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Research Article 研究报告

Effect of zinc-bearing palygorskite on rumen bacterial diversity *in vitro*

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Abstract: [Objective] The aim of this experiment was to study the effects of zinc-bearing palygorskite (Zn-Pal) on rumen bacterial diversity *in vitro*. **[Methods]** We prepared Zn-Pal by the ion-exchange, and evaluated the compositions of bacterial communities in 60 samples based on 16S rDNA genes. **[Results]** We obtained a total of 1490959 effective sequences and 87662 OTUs. The bacterial diversity in the treatment IV group increased at 24 h, and the abundance of the treatment IV group increased at 48 h. Bacterial community composition analysis shows that the dominant phyla were Bacteroidetes, Firmicutes, Proteobacteria and Lentisphaerae. Compared with the control group, Firmicutes in treatment groups significantly increased (P<0.05) at 24 h and 48 h, whereas Bacteroidetes in the treatment IV group was decreased (P<0.05) at 48 h. At the genus level, the sequences could be assigned to 124 different genera. The content of *Prevotella* had no significant difference between the control and treatments. The relative abundance of *Treponema* in the treatment IV group was significantly higher at 48 h than in the control group (P<0.05), whereas the relative abundance of *Victivallis* and *Pseudobutyrivibrio* in treatment IV was lower. **[Conclusion]** Zn-Pal may affect rumen fluid bacterial diversity in dairy cows, and the degrees of this influence varied with the dose and time of Zn-Pal.

Keywords: zinc-bearing palygorskite, 16S rDNA, rumen bacterial diversity, *in vitro*, dairy cow

The rumen of cattle is a complex ecosystem that harbors a wide variety of microorganisms, including bacteria, protozoa, archaea and fungi[1]. A principal function of the rumen microbiome is the conversion of plant materials into digestible compounds that can be used by the animal host[2]. Additionally, as the microbiome in the rumen undergoes long-term selection and evolution, the microbes and host form an interinhibitory and interdependent homeostatic relationship that has an important role in maintaining host health, improving performance, reducing environmental pollution, and ensuring food and animal product safety[3]. Therefore, studies of ruminal microbes represent a key area of nutrition research in ruminants, and it is important to improve our understanding of the

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complexity of microbial composition and their interactions.

Clay minerals can be used as a carrier loaded with metal ions, such as Ag^{+}, Cu^{2+} and Zn^{2+}, and are good inorganic antibacterial agents. Zinc has many physiological functions, not only is it an essential trace mineral which is required for growth, and enzyme structure and function in poultry[4], but it also can regulate microbial activity[5]. A previous study reported that dietary palygorskite inclusion would exert beneficial effects on the immunity, intestinal integrity and barrier function of broilers at early age[6]. Chalvatzi *et al.*[7] found that dietary inclusion of palygorskite can alter conditions within the caecum in favour of specific species. Zaid *et al.*[8] found that palygorskite was safe and effective in the treatment of mild-to-moderate acute diarrhea in humans. Zhang *et al.*[9] have reported that palygorskite was beneficial to intestinal integrity, which improved growth performance in weaned piglets. Xu *et al.*[10] have found that Zinc loads of 10.3% of clinoptilolite have an antibacterial rate greater than 99.9% for *Escherichia coli* and *Staphylococcus aureus*. Recently, Luo[11] prepared Zn-bearing palygorskitethrough the ion-exchange method and found that it effectively restrained the growth of *E. coli in vitro* and decreased its levels in the intestines of broilers, suggesting that Zn-Pal can be used as an antibiotic agent. Additionally, the presence of micropores and channels in palygorskite, together with the fine particle size and fibrous habit, accounts for its largesurface area[12]. Zn bearing zeolite and montmorillonite have been synthesized based on the high specific area and adsorption capacity of these agents[13–14]. It has also been demonstrated that dietary supplementation of these Zn-bearing nonmetallic minerals can promote growth performance, improve intestinal morphology, inhibit the growth of pathogenic bacteria and enhance intestinal antioxidant capacity[13–16]. Moreover, they could replace antibiotics widely used in poultry production due to their antimicrobial ability.

In this study, palygorskite was used to prepare Zn-Pal through the ion-exchange method, and we evaluated the composition and structure of bacterial communities in samples by Illumina MiSeq high-throughput sequencing of the V3-V4 region of the 16S rDNA genes.

1 Materials and Methods

1.1 Preparation of Zn-Pal

Palygorskite was kindly provided by Jiangsu Sinitic Biotech Co., Ltd. (Xuyi, Jiangsu, P. R. China). Zn-Pal was prepared according to the method described by Yan *et al.*[17]. The chemical composition of Zn-Pal claymineral is SiO_2, 53.07%; Al_2O_3, 10.43%; MgO, 6.11%; CaO, 1.85%; K_2O, 1.92% and Fe_2O_3, 7.75% (Measured using a Minipal 4 X-ray fluorescence spectrometer, PANalytical, Netherland). The amount of Zn adsorbed by palygorskite was 24.5 mg/g with inductively coupled plasma mass spectrometry (ICP-MS, Optima 2100 DV, Perkin Elmer, USA) according to the method described by Yan *et al.*[17].

1.2 Characterization of Zn-Pal

X-ray diffraction (XRD) patterns of palygorskite and zinc-bearing palygorskite were collected on an X'pert PRO X-ray power diffractometer, equipped with a Cu-Kα radiation source (40 kV, 40 mA) from 3 to 80° (2θ) at a scanning step time of 15.2 s, with a step interval about 0.017°, divergence slit of 0.5°. The Zeta potential of palygorskite and Zn-Pal were measured by a Malvern Zetasizer Nano system (Malvern Instruments, USA) at 25 °C, using a folded capillary cell. The specific surface area (SBET) was determined by the Brunauer-Emmett-Teller (BET) method. Determination of cation-exchange capacity and ethylene blue absorption were performed according to the method described by Qiao *et al.*[18].

1.3 Experimental design

The substrate used was a common total mixed

ration diet (with a 50:50 forage: concentrate diet, 54.60% dry matter, 17.15% crude protein, and 34.72% neutral detergent fiber), which was dried at 65 °C for 48 h and broken up by passing it through a 1 mm screen. The composition and nutrient content of the basal diet are shown in Table 1.

Levels of Zn-Pal in *in vitro* incubation fluid were 0% (Control), 0.2% (Treatment I), 0.4% (Treatment II), 0.6% (Treatment III), 0.8% (Treatment IV). The Zn-Pal was mixed with the substrate before the commencement of the experiment. Ruminal fluid was collected from three healthy dairy cows (650 kg mean body weight), which were killed after a 7 day adaptation to the diet. Ruminal fluid was collected from different locations of the rumen, then mixed and strained through four layers of cheese cloth into a pre-warmed thermos flask. The 100 mL of rumen fluid-buffer mixture and rumen fluid at a ratio of 4:1, was dispensed anaerobically into bottles containing 1g of TMR and added Zn-Pal. The composition and dosage of the rumen fluid-buffer mixture are performed according to the method described by Theodorou *et al.*[19]. The serum bottles were filled with O_2-free CO_2 gas, and then capped with a rubber stopper. The bottles were kept in an incubator (JSGI-250T, JSR, Gongju, Korea) at 39 °C. At 0, 12, 24 and 48 h, fluid was sampled to determine the microbial composition using 16S rDNA amplicon pyrosequencing.

Table 1. Composition and nutrient levels of basal diet in dairy cows after parturition (DM basis)

Ingredients	Content/%	Nutrient levels	Content/%
Corn	20.86	ME(MJ/kg)	10.71
Barley	1.89	NE_L(MJ/kg)	6.89
Soybean meal	12.49	CP	18.32
Cottonseed	3.41	EE	4.33
Premix①	2.70	Ash	9.22
DDGS	6.24	ADF	22.81
Wheat silage	20.75	NDF	34.58
Alfalfa hay	12.13	Ca	0.41
Chinensis wildrye	4.04	P	0.38
Beet meal	12.26		
Brewers grain, wet	3.23		

Content of per kilogram premix: 500 kIU vitamin A, 140 kIU vitamin D_3, 2000 kIU vitamin E, 2200 mg Cu, 4000 mg Fe, 2400 mg Mn, 5600 mg Zn, 80 mg I, 35 mg Se and 20 mg Co.

1.4 DNA extraction and 16S rDNA sequencing

Genomic DNA was extracted from thawed rumen fluid samples using a stool DNA Kit (Tiangen Biotech, Beijing) following the manufacturer's procedures. In the present study, the primers B341F 5′-CCTACGGGNGGCWGCAG-3′ and B785R 5′-GACTACHVGGGTATCTAATCC-3′ were used for monitoring bacterial and archaeal populations in the rumen. This primer target of the V3-V4 hypervariable region can be fully covered by the Illumina MiSeq. PCR amplification was performed using a KAPA HiFi HotStart ReadyMix PCR Kit. Each reaction (25 μL) contained 12.5 μL 2×KAPA HiFi HotStart ReadyMix, 0.25 mol/L of each primer and 10 ng of DNA template. The PCR reaction was carried out at 95 °C for 3 min, 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. PCR amplicon libraries were prepared by combining the PCR products for each sample. After purification, the PCR products from the different samples were quantified using the Agilent 2100 Bioanalyzer System (Santa Clara, CA, USA) and then pooled at equal concentrations. Amplicon sequencing was performed on Illumina Miseq platform at Beijing Ori-Gene Science and Technology Co., Ltd. (Beijing, China). The resulting sequences were then screened and filtered for quality and length. Sequences with a length shorter than 50 bp, having more than two primer mismatches, containing ambiguous characters or exhibiting a homopolymer run exceeding 6 bp, were removed[20].

1.5 Bioinformatics and statistical analysis

Using USEARCH[21], the high-quality sequences were clustered into operational taxonomic units (OTUs) defined by 97% similarity. These OTUs were used for diversity (Shannon and Simpson), richness (Ace[22] and Chao[23]), and

rarefaction curve analysis using MOTHUR[24]. Taxonomic assignments of OTUs that reached the 97% similarity level were made using RDP classifier[25] by comparison with the SILVA[26] database, classification based on Bergey's taxonomy, which is divided into kingdom, phylum, class, order, family and genus. The default threshold is 80%, and below this value is unclassified. The effect of different concentrations of zinc-bearing palygorskite on various parameters was evaluated with the one-way analysis of variance (ANOVA) protocol in SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). Means were compared by employing the LSD (least significant difference) multiple-range test at a significance level of $P<0.05$. All data are presented as mean±standard error (SE).

2 Results

2.1 Characterization of Zn-Pal

It can be seen from Table 2 that the BET surface area of Pal is 164.29 m^2/g, the cation exchange capacity of Pal is 28.01 mmol/100 g, the Zeta potential of Pal is –13.60 mV, and the BET surface area of Zn-Pal is 136.60 m^2/g, the cation exchange capacity of Zn-Pal is 35.52 mmol/100 g, the Zeta potential of Zn-Pal is –15.5 mV.

Table 2. Physico-chemical properties of the palygorskites

Items	Palygorskite	Zinc-bearing palygorskite
BET surface area/(m^2/g)	155.74	136.60
Cation exchange capacity/(mmol/100 g)	28.01	35.52
Ethylene blue absorption/(mmol/100 g)	15.00	15.00
Zeta potential/(mV)	−13.60	−15.50

These data were measured by Qiao *et al*. (2015) and the determinations of cation exchange capacity, ethylene blue absorption, specific surface area (BET method) were performed according to the method described by Yongduo and Gaoxiang (2004). Zeta potential was measured by a Malvern Zetasizer Nano system (Malvern Instruments, USA).

2.2 OTU statistics and analysis of alpha diversity

The effect of Zn-Pal on OTUs and alpha diversity after different times of *in vitro* incubation is shown in Table 3. Indices of bacterial richness based on OTUs were estimated by the method of Ace and Chao, and indices of bacterial diversity were determined using the method of Simpson and Shannon. A total of 1490959 quality effective sequences and 1425181 high-quality sequences were obtained from the 60 samples. These sequences included an average of 23753 reads per rumen sample. Among the 60 samples, the total number of OTUs detected by our analysis was 87662 with an average of 1461 OTUs per sample. Simpson in the treatment IV was lower compared with control at 24 h ($P<0.05$), and OTUs in the treatment IV became higher than control at 48 h ($P<0.05$), while alpha diversity indexs in other treatments has no significant differences compared with control ($P>0.05$). Compared with treatment II group, OUTs and Chao in treatment IV group were higher at 48 h ($P<0.01$). Most rarefaction curves for each sample approached the saturation plateau, which indicated that the sampling effort had sufficient sequence coverage to accurately describe the bacterial composition of each group.

2.3 Relative abundance of Bacterial Phyla

In this study, the composition of the bacterial community in rumen fluids was examined to determine the effect of Zn-Pal on microbiota in dairy cattle. Twenty-five different phyla were detected in these samples (including unclassified). The relative abundances of the 10 most abundant phyla are presented in Figure 1 and the relative abundances of the most abundant phyla are presented in Table 4. The five groups showed very similar taxonomic compositions at the phylum-level, even at different time points. At 0 h, Bacteroidetes (range 39%–41%) and Firmicutes (range 30%−32%) were the dominant phyla in the five groups ($P>0.05$). At 12 h, Bacteroidetes, Firmicutes and Proteobacteria were

the most common groups and accounted for 35.33%−37.00%, 31.33%−33.00% and 16.67%−19.00% of the reads, respectively. There were no significant differences between groups (P>0.05) except for Proteobacteria which were higher in the four treatment groups compared with controls (P<0.05). At 24 h, the five groups were dominated by Bacteroidetes, Firmicutes and Proteobacteria, which represented 32.00%–36.33%, 27.00%–29.33% and 15.00%–20.00% of the reads, respectively. And the three phyla in all treatment groups had no significant differences compared with controls except Firmicutes in the treatment IV group, which was higher than in controls, and Proteobacteria in the treatment IV group was lower than in the controls (P<0.05). At 48 h, Firmicutes (37%–39%), Bacteroidetes (32.33%–36.00%), Proteobacteria (10.67%–12.33%) and Lentisphaerae

Table 3. Influence of zinc-bearing palygorskite on the Alpha diversity

t/h	Item	Control	Treatment Ⅰ	Treatment Ⅱ	Treatment Ⅲ	Treatment Ⅳ	SEM	*P*
0	Reads	24721.33±3275.87	24319.00±4182.84	25155.67±2301.31	25028.33±5272.50	14230.00±1165.33	1752.81	0.21
	OTUs	1402.08±6.88	1402.22±20.69	1406.59±17.18	1384.00±11.73	1383.60±13.52	6.17	0.70
	Chao	1954.03±16.95	1959.01±69.40	1979.60±30.82	1854.73±42.47	1866.60±31.38	21.04	0.19
	Ace	1973.69±25.86	1970.73±71.48	1997.06±45.25	1877.97±31.69	1879.43±28.82	21.44	0.23
	Coverage	0.96±0.00	0.96±0.00	0.96±0.00	0.96±0.00	0.96±0.00	–	0.24
	Shannon	6.08±0.01	6.07±0.02	6.09±0.01	6.04±0.02	6.05±0.01	0.01	0.25
	Simpson	0.01000±0.00008	0.01±0.00	0.01±0.00	0.0100±0.0041	0.01±0.00	0.00	0.02
12	Reads	15745.67±1593.64	18127.67±1174.31	17844.33±2533.88	16130.67±2968.67	17468.67±2302.93	873.62	0.91
	OTUs	1096.78±22.64	1108.78±15.98	1108.93±13.75	1082.10±8.33	1125.56±9.40	6.84	0.38
	Chao	1560.89±63.47	1622.42±25.51	1596.18±9.60	1601.57±33.62	1649.23±54.77	17.77	0.67
	Ace	1579.29±65.76	1730.55±77.05	1612.13±16.53	1618.62±46.70	1716.89±71.66	27.79	0.34
	Coverage	0.97±0.00	0.96±0.00	0.96±0.00	0.96±0.00	0.96±0.00	–	0.47
	Shannon	4.86±0.03	4.86±0.06	4.89±0.06	4.73±0.02	4.94±0.03	0.02	0.05
	Simpson	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.04±0.00	–	0.10
24	Reads	19488.33±5732.20	24783.67±1075.16	21021.33±3104.38	16567.00±2996.16	22184.00±8126.50	1633.37	0.65
	OTUs	1140.43±12.71	1119.77±16.43	1109.15±10.15	1114.16±6.35	1110.84±7.04	5.20	0.34
	Chao	1643.39±30.46	1615.66±33.99	1616.99±18.76	1657.90±15.52	1582.07±28.28	12.16	0.36
	Ace	1690.34±60.73	1689.97±66.01	1662.54±50.13	1848.33±101.47	1673.73±34.04	31.05	0.32
	Coverage	0.96±0.00	0.96±0.00	0.96±0.00	0.96±0.00	0.96±0.00	–	0.47
	Shannon	$5.09±0.03^{ab}$	$5.07±0.02^{ab}$	$5.05±0.09^{ab}$	$5.00±0.05^{b}$	$5.20±0.01^{a}$	0.03	0.13
	Simpson	$0.04±0.00^{b}$	$0.04±0.00^{b}$	$0.04±0.00^{b}$	$0.04±0.00^{b}$	$0.03±0.00^{a}$	–	0.04
48	Reads	$20921.33±3534.80^{ab}$	$25927.67±2781.81^{a}$	$16930.67±1224.23^{b}$	$29444.33±2443.74^{a}$	$28355.67±3333.49^{a}$	1642.80	0.04
	OTUs	$1155.65±13.01^{B}$	$1222.97±41.64^{AB}$	$1161.95±12.85^{B}$	$1244.42±22.34^{AB}$	$1296.55±33.16^{A}$	17.42	0.02
	Chao	$1623.53±28.87^{Bbc}$	$1792.55±66.42^{ABab}$	$1579.31±27.03^{Bc}$	$1778.65±41.18^{ABab}$	$1868.33±82.88^{Aa}$	35.56	0.02
	Ace	$1634.97±23.82^{Bb}$	$1968.60±130.21^{Aba}$	$1618.91±16.63^{Bb}$	$1882.83±70.62^{ABab}$	$2089.41±144.00^{Aa}$	60.77	0.02
	Coverage	$0.97±0.00^{Aa}$	$0.96±0.00^{ABb}$	$0.97±0.00^{Aa}$	$0.96±0.00^{ABb}$	$0.96±0.00^{Bb}$	–	0.02
	Shannon	5.56±0.05	5.56±0.05	5.56±0.07	5.58±0.07	5.69±0.02	0.02	0.41
	Simpson	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.01±0.00	–	0.62

With the same row different lowercase letters mean significant at P<0.05 and different capital letters mean significant at P<0.01.

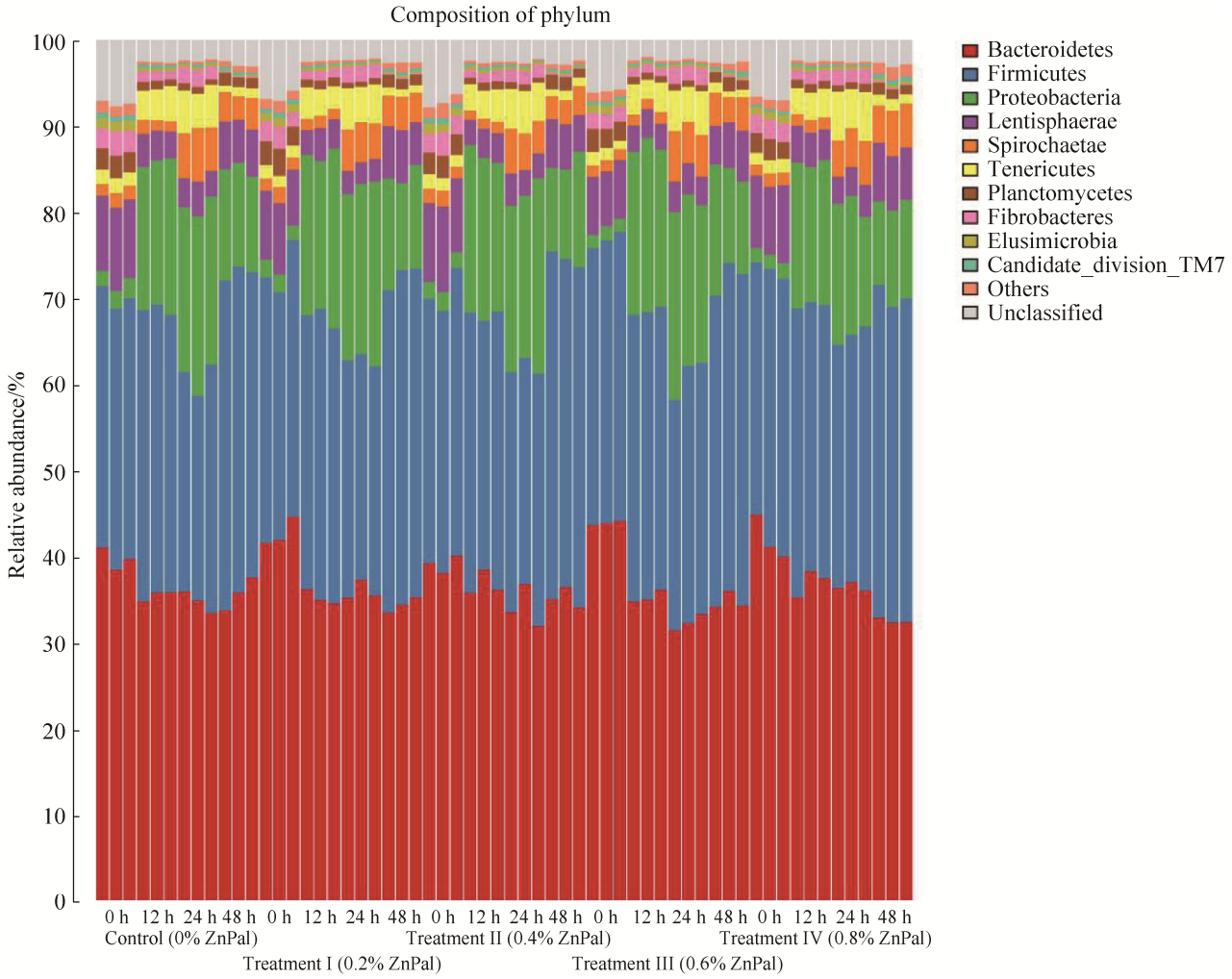

Figure 1. Phylum-level composition of the rumen microbiome. The relative abundances of the 10 most abundant phylum.

(5.00%–6.33%) were the dominant bacterial phyla in cattle rumen fluid. Firmicutes numbers in the treatment II group and in the treatment IV group were increased compared with controls (P<0.05) while Bacteroidetes in the treatment IV group was decreased compared with controls (P<0.05).

At the genus level, the sequences could be assigned to 124 different genera. The relative abundances of the 14 most abundant genera are presented in Figure 2 and Table 5. At 0 h, *RC9_gut_group* was predominant with an abundance of 8.09%–9.54%, followed by *Prevotella*, *Victivallis*, *Fibrobacter*, *Anaeroplasma*, *Oligosphaera* and *Incertae_Sedis* in all five groups. At 12 h, the most abundant genera were *Prevotella*, *RC9_gut_group*, *Anaeroplasma*, *Succinivibrio*, *Incertae-Sedis* and *Fibrobacter*, which together accounted for 30.85%, 29.99%, 32.26%, 30.94% and 31.36% of the reads, respectively. The relative abundances of *RC9_gut_group* and *Succiniclaticum* in treatment IV significantly increased; however, the relative abundance of *Fibrobacter* in treatment group III significantly declined compared with controls (P<0.05). At 24 h, the most abundant sequences were those related to *Prevotella*, *Treponema*, *RF9_gut_group*, *Anaeroplasma*, *Succinivibrio*, *Fibrobacter* and *Incertae_Sedis* in all

Table 4. Influence of zinc-bearing palygorskite on the relative abundance (%) of bacterial groups (phylum level)

t/h	Item	Control	Treatment Ⅰ	Treatment Ⅱ	Treatment Ⅲ	Treatment Ⅳ	SEM	*P*
0	Bacteroidetes	39.67±0.88	40.07±0.67	39.70±0.58	40.33±0.88	40.80±0.58	0.59	0.14
	Firmicutes	30.00±0.00	30.67±0.88	31.33±0.88	32.00±0.58	31.00±1.00	0.38	0.20
	Lentisphaerae	8.83±0.33	8.67±0.67	8.93±0.33	8.67±0.33	8.93±0.33	0.30	0.24
	Proteobacteria	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00	–	–
12	Bacteroidetes	35.67±0.33	35.33±0.33	37.00±1.00	35.33±0.33	36.67±0.88	0.32	0.29
	Firmicutes	33.00±0.58	32.67±0.67	31.33±1.20	33.00±0.00	32.33±0.88	0.33	0.55
	Lentisphaerae	3.33±0.33^{a}	3.33±0.33^{a}	3.00±0.00^{a}	3.00±0.00^{a}	4.00±0.00^{b}	0.10	0.04
	Proteobacteria	17.33±0.33^{ab}	19.00±1.16^{a}	18.33±0.67^{ab}	19.00±0.58^{a}	16.67±0.33^{b}	0.38	0.13
24	Bacteroidetes	34.67±0.88^{ab}	36.00±0.58^{a}	34.33±1.45^{ab}	32.00±0.58^{b}	36.33±0.33^{a}	0.50	0.03
	Firmicutes	26.00±1.53^{a}	27.00±0.58^{ab}	27.67±0.88^{ab}	28.67±0.88^{ab}	29.33±0.88^{b}	0.48	0.22
	Lentisphaerae	3.33±0.33	3.00±0.00	3.33±0.33	3.67±0.33	3.33±0.33	0.12	0.65
	Proteobacteria	19.67±0.67^{a}	20.00±0.58^{a}	20.33±1.33^{a}	20.00±1.16^{a}	15.00±1.00^{b}	0.65	0.02
48	Bacteroidetes	36.00±1.16^{a}	34.33±0.33^{ab}	35.00±0.58^{a}	34.67±0.67^{a}	32.33±0.33^{b}	0.39	0.04
	Firmicutes	37.00±1.00^{a}	38.00±0.58^{ab}	39.33±0.67^{b}	37.33±0.67^{ab}	38.00±0.58^{b}	0.32	0.26
	Lentisphaerae	5.33±0.33	5.67±0.33	5.00±0.58	5.00±0.58	6.33±0.33	0.18	0.26
	Proteobacteria	12.00±0.58	11.67±0.88	11.00±1.00	12.33±1.33	10.67±0.33	0.40	0.67

With the same row different lowercase letters mean significant at $P<0.05$ and different capital letters mean significant at $P<0.01$.

groups. The *Treponema* genus was significantly lower in treatment II and treatment IV groups than that of the controls ($P<0.05$). Also, in comparison with controls, *Succiniclasticum* in the treatment II group became lower, however levels of *Succiniclasticum* in treatment IV were higher ($P<0.05$). At 48 h, the genera were numerically dominated by sequences related to *Prevotella*, *RC9_gut_group*, *Succiniclasticum*, *Incertae_Sedis*, *Treponema*, *Pseudobutyrivibrio* and *Victivallis* in all groups. The relative abundances of *RC9_gut_group* in all treatment groups were lower than in controls ($P<0.05$). The relative abundances of *Succiniclasticum* and *Pseudobutyrivibrio* were decreased, while the relative abundance of *Incertae_sedis* was increased in the treatment II group compared with controls ($P<0.05$). The relative abundance of *Treponema* in the treatment IV group was significantly higher, however the relative abundance of *Victivallis* and *Pseudobutyrivibrio* in treatment IV was significantly lower than in controls ($P<0.05$).

3 Discussion

Zinc-bearing palygorskite (Zn-Pal) could replace antibiotics widely used in poultry production due to their antimicrobial activity. Here we tested the effects of Zn-Pal on microbial composition from dairy cattle, in order to evaluate its potential application in this setting. The change of BET surface area and cation exchange capacity is due to the process of zinc-bearing. The change of zeta potential is due to the dissociation and dispersion of the palygorskite crystal beam and the exchange of sodium ions with metal ions on the surface of palygorskite. In our study, the Zeta potentials of palygorskite were negative, and Zn-Pal was more negative that palygorskite alone. The palygorskite particle can be considered as an anion with large size and high charge density, which attracts the ions of opposite sign (counterions) and repels the ions of the same sign (co-ions)[27]. While at the same ion valency, the thickness of the diffuse layer might reduce due to increase in the concentration of electrolyte[28]. Therefore, the difference in the surface

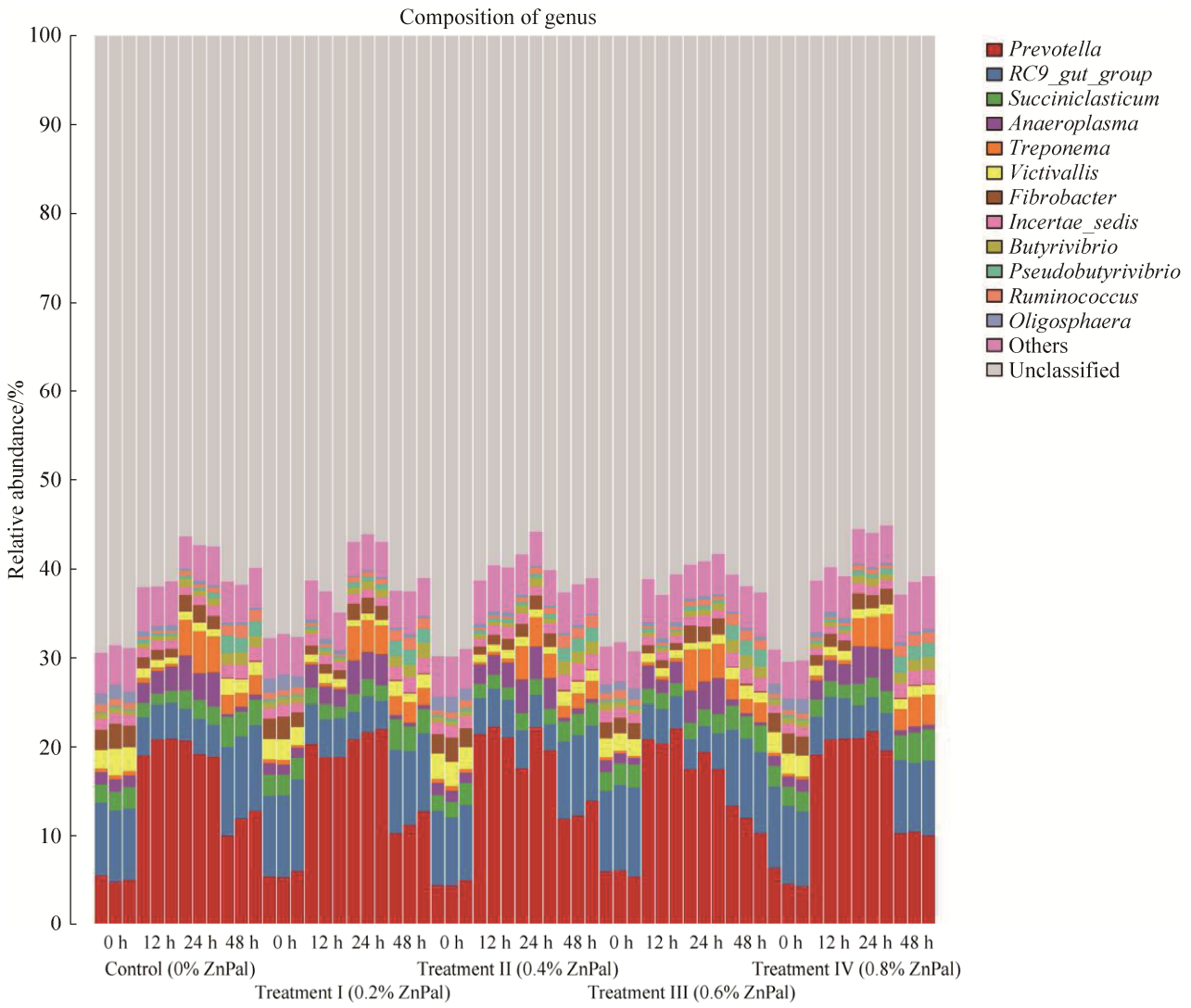

Figure 2. Genera-level composition of the rumen microbiome. The relative abundances of the 12 most abundant genera.

structures between palygorskite and Zn-Pal might indicate that they would have differential effects on microbial composition.

The ruminant rumen has abundant bacterial population and is similar to other vertebrate digestive tract microflora. Bacteria account for about 50% to 80% of the rumen biomass. Bacteroides and Firmicutes are the two major categories of rumen bacteria, accounting for about 80% of the total, in addition to the less abundant Spirochaetes, Actinobacteria and Proteobacteria[29–30]. Bacteroides plays an important role in the degradation of non-fibrous materials, and the thick-walled bacteria are mainly decomposing Firmicutes[31]. In our study, the Bacteroidetes and Firmicutes also were the most abundant in samples obtained from all treatment groups and this dominant position is not altered with changes of Zn-Pal levels in the diet. Proteobacteria is a gram-negative bacterium whose outer membrane is mainly composed of LPS. Many bacterial belong to the micro-aerobic and facultative anaerobic types. There are five main categories of which Proteobacteria contains a variety of pathogens, such as *E. coli*, *Salmonella*. Li[32] have reported that

Table 5. Influence of zinc-bearing palygorskite on the relative abundance (%) of bacterial groups (genus level)

t/h	Item	Control	Treatment Ⅰ	Treatment Ⅱ	Treatment Ⅲ	Treatment Ⅳ	SEM	*P*
0	*RC9_gut_group*	8.09±0.20	8.53±0.22	8.16±0.18	8.54±0.22	8.45±0.64	0.20	0.15
	Prevotella	5.20±0.06	5.67±0.38	4.70±0.26	5.92±0.28	5.20±0.22	0.17	0.18
	Victivallis	2.57±0.10	2.23±0.02	2.68±0.23	2.48±0.14	2.30±0.06	0.08	0.13
	Fibrobacter	2.51±0.02	2.17±0.06	2.36±0.05	1.78±0.17	2.19±0.08	0.09	0.09
	Succiniclasticum	2.18±0.03	2.39±0.07	2.00±0.04	2.39±0.04	2.24±0.02	0.06	0.26
	Anaeroplasma	1.38±0.25	1.17±0.09	1.25±0.12	1.07±0.06	1.20±0.02	0.04	0.25
	Oligosphaera	1.38±0.15	1.52±0.26	1.56±0.20	1.07±0.02	1.36±0.04	0.07	0.17
	Incertae_Sedis	1.37±0.03	1.37±0.13	1.47±0.02	1.44±0.05	1.36±0.04	0.04	0.09
	Ruminococcus	0.77±0.04	0.74±0.03	0.68±0.02	0.80±0.10	0.57±0.05	0.03	0.20
	Butyrivibrio	0.56±0.03	0.66±0.02	0.55±0.05	0.66±0.03	0.63±0.04	0.02	0.53
	Treponema	0.44±0.06	0.41±0.04	0.45±0.07	0.30±0.11	0.36±0.02	0.02	0.10
	Pseudobutyrivibrio	0.22±0.12	0.30±0.16	0.33±0.12	0.37±0.11	0.24±0.15	0.20	0.07
12	*Prevotella*	20.33 ± 0.62^{ab}	19.38 ± 0.49^{b}	21.64 ± 0.35^{a}	21.15 ± 0.51^{a}	20.36 ± 0.59^{ab}	0.28	0.09
	RC9_gut_group	4.11 ± 0.10^{bc}	4.41 ± 0.08^{ab}	4.20 ± 0.06^{abc}	3.86 ± 0.12^{c}	4.53 ± 0.16^{a}	0.75	0.01
	Anaeroplasma	2.53±0.12	2.16±0.16	2.39±0.05	2.16±0.08	2.29±0.11	0.09	0.71
	Succiniclasticum	1.38 ± 0.13^{a}	1.63 ± 0.21^{ab}	1.54 ± 0.20^{ab}	1.66 ± 0.35^{ab}	1.75 ± 0.06^{b}	0.05	0.24
	Incertae_Sedis	1.38±0.07	1.35±0.04	1.39±0.05	1.21±0.01	1.16±0.04	0.04	0.32
	Fibrobacter	1.12 ± 0.11^{a}	1.05 ± 0.07^{ab}	1.10 ± 0.05^{ab}	0.89 ± 0.01^{b}	1.26 ± 0.04^{a}	0.41	0.05
	Victivallis	0.85±0.11	0.90±0.07	0.87±0.05	0.94±0.03	1.04±0.05	0.03	0.37
	Oligosphaera	0.59±0.07	0.52±0.02	0.47±0.09	0.47±0.05	0.60±0.09	0.02	0.05
	Butyrivibrio	0.47±0.02	0.41±0.14	0.41±0.12	0.47±0.06	0.48±0.05	0.02	0.55
	Treponema	0.42±0.05	0.32±0.04	0.35±0.02	0.38±0.09	0.49±0.04	0.02	0.14
	Pseudobutyrivibrio	0.37±0.01	0.36±0.04	0.42±0.01	0.29±0.04	0.39±0.04	0.02	0.53
	Ruminococcus	0.30±0.03	0.31±0.06	0.29±0.02	0.32±0.04	0.26±0.01	0.01	0.70
24	*Prevotella*	19.65 ± 0.56^{ab}	21.58 ± 0.34^{a}	19.87 ± 1.34^{ab}	18.20 ± 0.65^{b}	20.85 ± 0.63^{a}	0.43	0.09
	Treponema	4.26 ± 0.12^{a}	3.56 ± 0.29^{ab}	3.26 ± 0.41^{b}	4.03 ± 0.34^{ab}	3.47 ± 0.14^{b}	0.14	0.09
	RC9_gut_group	3.73±0.02	3.41±0.07	3.62±0.06	3.45±0.10	3.92±0.09	0.12	0.71
	Anaeroplasma	3.59±0.28	3.43±0.21	3.64±0.10	3.61±0.28	4.14±0.37	0.12	0.44
	Succiniclasticum	2.09 ± 0.24^{b}	1.92 ± 0.17^{bc}	1.82 ± 0.31^{c}	1.97 ± 0.28^{bc}	2.38 ± 0.23^{a}	0.06	0.02
	Fibrobacter	1.79 ± 0.04^{ab}	1.75 ± 0.03^{ab}	1.53 ± 0.07^{b}	1.85 ± 0.08^{a}	1.69 ± 0.04^{ab}	0.05	0.26
	Incertae_Sedis	1.52±0.15	1.54±0.09	1.63±0.03	1.69±0.08	1.48±0.10	0.04	0.49
	Victivallis	0.88±0.14	0.67±0.05	0.88±0.07	0.86±0.12	1.00±0.01	0.03	0.02
	Butyrivibrio	0.77±0.10	0.86±0.06	0.79±0.14	0.67±0.06	0.82±0.06	0.04	0.63
	Ruminococcus	0.52±0.10	0.59±0.03	0.61±0.04	0.55±0.04	0.44±0.04	0.03	0.25
	Pseudobutyrivibrio	0.43±0.06	0.55±0.03	0.46±0.04	0.44±0.08	0.55±0.05	0.03	0.34
	Oligosphaera	0.30±0.06	0.20±0.03	0.28±0.01	0.33±0.04	0.28±0.03	0.02	0.17

(待续)

(续表 5)

48	*Prevotella*	11.67±0.83^{ab}	11.49±0.71^{ab}	12.80±0.62^{a}	12.00±0.89^{ab}	10.36±0.13^{b}	0.34	0.24
	RC9_gut_group	9.59±0.21^{a}	8.84±0.32^{b}	8.71±0.18^{bc}	8.85±0.16^{b}	8.10±0.20^{c}	0.15	0.01
	Succiniclasticum	3.02±0.19^{a}	2.96±0.24^{b}	2.40±0.08^{b}	2.72±0.14^{ab}	3.24±0.23^{a}	0.10	0.07
	Incertae_Sedis	2.05±0.10^{b}	2.11±0.01^{b}	2.36±0.07^{a}	2.01±0.11^{b}	2.08±0.05^{b}	0.04	0.01
	Treponema	1.97±0.21^{a}	2.11±0.12^{a}	1.68±0.20^{a}	2.02±0.21^{a}	3.04±0.33^{b}	0.15	0.02
	Pseudobutyrivibrio	1.75±0.10^{a}	1.66±0.22^{ab}	1.47±0.13^{b}	1.71±0.11^{a}	1.64±0.04^{b}	0.04	0.14
	Victivallis	1.58±0.02^{a}	1.38±0.01^{ab}	1.38±0.02^{ab}	1.34±0.04^{ab}	1.14±0.02^{b}	0.06	0.31
	Butyrivibrio	1.32±0.04	1.44±0.07	1.45±0.07	1.41±0.06	1.31±0.05	0.04	0.67
	Ruminococcus	1.29±0.06	1.23±0.16	1.31±0.06	1.15±0.05	1.22±0.06	0.03	0.31
	Anaeroplasma	0.49±0.10	0.46±0.04	0.50±0.01	0.46±0.07	0.59±0.08	0.03	0.72
	Oligosphaera	0.27±0.08	0.31±0.05	0.27±0.05	0.30±0.04	0.39±0.02	0.02	0.11
	Fibrobacter	0.13±0.01	0.14±0.04	0.15±0.05	0.17±0.01	0.15±0.02	0.01	0.78

With the same row different lowercase letters mean significant at $P<0.05$ and different capital letters mean significant at $P<0.01$.

zinc-bearing palygorskite (ZnBP) has an antibacterial activity on *E. coli* K88 in an artificial gastroinestinal pH environment from piglets. Xu *et al*.[10] proved the antibacterial rates of the coatings for *E. coli* and *Staphylococcus aureus* exceeded 99.0% when the content of zinc-typed antibacterial agent was at 3% in coatings. Wang *et al*.[33] suggested that the addition of zinc-bearing clinoptilolite (ZnCP) to feed exerted protective effects on performance and gut health of broilers against *S. pullorum* infection. Moreover, Tang *et al*.[15] indicated that zinc-bearing clinoptilolite (ZnCP) supplementation may modulate digestive enzyme activities and intestinal structure and function of broiler chickens. In our study, Proteobacteria in treatment IV group was lower than control at all times, while Proteobacteria in treatment groups I, II and III became higher than in the control. This can be partially explained by the fact that a low dose of Zn-Pal may benefit the growth of beneficial bacteria, and a high dose of Zn-Pal may inhibit the propagation of harmful bacteria. This could be caused by fact that the Zn^{2+} content on the surface of the embankment is higher than that of the bacteria, and therefore the antibacterial activity is improved. The slow release of Zn^{2+} penetrates the negatively charged microbial cell membrane, destroys the activity of the cell synthetic machinery, interferes with DNA synthesis, and blocks division and proliferation [16,34].

Prevotella degrades starch and is the major flora of proteolytic bacteria in the rumen[35]. This study also detected that *Prevotella* content was the highest in the rumen of all treatment groups, and it was confirmed that *Prevotella* was the dominant flora of the rumen based on traditional culture and various molecular biology methods[36–37]. In our study, the number of *Prevotella* increased gradually with the increase in the amount of Zn-Pal. *Fibrobacter*, *Ruminococcus* and *Butyrivibrio* are the major fiber degrading bacteria. In our study, compared with control group, in all treatment groups the three types of bacteria are reduced. We speculated that this change could be due to the inhibitory effect of Zn on the digestion of fiber by rumen microbes[38]. Arelovich *et al*.[39] have proved that Zn tends to selectively inhibit growth, or metabolic activities, of rumen microbes. Vázquezarmijo *et al*.[40] have found that Zn can reduce the cellulolytic activity of bacteria in the inoculation medium. In conclusion, Zn-Pal affected rumen fluid bacterial diversity *in vitro*, and its impact varied with the application dose and time. Zn-Pal might be a good alternative to antibotics in dairy cattle.

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载锌凹凸棒石黏土对瘤胃体外发酵细菌多样性的影响

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摘要:【目的】研究载锌凹凸棒石黏土对瘤胃体外发酵细菌多样性的影响。【方法】本试验采用离子交换法制备载锌凹凸棒石黏土，利用 16S rDNA 测序技术分析了载锌凹凸棒石黏土对奶牛瘤胃体外发酵细菌菌群和多样性的影响。【结果】研究共获得 1490959 个有效序列和 87662 个 OTUs。测序结果表明，与对照组相比，试验Ⅳ组中的细菌多样性在体外发酵 24 h 时提高，试验Ⅳ组中的细菌丰度在体外发酵 48 h 时提高。细菌菌群组成分析表明，60 个样本中的优势菌门主要是厚壁菌门、拟杆菌门、变形菌门和黏胶球形菌门。与对照组相比，各试验组的厚壁菌门在体外发酵 24 h 和 48 h 时均显著增加(P<0.05)，试验Ⅳ组中拟杆菌门在发酵 48 h 时显著降低(P<0.05)；60 个样本中共获得 124 个细菌菌属，其中对照组与试验组中普氏菌属含量均没有显著变化(P>0.05)。在体外发酵 48 h 时，试验Ⅳ组中密螺旋体属含量与对照组相比显著增加(P<0.05)，而食物谷菌属和假丁酸弧菌属含量与对照组相比均显著降低(P<0.05)。【结论】载锌凹凸棒石黏土对奶牛瘤胃体外发酵中细菌多样性产生一定影响，其影响随发酵时间和添加剂量而不同。

关键词: 载锌凹凸棒石黏土，16S rDNA，瘤胃细菌多样性，体外，奶牛

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