that in AOC0 group ($p = 0.040$). The total concentration of VFA ($p = 0.023$) and molar proportion of propionate ($p = 0.026$) were decreased in the low AS pH and tended to increase the molar proportion of butyrate ($p = 0.086$) and the ratio of acetate to propionate ($p = 0.088$). The molar proportion of acetate, iso-buyrate, iso-valerate, and n-valerate were not affected by the AS pH ($p > 0.05$). Similarly, adding AOC in diets had no effect on the molar proportion of individual VFA ($p > 0.05$).

Table 4. Effects of AS pH and supplementation of AOC on nutrient digestion in RUSITEC system.

pH	6.8	5.5	5.5	5.5	SEM	*p* Value		
AOC, %	0	0	1.25%	2.5%		pH 6.8 vs. 5.5	Added AOC Level	
							Liner	Quadratic
DM degradability, %	66.67	57.57	58.44	59.97	0.775	<0.001	0.123	0.808
OM degradability, %	66.01	56.24 ^{b}	57.10 ^{ab}	58.74 ^{a}	0.804	<0.001	0.040	0.629
NDF degradability, %	31.98	21.10	23.96	23.17	1.320	0.005	0.652	0.511
ADF degradability, %	25.49	15.22	18.46	14.78	1.191	0.002	0.310	0.132
CP degradability, %	63.98	41.84	48.05	47.88	2.061	0.006	0.217	0.355
pH	6.69	5.44	5.47	5.46	0.142	<0.001	0.951	0.861
TVFA, mmol/L	104.82	67.66	58.01	66.42	5.487	0.023	0.328	0.149
VFA molar ratios, mol/100 mol								
Acetate, %	47.76	47.84	49.74	47.67	3.269	0.504	0.884	0.630
Propionate, %	18.03	13.22	14.51	14.45	0.636	0.026	0.342	0.659
Iso-buyrate, %	0.65	0.34	0.27	0.21	0.080	0.312	0.589	0.932
Butyrate, %	17.01	21.23	19.87	20.59	0.885	0.086	0.873	0.655
Iso-valerate, %	3.56	3.02	3.19	3.96	0.251	0.510	0.472	0.668
Valerate, %	5.61	4.97	4.63	4.89	0.185	0.241	0.806	0.534
Acetate/Propionate	2.73	3.62	3.71	3.30	0.516	0.088	0.465	0.404

Note: a, b differences in level <0.05.

3.2. Rumen Bacteria

The results of AS pH and supplementation of AOC on the copy number of rumen bacteria is presented in Table 5. Compared with the high AS pH, low AS pH had the higher copy numbers of *Selenomonas ruminantium* ($p < 0.001$) and *Prevotella brevis* ($p < 0.001$)

but decreased the copy number of *Ruminococcus albus* ($p < 0.001$). The copy numbers of *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* were similar between the high AS pH and low AS pH treatment ($p > 0.05$). The AOC2 group tended to increase the mounts of *Selenomonas ruminantium* ($p = 0.058$) and *Fibrobacter succinogene* ($p = 0.089$) compared with the AOC0 group.

Table 5. The effect of AS pH and supplementation of AOC on rumen bacteria in RUSITEC system (Log10 16S rRNA copy number/mL rumen fluid).

AS pH	6.8	5.5	5.5	5.5	SEM	*p*-Value		
AOC, %	0	0	1.25%	2.5%		pH 6.8 vs. 5.5	Added AOC Level	
							Liner	Quadratic
Butyrivibrio fibrisolvens	5.90	6.24	6.22	6.32	0.130	0.604	0.686	0.547
Selenomonas ruminantium	9.53	10.27	10.15	10.36	0.044	<0.001	0.058	0.031
Ruminococcus albus	5.02	3.84	3.84	3.92	0.109	<0.001	0.959	0.865
Fibrobacter succinogenes	4.74	5.13	5.88	6.53	0.195	0.120	0.089	0.927
Prevotella brevis	10.41 [b]	10.80 [a]	10.79	10.88	0.024	<0.001	0.228	0.356

Note: small letters indicate differences between high AS pH and low AS pH.

3.3. Alpha Diversity and Rumen Bacteria at the Phylum

The effect of AS pH and supplementation of AOC on alpha diversity and rumen bacteria at the phylum level are shown in Table 6. Low AS pH decreased the indexes of Chao1, ACE, and Shannon compared with high AS pH ($p < 0.01$) and had no difference on Simpson index ($p = 0.389$). However, adding AOC in diets had no effect on alpha diversity ($p > 0.05$). The abundance of *Firmicutes* ($p = 0.065$) tended to be greater in low AS pH group than high AS pH group, and the abundance of *Proteobacteria* tended to be lower ($p = 0.063$). Low AS pH also increased the abundance of *Actinobacteria* ($p = 0.002$) compared with the high AS pH, while decreased the abundance of *Spirochaetes* ($p = 0.032$). Nevertheless, the alpha diversity and rumen bacteria at the phylum level did not differ among supplementation of AOC groups under a severe SARA in RUSITEC ($p > 0.05$).

Table 6. The effect of AS pH and supplementation of AOC on rumen bacteria at the phylum in RUSITEC system.

AS pH	6.8	5.5	5.5	5.5	SEM	*p*-Value		
AOC, %	0	0	1.25%	2.5%		pH 6.8 vs. 5.5	Added AOC Level	
							Liner	Quadratic
Chao1	551.29	260.39	259.51	283.81	11.123	<0.001	0.697	0.479
Ace	553.82	277.40	262.83	308.66	10.132	<0.001	0.312	0.304
Shannon	3.70	3.39	3.32	3.50	0.029	<0.001	0.174	0.237
Simpson	0.09	0.07	0.08	0.06	0.004	0.389	0.102	0.190
Phylum								
Bacteroidetes	54.88	58.97	65.67	56.59	1.785	0.400	0.261	0.665
Firmicutes	23.24	29.93	22.99	32.87	1.467	0.065	0.148	0.542
Proteobacteria	14.55	0.96	1.41	1.12	1.185	0.063	0.497	0.689
Spirochaetes	4.34	0.32	0.27	0.28	0.267	0.032	0.848	0.676
Actinobacteria	1.51	9.51	9.20	8.68	0.045	0.002	0.946	0.749
Others	1.47	0.31	0.48	0.45	0.770	<0.001	0.354	0.252

3.4. Beta Diversity

The beta diversity of rumen bacteria within different treatment for each fraction were calculated and visualized through the two-dimensional PCoA analysis based on

weight_unifrac. A significant difference between the bacterial communities in the AS pH and supplementation of AOC treatment was noted (Figure 1). Both principal components accounted for 66.62% (PC1) and 16.07% (PC2) of the explained variance.

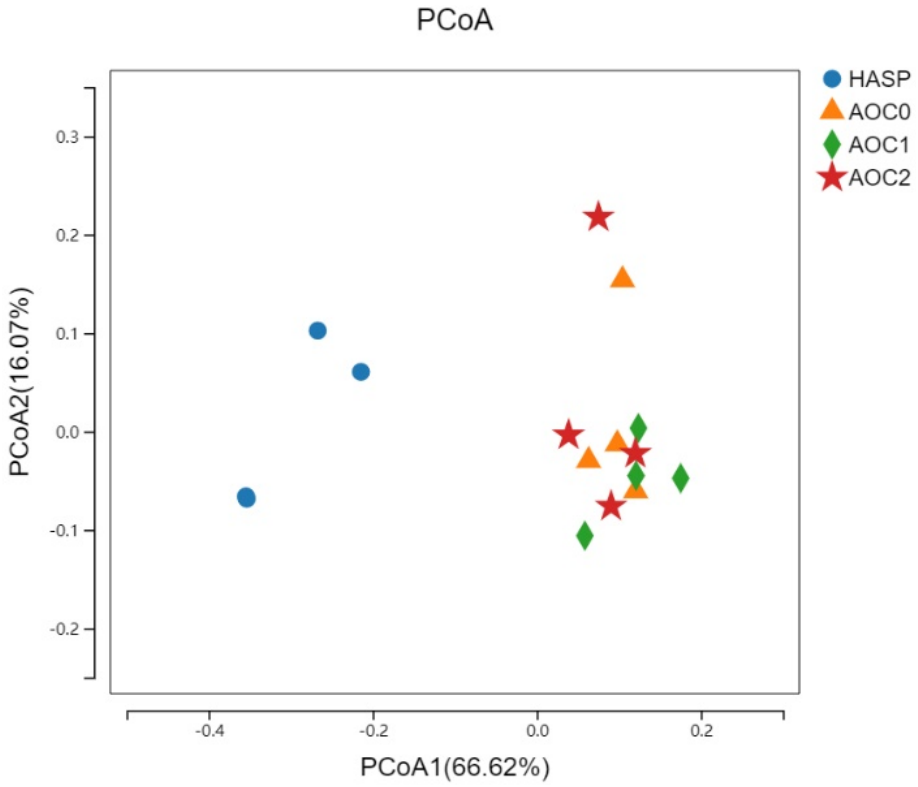

Figure 1. Two dimensional PCoA plots based on weighted_unifrac distance.

3.5. Rumen Bacteria at the Genus

The effect of AS pH and supplementation of AOC on rumen bacteria at the genus in RUSITEC is shown in Table 7. Compared with the high AS pH, low AS pH increased the abundances of *Prevotella* ($p = 0.003$), *Pseudoscardovia* ($p = 0.001$), *Mitsuokella* ($p = 0.005$), and *Dialister* ($p = 0.047$), and decreased the abundances of *Olivibacter* ($p = 0.026$), *Ruminobacter* ($p = 0.025$), *Treponema* ($p = 0.037$), and *Sphaerochaeta* ($p = 0.027$). Low AS pH tended to increase the abundance of *Lactobacillus* ($p = 0.055$). Under a severe SARA in RUSITEC, adding AOC in diets tended to increase the abundance of *Megasphaera* ($p = 0.094$).

Table 7. The effect of AS pH and supplementation of AOC on rumen bacteria at the genus in RUSITEC system.

AS pH	**6.8**	**5.5**	**5.5**	**5.5**	**SEM**	***p*-Value**		
AOC, %	**0**	**0**	**1.25%**	**2.5%**		**pH 6.8 vs. 5.5**	**Added AOC Level**	
							Liner	**Quadratic**
Prevotella	24.13	57.74	64.72	55.29	2.207	0.003	0.249	0.662
Pseudobutyrivibrio	1.13	8.26	8.41	7.52	0.684	0.001	0.911	0.744
Megasphaera	2.13	6.70	3.98	9.96	0.774	0.126	0.094	0.207
Selenomonas	5.56	5.03	5.04	6.80	0.758	0.734	0.687	0.461
Olivibacter	22.11	0.01	0.004	0.002	1.651	0.026	0.496	0.328
Lactobacillus	1.52	5.10	4.69	6.03	0.950	0.055	0.908	0.771
Mitsuokella	0.61	2.26	2.10	2.51	0.229	0.005	0.953	0.736
Succinivibrio	7.8	0.10	0.09	0.10	0.931	0.130	0.955	0.911
Ruminobacter	6.37	0.01	0.01	0.002	0.626	0.025	0.697	0.432
Butyrivibrio	1.17	1.49	1.15	1.33	0.190	0.592	0.775	0.746

Table 7. *Cont.*

AS pH	6.8	5.5	5.5	5.5	SEM	*p*-Value		
AOC, %	0	0	1.25%	2.5%		pH 6.8 vs. 5.5	Added AOC Level	
							Liner	Quadratic
Dialister	0.58	1.12	0.94	1.30	0.118	0.047	0.625	0.640
Treponema	2.66	0.54	0.08	0.45	0.182	0.037	0.970	0.815
Bifidobacterium	0.27	0.82	0.62	0.82	0.150	0.140	0.887	0.989
Lachnospiracea_incertae_sedis	0.61	0.62	0.53	0.60	0.077	0.976	0.841	0.922
Sphaerochaeta	1.69	0.27	0.22	0.24	0.095	0.027	0.894	0.749
Others	21.66	10.48	7.46	7.46	1.409	0.087	0.578	0.375

3.6. LefSe Analysis

Using LefSe analysis, we compared the difference of bacteria taxa in the addition of AOC under a severe SARA at the phylum and genus level (Figure 2). The abundances of *Actinobacteria*, *Bifidobacteriaceae*, and *Bifidobacteriales* were enriched in the low AS pH group compared with the high AS pH group. Under a severe SARA in RUSITEC, supplementation of 1.25% AOC in diets had a higher abundance of *Prevotella*, *Bacteroidales*, and *Bacteroidla*, and the abundances of *Fimicutes* and *Megasphaera* were more abundant in the addition of 2.5% AOC group.

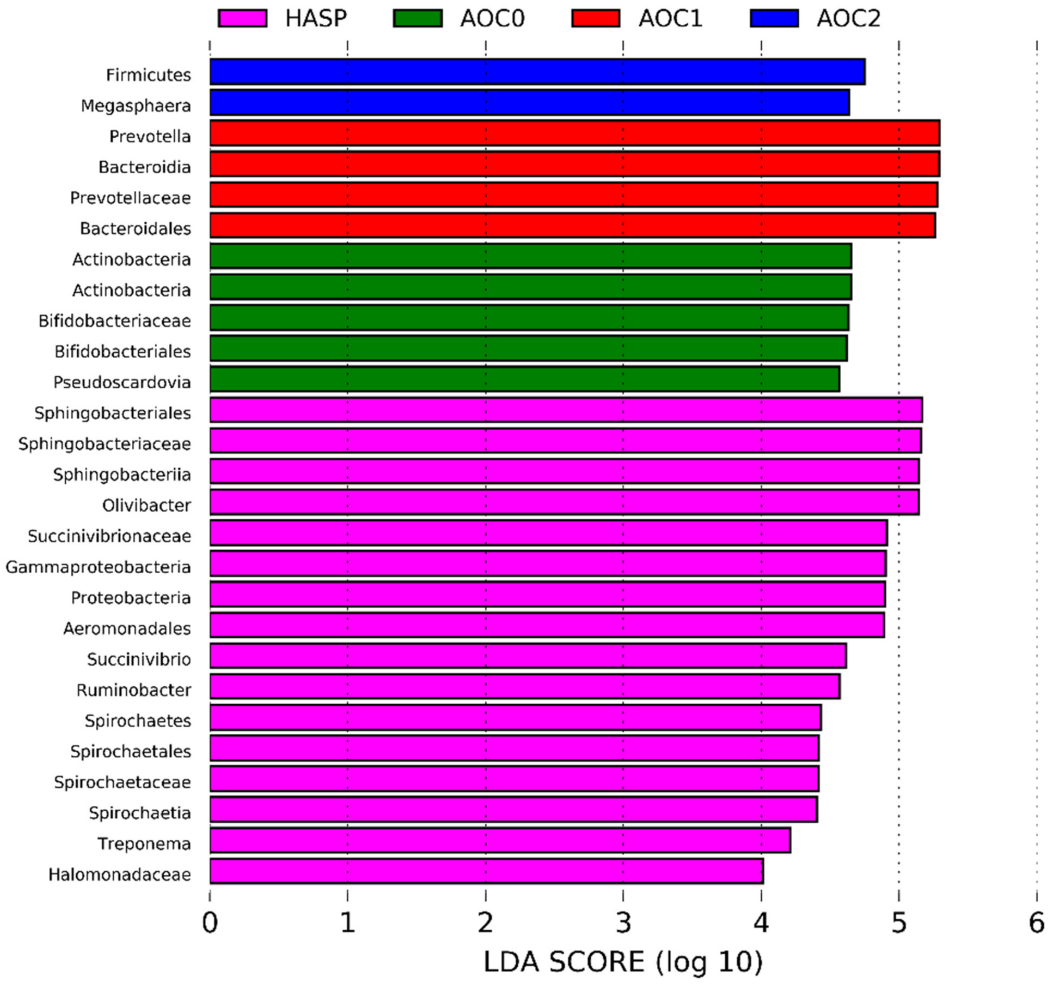

Figure 2. The difference of bacteria taxa in the addition of AOC under a severe SARA with Linear discriminant analysis (LDA) effect size (LEfSe) analysis.

3.7. Correlation Analysis

Some rumen bacteria were associated with metabolites (Figure 3). *Olivibacter* showed positive correlation with TVFA, propionate, and iso-butyrate ($r > 0.50$, $p < 0.05$). *Pseudoscardovia* was negatively associated with TVFA and propionate ($r < -0.50$, $p < 0.05$), and positively associated with butyrate ($r = 0.58$, $p < 0.05$) and the ratio of acetate to propionate ($r = 0.72$, $p < 0.05$). *Succinivibrio* showed correlation with TVFA and propionate ($r > 0.50$, $p < 0.01$). *Butyrivibrio* was also positive correlated with acetate ($r = 0.60$, $p < 0.05$) and the ratio of acetate to propionate ($r = 0.53$, $p < 0.05$). *Treponema* and *Sphaerochaeta* were positively associated with TVFA, propionate, and iso-butyrate ($r > 0.50$, $p < 0.05$).

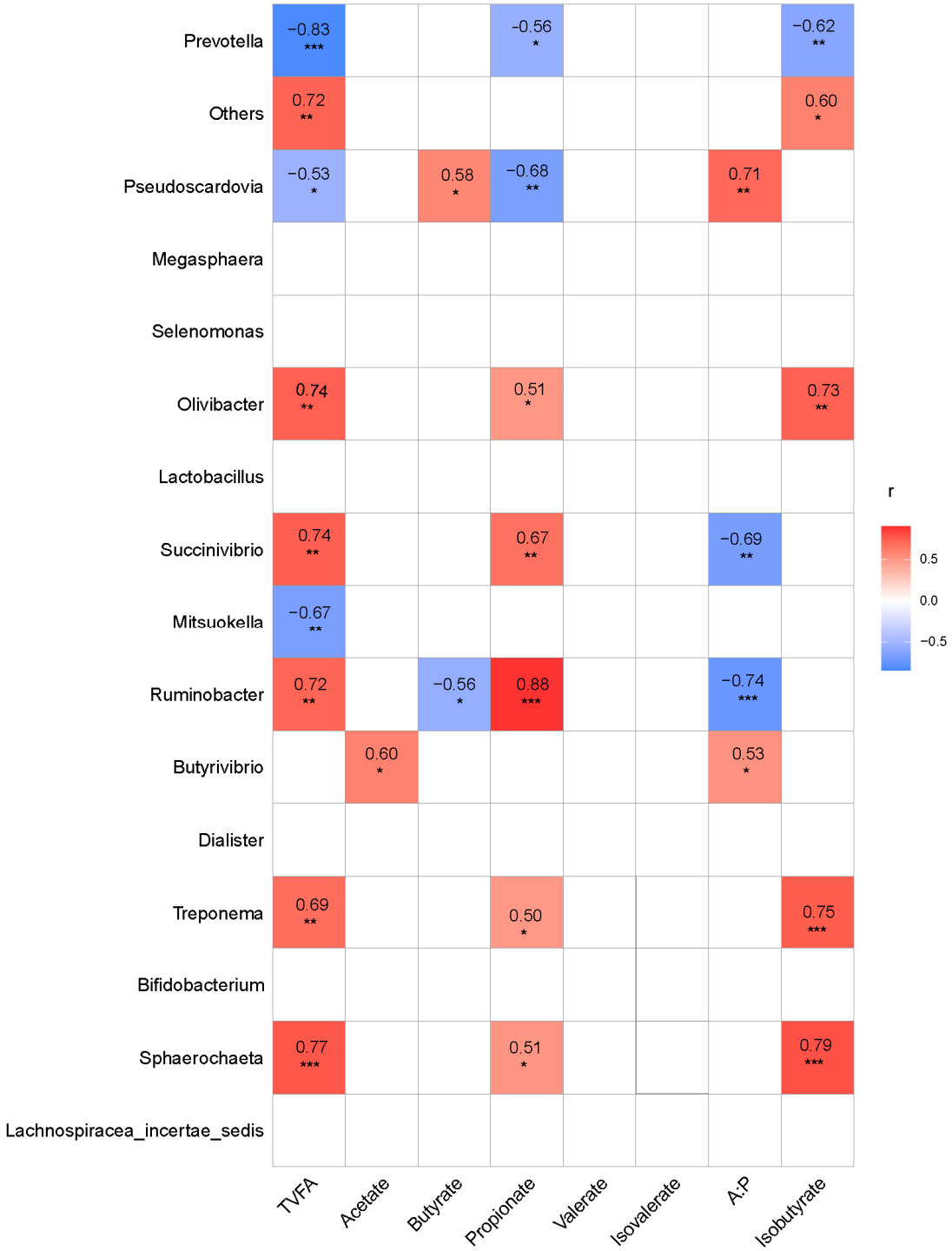

Figure 3. Correlation heat map. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Low AS pH with reducing buffer capacity decreased the degradabilities of DM, OM, NDF, ADF, and CP, which were similar with those of Calsamiiglia et al. and Cerrato-Sánchez et al. [37,38]. The decreased degradabilites of NDF and ADF in low AS pH group were likely the result of reducing the fibrolytic population in the fermenters. Calsamiiglia et al. identified that the fibrolytic bacteria could survive transitory at low rumen pH [37]. Nevertheless, in this experiment, the rumen bacteria in the fermenters were exposed to a low pH of 5.5 for a long time, which inhibited the growth of fibrolytic bacteria and reduced their activity. In contrast, low pH can facilitate the growth and activity of amylolytic bacteria [8]. This result was consistent with our findings—that the amylolytic bacteria of *Selenomonas ruminantium* and *Prevotella brevis* were increased in the low AS pH.

We found adding AOC in diets tended to increase the copy numbers *Selenomonas ruminantium* and *Fibrobacter succinogenes* under a severe SARA in RUSITEC. Similarly, Beharka et al. found the filtrate AO increased the growth rates of *Selenomonas ruminantium* and *Fibrobacter succinogenes* [16]. Nisbet et al. demonstrated that soluble components in AOC and another culture filtrate stimulated lactate uptake by *Selenomonas ruminantium* [39]. The comprehensive enzymes in the AOC stimulated the growth of microorganisms by converting nutrients into micromolecular nutrients for microorganisms. These results demonstrated that the AOC provides factors that are needed for growth by *Fibrobacter succinogenes* and *Selenomonas ruminantium*.

In the present study, the reduction in CP degradation in low AS pH group was consistent with Hoover [40]. A possible explanation is that the reduction in protein degradation is related to the reduction in the digestibility of fiber. The undigested fiber within feed would reduce the access of bacteria and enzymes to the protein, therefore reducing the degradation of protein in feed [38].

The *Aspergillus oryzae* (AO) secretes cellulases and proteases, which should increase the digestibility of feed [41]. In the present study, the addition of 2.5% AOC in diets increased the degradation of OM, but had no difference on DM, NDF, ADF, and CP degradation under a severe SARA in RUSITEC. The main reason for this is that the AOC increased the number of *Selenomonas ruminantium* utilizing the carbohydrates. Previous studies also obtained similar results. Manslield et al. found supplementation of AOC had no effect on the digestion of DM, OM, NDF, and ADF in vitro [14]. Oellermann et al. reported that the digestibility of CP, NDF, and ADF did not affect supplementation of AOC in diets for cattle [15]. The inconsistent results among studies could be due to the different fungi secreting different types of cellulase enzymes.

The total concentration of VFA was decreased from 104.8 mmol/L to 67.6 mmol/L in the low AS pH group compared with the high AS pH group, which was consistent with the results of OM digestion. The molar proportion of propionate was decreased in the low AS pH group, but the molar proportion of acetate was not affected, which resulted in increasing the ratio of acetate to propionate. These results are similar to an earlier study in our lab [42]. The difference in the molar proportion of propionate may be relate to the reduction of starch digestion. Low AS pH group tended to increase the molar proportion of butyrate in our study, which was consistent with the results of other studies [43,44]. Previous studies showed that the concentration of butyrate was only affected by the changes of pH and not affected by the diet composition in vitro [6]. The higher molar proportion of butyrate in the low AS pH group could be explained the increased abundance of *Prevotella* in the fermenters.

The total concentration of VFA and molar proportions of acetate, propionate, and butyrate were similar among the treatments under a severe SARA in a RUSITEC system. Newbold et al. reported the addition of AOC in the high barely diets for sheep decreased the molar proportion of propionate [45]. However, Mansfiled et al. found that addition of AO did not affect the molar proportion of individual VFA in vitro [14]. The inconsistent results among studies were likely due to difference in experiment treatment, effective

dosage of AOC, and experiment animals. The reason in the present study was that the activity of AOC was inhibited with a severe SARA in the fermenters.

As expected, low AS pH significantly decreased the alpha diversity and richness, including the indexes of Chao1, Ace, and Shannon compared with the high AS pH. This result was in accordance with Brede et al. [46]. The PCoA analysis based on uniFrac distance analysis showed that bacterial community clustered separately [47], which indicated that the overall community was changed by the AS pH and supplementation of AOC. The *Actinobacteria*, *Bifidobacteriaceae*, and *Bifidobacteriales* were enriched in the low AS pH group. Under a severe SARA in RUSITEC, supplementation of 1.25% AOC in diets showed a higher abundance *Prevotella*, while the abundances of *Fimicutes* and *Megasphaera* were higher in the addition of 2.5% AOC. The results indicated that supplementation of AOC increased the growth rate of certain rumen bacteria that digest starch (*Prevotella*) or utilize lactate (*Fimicutes* and *Megasphaera*) under a severe SARA.

In the present study, the three main phylum *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were altered by the low AS pH. The abundance of *Bacteroidetes* did not differ among the treatments according to Brede et al. and Guo et al. [42,46], who found the abundance of *Bacteroidetes* was not altered by the SARA challenge or low AS pH at 6.0 in RUSITEC system. However, these results were in contrast to some previous studies, which reported a decrease in the abundance of the phylum *Bacteroidetes* during SARA challenges [3,7]. *Bacteroidetes* are the most abundant Gram-negative bacteria found in the anaerobic communities of the rumen, and low pH led to the death and lysis of Gram-negative bacteria [48]. *Bacteroidetes* were not altered in our study by a severe SARA in RUSITEC and may be related to the diet type. *Firmicutes* is the largest bacterial phylum and contains more than 200 genera, which are mainly comprised of Grampositive bacteria [49]. Mao et al. reported that the *Firmicutes* was more abundant in cattle fed SARA diets compared with the control diets [3]. Guo et al. found that the abundance of *Firmicutes* was not affected by AS pH at 6.02 in RUSITEC [42]. In this experiment, decreasing AS pH at 5.5 increased the abundance of *Firmicutes*. This result indicated that *Firmicutes* have different responses to the vary rumen pH. Low AS pH group significantly decreased the abundance of *Proteobacteria* from 14.6% to 0.96% compared with the high AS pH group, which was an agreement with Mao et al. [3], who found that feeding SARA diets in cattle decreased the abundance of *Proteobacteria*. Nevertheless, this is in contrast with findings of Guo et al. [42], who found that the abundance of *Proteobacteria* was greater in low AS pH at 6.02 in RUSITEC system. Combined with other studies, the results indicated that *Proteobacteria* was more sensitive to rumen pH; the growth and activity of *Proteobacteria* was inhibited by low AS pH 5.5, while it increased in low AS pH 6.02. In the current study, we found the abundance of *Spirochaetes* was decreased in low AS pH. In contrast with earlier studies, Petri et al. found that the higher abundance of *Spirochaetes* was observed in SARA cows [50]. The results indicated that the abundance of *Spirochaetes* could be affected by the combination of rumen pH and diets type. The Gram-positive *Actinobacteria* are colonized and actively influenced the digestive system of ruminants [51]. We found the abundance of *Actinobacteria* increased from 1.51% to 9.51% in low AS pH group compared with the high AS pH group. Similarly, the SARA challenge increased the relative abundance of *Actinobacteria* in the rumen fluid. However, the information is limited on the ecology and biology of *Actinobacteria* [51]. The results indicated that *Actinobacteria* could survive and increase under a SARA condition and low AS pH stimulated their growth and activity.

This study detected a significant difference in the population structure at the genus level between the high AS pH and low AS pH group. Correlation analysis revealed the potential relationship between rumen bacteria and individual VFA. *Prevotella* (*Bacteroidetes*) has been reported to be the most predominant rumen genus. It produces succinate and acetate [52], and plays an important role in the degradation cellulose, protein, and starch. In the present study, low AS pH increased the abundance of *Prevotella*. The result was consistent with Bekele et al. [52], and the change in the abundance of *Prevotella* was associated with the rumen pH [50]. *Pseudobutyrivibrio* (*Firmicutes*) is dominated in anaerobic gastroin-

testinal microbiomes, particularly in the rumen [53]. The rumen genus of *Pseudobutyrivibrio* is an important degrader and utilizer of lignocellulosic plant material [54]. Similarly, the abundance of *Pseudoscardovia* increased from 1.13% to 8.26% in the low AS pH compared with the high AS pH. Correlation analysis showed that *Pseudoscardovia* was negatively associated with TVFA and propionate, and positively associated with butyrate and the ratio of acetate to propionate. These results suggested that low pH facilized the growth and activity of *Pseudoscardovia*, which also utilized the dietary energy and degraded lignocellulosic plant material under a severe SARA. Low AS pH increased the abundance of *Megasphaera* (*Firmicutes*) from 2.13% to 6.70% at the genus level compared with the high AS pH in this research. The genome analysis and physiological characterization of these *Megasphaera* isolates highlighted their ability to produce short chain fatty acids via propionate, acetate, and butyrate [55]. Considering the metabolic features of *Megasphaera* explained earlier and the effect of different environmental factors, it is possible that the acid condition in the fermenters was suitable for growth, and increased the abundance of *Megasphaera* in this experiment. However, low AS pH decreased the abundance of *Olivibacter* (*Bacteroidetes*) (22.11% vs. 0.01%) compared with the high AS pH. *Olivibacter* existed almost solely in the high AS pH, which indicated that this genus was constrained by low rumen pH. The application of *Lactobacillus* (*Firmicutes*) strains as silage inoculants has been widely studied in the field of ruminant nutrition (Sarong et al., 2020) [56]. In the current study, the relative abundance of *Lactobacillus* was greater in the fermenters when the AS pH was decreased at 5.5. These results were similar with findings by Guo et al. [42], who reported the abundance of *Lactobacillus* was increased when the AS pH decreased to 6.02. This result was likely due to rapid growth at low pH in *Lactobacillus*. In the present study, the abundance of *Ruminobacter* (*Proteobacteria*) was decreased in the low AS pH compared with the high AS pH. These results were consistent with Guo et al. [42], who also found that reduction of rumen pH at 6.02 decreased the abundances of *Ruminobacter* in RUSITEC system. Correlation analysis showed that *Ruminobacter* was negatively associated with butyrate and the ratio of acetate to propionate, while being positively associated with propionate and TVFA. *Ruminobacter* is mainly known for amylolytic activities. The decreased abundance of *Ruminobacter* in low AS pH group indicated that the degradation rate of starch might decline. Some rumen bacteria always co-occur with metabolites, and this result has important significance for regulating animal nutrition and health by microbiome intervention [57].

5. Conclusions

In conclusion, decreasing rumen pH at 5.5 significantly decreased the nutrients degradability. For the rumen fermentation, the reduction of pH at 5.5 increased the molar proportion of butyrate and ratio of acetate to propionate, while it decreased the total concentration of VFA and the molar proportion of propionate. Low AS pH increased the abundances of phylum *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, while also decreasing the abundance of *Spirochaetes*. Compared with the high AS pH, low AS pH also increased the abundances of *Prevotella*, *Pseudoscardovia*, *Mitsuokella*, and *Dialister*, while decreasing the abundances of *Olivibacter*, *Ruminobacter*, *Treponema*, and *Sphaerochaeta* at the genus. These findings indicated that the reduction AS pH at 5.5 caused a strong shift in bacterial composition in the rumen. In addition, the addition of AOC in diets increased the growth rate of certain rumen bacteria that digest fiber or utilize lactate under SARA conditions in RUSITEC system.

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