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ENVIRONMENTAL MICROBIOLOGY

Age-Related Response of Rumen Microbiota to Mineral Salt and Effects of Their Interactions on Enteric Methane Emissions in Cattle

C. Liu[1] · X. H. Li[2,3] · Y. X. Chen[1] · Z. H. Cheng[4] · Q. H. Duan[3] · Q. H. Meng[1] · X. P. Tao[1] · B. Shang[1] · H. M. Dong[1]

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Abstract Mineral salt bricks are often used in cow raising as compensation for mineral losses to improve milk yield, growth, and metabolic activity. Generally, effects of minerals are partially thought to result from improvement of microbial metabolism, but their influence on the rumen microbiota has rarely been documented to date. In this study, we investigated the response of microbiota to mineral salt in heifer and adult cows and evaluated ruminal fermentation and enteric methane emissions of cows fed mineral salts. Twelve lactating Holstein cows and twelve heifers fed a total mixed ration (TMR) diet were randomly allocated into two groups, respectively: a treatment group comprising half of the adults and heifers that were fed mineral salt and a control group containing the other half fed a diet with no mineral salt supplement. Enteric methane emissions were reduced by 9.6% ($P < 0.05$) in adults ingesting a mineral salt diet, while concentrations of ruminal ammonia, butyrate, and propionate were increased to a significant extent ($P < 0.05$). Enteric methane emissions were also reduced in heifers ingesting a mineral salt diet, but not to a significant extent ($P > 0.05$). Moreover, the concentrations of ammonia and volatile fatty acids (VFAs) were not significantly altered in heifers ($P > 0.05$). Based on these results, we performed high-throughput sequencing to explore the bacterial and archaeal communities of the rumen samples. *Succiniclasticum* and *Prevotella*, two propionate-producing bacteria, were predominant in samples of both adults and heifers. At the phylotype level, mineral salt intake led to a significant shift from *Succiniclasticum* to *Prevotella* and *Prevotellaceae* populations in adults. In contrast, reduced abundance of *Succiniclasticum* and *Prevotella* phylotypes was observed, with no marked shift in propionate-producing bacteria in heifers. Methanogenic archaea were not significantly abundant between groups, either in adult cows or heifers. The shift of *Succiniclasticum* to *Prevotella* and *Prevotellaceae* in adults suggests a response of microbiota to mineral salt that contributes to higher propionate production, which competes for hydrogen utilized by methanogens. Our data collectively indicate that a mineral salt diet can alter interactions of bacterial taxa that result in enteric methane reduction, and this effect is also influenced in an age-dependent manner.

Keywords Mineral salt · Rumen microbiota · Enteric methane · VFAs · High-throughput sequencing · Methanogenic archaea

C Liu and XH Li are co-first authors.

Author contributions Conceived and designed the experiments: C Liu. Performed the experiments: C Liu, XH Li, YX Chen, ZH Cheng, QH Duan, QH Meng, XP Tao and B Shang. Analyzed the data: C Liu, XH Li, YX Chen. Contributed reagents/materials/analysis tools: XH Li and HM Dong. Wrote the paper: C Liu and XH Li. All authors agree to be accountable for all aspects of the work.

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✉ H. M. Dong
donghonmin@163.com

[1] Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Zhongguancun Southern St. No. 12, 100081, Haidian District Beijing, China

[2] Agro-environmental Protection Institute, Ministry of Agriculture, Tianjin 300191, China

[3] Rural Energy and Environment Agency, Ministry of Agriculture, Beijing 100125, China

[4] Tianjin Agricultural Environmental Protection Management and Monitoring Station, Tianjin 300061, China

Introduction

Enteric fermentation in ruminants generates methane, regarded as a byproduct of the digestive process, with 2–12% loss of ingested gross energy (GE) [1, 2] and significantly contributes to global warming [3, 4]. Several studies are underway to develop nutritional or additive-inhibiting strategies based on the principle of anti-methanogenesis to reduce methane emission in ruminants, including the use of electron receptors, such as fumarate, nitrates, sulfates, and nitroethane [5, 6]; chemical inhibitors, such as cyclodextrin [7]; ionophores, such as monensin [8]; plant bioactive compounds, such as tannin and saponin [9, 10]; and biotic agents [11, 12]. However, the effects of these additives are inconsistent, with some exerting toxicity in host animals at sufficiently high doses to reduce enteric methane emissions, in addition to concerns about toxic residuals in animal products sold to the public. Consequently, these techniques have yet to achieve widespread applicability. A strategy of nutritional regulation is believed to present an effective solution.

Mineral salt bricks are used to supply necessary mineral nutrients to animals as a feed supplement and generally aid in improving growth, fattening, milk yield, metabolism, and even disease prevention [13–15]. The mineral residual is partially taken up by the systemic circulation, and the remainder is absorbed by microbes, potentially resulting in changes in microbiota in the rumen. Methane is a byproduct released by methanogens during metabolic activity, a process known as methanogenesis [16], which also involves other microbial participants within the network of metabolic substrates [17]. So, it is very interesting to question if these related microbe responds to minerals and what do they have an effect on ruminal fermentation and enteric methane production. In a previous study by our group, ingestion of diets mixed with mineral salt brick powder led to reduced enteric methane emission from lactating cows (unpublished). Accordingly, we speculate that the effects of mineral salt on microbial communities may be related to methane mitigation. However, to our knowledge, no reports to date have focused on the response of the microbial community in the rumen to mineral salt intake, and the shifts in microbial composition, interactions, and abundance require further investigation.

In the present study, we applied high-throughput sequencing to analyze the effects of mineral salt intake on the diversity and composition of bacterial and archaeal communities in the rumen. Age was additionally included as a factor in a simultaneous investigation on heifers and lactating cows. Our main objective was to establish the responses of microbiota to mineral salt, based on key taxa classification and interaction patterns, with a view to explaining the mechanisms underlying the effects of mineral salt on ruminal fermentation and enteric methane mitigation.

Materials and Methods

Experimental Design

Twelve healthy lactating Holstein cows (3 to 4 years old, 682.3 ± 12.7 kg body weight) and twelve heifers (10 months old, 304.9 ± 11.8 kg body weight) were divided into two groups, with those in the treatment group fed a fresh diet containing mineral salts for 1 month. This study was performed in Shandong Province (118° 41′ N, 37° 04′ E) from September 2014 to October 2014, when the ambient temperature ranged from 25 to 30 °C inside the barn. The experimental period continued for 40 days, with the initial 10 days as the adaptive phase to comfort these animals. All the lactating cows chosen for experiment have produced two or three offspring and were 4 or 5 months postpartum. A commercial mineral brick (Tithebarn, Cheshire, UK) was ground into powder to produce mineral salt. The dose of minerals supplied was calculated according to the recommendation of the producer with 2 g minerals contained in 1 kg fresh diet. The mineral salt brick mainly contained Mg (5000 mg/kg), Co (75 mg/kg), Cu (1000 mg/kg), Fe (1500 mg/kg), Mn (500 mg/kg), Se (30 mg/kg), Zn (1000 mg/kg), I (150 mg/kg), and Na (38%). All animals were fed a TMR diet based on corn silage and alfalfa, had free access to food and water, and had no metabolic disorders or antibiotic treatments. During the whole experimental period, we canceled the premix supply, and the requirement for minerals just met with the diet. The ingredients and compositions of the diet are shown in supplementary Table S1.

Feed Sample, Enteric Methane, and Ruminal Fluid Analysis

The feed samples were dried in an oven at 105 °C for 24 h prior to grinding. The drying samples were ground through a 1-mm sieve before further analysis. Dry matter (DM), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), ether extract (EE), gross energy (GE), and total phosphorus (TP) were analyzed following the national standard methods in China. Total nitrogen (TN) and total carbon (TC) contents were analyzed by element analyzer (Elementar, Heraeus, Germany).

Enteric methane emissions were measured using the sulfur hexafluoride tracer technique according to the protocol of Lassey [18]. In the adaptive phase, permeation tubes filled with 1.5 g of sulfur hexafluoride were placed into the rumen for equilibrium. The release rate of each permeation tube was determined by weighing the tubes over time (about 4 mg/day). The sampling apparatus consisted of a U-shaped stainless steel vessel and a filter (50 μm) connected by a capillary tube. The U-shaped canister was fixed around the neck, while the filter was placed near to the cattle's mouth and nostrils. The filter was used to keep the capillary line from plugging. Before use,

the U-shaped vessel was first vacuumed, and the gas sampling time was regulated by the valve and the length of capillary tube. After a 24-h collection, the pressure inside was tested. If normal, pure N_2 (99.9999%) was flushed into the vessel with a final pressure of 0.15 MPa. Thereafter, the gas samples were transferred to gas bags. In this study, eructated gas was collected in five consecutive days. A gas chromatograph fitted with an electron capture detector and flame ionization detector (Shimadzu, Kyoto, Japan) was used for determining sulfur hexafluoride and methane, respectively, according to the method reported by Boadi et al. [19]. The column and injector temperatures were 100 and 200 °C, respectively. Nitrogen was used as the carrier gas with a flow rate of 200 mL/s, and air and hydrogen flow rates set at 50 and 60 mL/s, respectively. The final methane output was calculated with the following equation: $Q = R \times C_1/C_2$, where Q represents the methane emission volume per day, R the sulfur hexafluoride release rate, C_1 the methane concentration in samples, and C_2 the sulfur hexafluoride concentration in samples.

Ruminal fluid was aspirated using a flexible plastic tube and the first 100 mL discarded to avoid contamination of saliva. The remaining fluid was strained through a four-layer cheesecloth and transferred to a sterile centrifuge tube. Following immediate measurement of pH with a portable pH meter (IQ, CO, USA), the fluid was transferred into 2-mL tubes and immediately frozen by dipping of liquid nitrogen. Thereafter, the ruminal fluid samples were stored in −80 °C. Volatile fatty acid (VFA) concentrations were analyzed using a gas chromatograph (GC-14B, Shimadzu, Japan) according to the method of Hoskin et al. [20]. Ammonia-N levels were determined with a colorimetric technique using a spectrophotometer (Hash DR 6000, CO, USA) following the method of Chaney and Marbach [21].

DNA Extraction, MiSeq Sequencing and Bioinformatics Analysis

Total microbial DNA was extracted using a commercially available kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions, and the concentrations detected at A260/A280 nanometer with a spectrophotometer (Bio-Rad, CA, USA). Amplification and sequencing of the hypervariable region of the 16S ribosomal RNA (rRNA) gene was performed using validated, region-specific primers optimized for the Illumina MiSeq platform [22, 23]. For bacterial 16S rRNA gene amplification, the primers used were 338F (5′–ACTCCTACGGGAGGCAGCA–3′) and 806R (5′–GGACTACHVGGGTWTCTAAT–3′). For archaeal 16S rRNA gene amplification, the primers Arch524F (5′–TGYCAGCCGCCGCGGTAA–3′) and Arch958R (5′–YCCGGCGTTGAVTCCAATT–3′) were employed. PCR reactions were performed in a triplicate 20-μL mixture containing 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, USA) according to the manufacturer's instructions, and quantified using QuantiFluor™-ST (Promega, WI, USA). Purified amplicons were pooled and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to standard protocols.

Raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.17) using the following criteria: (i) 250-bp reads were truncated at any site receiving an average quality score of <20 over a 10-bp sliding window, discarding the truncated reads shorter than 50 bp; (ii) exact barcode matching, two-nucleotide mismatches in primer matching, and reads containing ambiguous characters were removed; and (iii) only sequences that showed >10 bp overlaps were assembled according to the overlap sequence. Reads that could not be assembled were discarded. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeric sequences identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed via RDP classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU119) 16S rRNA database using a confidence threshold of 70% [24].

Statistical Analysis

Statistical analyses were performed using analysis of variance (ANOVA) in SPSS 20.0 (IBM Statistics, NY, USA). Mean values were compared using the Bonferroni test for multiple comparisons. Differences between mean values were considered significant at $P < 0.05$. Bioinformatic analyses were performed using the R package. Non-metric multidimensional scaling (NMDS) was conducted to show the separation of samples, and the significance between the groups was tested using a vegan package based on the Bray-Curtis distance matrix. The correlation matrix was performed using the gpairs package, Venn diagram was plotted using the gplot package, and network analysis was performed using the igraph package. Linear discriminant analysis effect size (LEfSe) was performed using the LEfSe tool (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload) [25]. Redundancy analysis (RDA) was conducted with the vegan package to determine the relationships of microbial taxa, sample separation, and ruminal fermentation.

Table 1 The effect of mineral salt on performance, methane emission, and fermentation in adult and heifer

Item	Lactating cow		Heifer	
	CK	Treatment	CK	Treatment
DMI (kg/head/day)	16.43 ± 0.12^{b}	16.49 ± 0.09^{b}	6.16 ± 0.08^{a}	6.14 ± 0.07^{a}
Milk yield (kg/head/day)	23.79 ± 0.63	23.84 ± 0.89	–	–
Initial body weight (kg)	671 ± 35^{b}	696 ± 49^{b}	322 ± 11^{a}	288 ± 21^{a}
Final body weight (kg)	689 ± 16^{b}	695 ± 16^{b}	343 ± 16^{a}	312 ± 15^{a}
CH_4 (g/head/day)	447.3 ± 14.3^{b}	404.4 ± 25.3^{b}	215.9 ± 17.4^{a}	203.2 ± 9.3^{a}
CH_4 (g/kg DMI)	27.2 ± 0.9^{a}	24.5 ± 1.5^{a}	35.1 ± 2.8^{b}	33.1 ± 1.5^{b}
CH_4 (g/kg milk)	18.9 ± 1.0	17.1 ± 1.3	–	–
CH_4/GE (%)	5.0 ± 0.2^{a}	4.5 ± 0.3^{a}	9.1 ± 0.7^{b}	8.6 ± 0.4^{b}
pH	6.5 ± 0.0	6.4 ± 0.0	6.4 ± 0.1	6.3 ± 0.1
Ammonia-N (mmol/dL)	26.32 ± 3.35^{a}	36.57 ± 2.80^{b}	27.47 ± 3.08^{a}	28.63 ± 3.47^{a}
Acetate (mmol/L)	78.71 ± 9.65	85.94 ± 5.83	60.71 ± 9.25	61.83 ± 6.49
Propionate (mmol/L)	19.41 ± 2.62^{a}	28.89 ± 2.96^{b}	15.85 ± 1.75^{a}	17.82 ± 1.75^{a}
Butyrate (mmol/L)	10.44 ± 2.97	15.10 ± 1.40	9.96 ± 1.96	10.38 ± 1.24
A:P ratio	4.11 ± 0.21^{b}	3.07 ± 0.23^{a}	3.83 ± 0.43^{ab}	3.47 ± 0.14^{ab}

Within a row, the means without a common superscript letter differ at $P < 0.05$, and the means without superscript letter indicate no significance, $P > 0.05$.

A:P ratio ratio of acetate to propionate

Accession Number

The sequencing data [BioProject ID: PRJNA348656] were deposited in GenBank database with sample accession numbers from SAMN05913198 to SAMN05913245.

Results

Animal Performance, Enteric Methane Emissions, and Ruminal Fermentation

The effects of mineral salt intake on performance, enteric methane emission, and fermentation are shown in Table 1. We observed no significant differences in DM intake and body weight between the treatment and control groups of heifers ($P > 0.05$) and adults ($P > 0.05$). The body weight gain of heifers was not significantly different between groups ($P > 0.05$).

Emissions of enteric methane (g/day) were reduced for both heifers and adults fed a diet containing mineral salt, although these differences did not appear significant between groups. The differences between the adult groups were moderately significant ($P = 0.10$), compared with those between the heifer groups ($P = 0.61$). Similar trends were observed for calculated methane emission (g/kg DM) and methane energy loss. Based on DM intake, higher methane emission and energy loss were observed in heifers, indicating distinct

Table 2 Comparison of coverage and diversity estimation of the bacterial and archaeal 16S rRNA gene

Phyla	Stage	Group	OTUs	ACE	Chao1	Shannon	Simpson	Coverage
Bacteria	Adult	CK	908^{b}	1067^{b}	1074^{b}	5.17	0.02	0.99
		Treatment	945^{b}	1120^{c}	1123^{c}	5.12	0.02	0.99
	Heifer	CK	789^{ab}	986^{ab}	982^{ab}	4.81	0.03	0.99
		Treatment	774^{a}	901^{a}	905^{a}	4.95	0.02	0.99
Archaea	Adult	CK	71	87^{b}	85^{b}	1.20^{b}	0.43^{a}	1.00
		Treatment	60	65^{ab}	64^{ab}	1.09^{ab}	0.51^{ab}	1.00
	Heifer	CK	48	57^{a}	58^{a}	0.89^{a}	0.57^{ab}	1.00
		Treatment	54	65^{ab}	66^{ab}	0.83^{a}	0.64^{b}	1.00

Within a row, the means without a common superscript letter differ at $P < 0.05$, and the means without superscript letter indicate no significance, $P > 0.05$

Table 3 Average abundance of predominant taxa at the genus level

Phyla	Genera	Adult		Heifer		SE	*P* value
		CK	Treatment	CK	Treatment		
Bacteroidetes	*Prevotella*_1	26.90^{a}	48.14^{b}	23.08^{a}	22.68^{a}	5.80	0.001
	Rikenellaceae_RC9_gut	5.04^{ab}	3.39^{a}	5.70^{ab}	7.72^{b}	1.31	0.05
	*Bacteroidales*_S24-7	5.36^{b}	3.01^{a}	2.76^{a}	3.77^{ab}	1.10	NS
	*Bacteroidales*_BS11_gut	1.63	2.05	1.30	2.64	0.88	NS
	*Bacteroidetes*_VC2.1_Bac22	0.79^{a}	0.52^{a}	1.42^{a}	4.65^{b}	1.53	0.05
	Prevotellaceae_UCG-003	1.15	1.06	1.41	1.19	0.41	NS
	Prevotellaceae_UCG-001	0.99	1.15	1.25	1.15	0.43	NS
	*Prevotella*_7	2.47	0.23	1.66	0.00	1.99	NS
	Prevotellaceae_NK3B31	0.49^{a}	0.39^{a}	0.63^{a}	2.69^{b}	1.00	NS
	*Bacteroidetes*_unclassified	0.18	0.35	0.32	1.07	0.52	NS
Firmicutes	*Succiniclasticum*	12.61^{b}	2.62^{a}	14.04^{b}	3.30^{a}	2.72	0.001
	Ruminococcaceae_NK4A214	5.19	4.51	4.41	5.91	0.92	NS
	Christensenellaceae_R-7	2.37^{a}	2.45^{a}	4.67^{b}	6.84^{b}	1.36	0.01
	*Ruminococcus*_2	3.22	4.22	1.63	3.49	1.47	NS
	Ruminococcaceae_UCG-014	2.14	3.03	3.09	1.56	1.39	NS
	Saccharofermentans	1.48	0.92	1.33	1.49	0.45	NS
	*Butyrivibrio*_2	1.29^{b}	0.45^{a}	1.60^{b}	1.38^{b}	0.43	NS
	Lachnospiraceae_NK3A20	1.17^{ab}	0.74^{a}	0.98^{ab}	1.80^{b}	0.43	NS
	*Eubacterium*_coprostanoligenes	0.83^{a}	0.55^{a}	1.07^{ab}	1.85^{b}	0.47	NS
	Lachnospiraceae_AC2044	0.78	0.31	2.69	0.30	1.33	NS
	Lachnospiraceae_XPB1014	0.59^{a}	0.31^{a}	0.99^{a}	2.06^{b}	0.40	0.002
	Lachnospiraceae_unclassified	0.53^{ab}	0.34^{a}	0.92^{b}	1.16^{bc}	0.27	0.05
	Roseburia	1.17	0.37	0.48	0.89	0.53	NS
	Lachnospiraceae_ND3007	0.07^{a}	0.19^{a}	0.48^{a}	1.21^{b}	0.26	0.001
Proteobacteria	*Desulfovibrio*	0.66	0.59	0.66	0.48	0.18	NS
Spirochaetes	*Treponema*_2	1.12	1.54	1.54	1.47	0.52	NS
Tenericutes	*Mollicutes*_RF9	1.41	1.18	1.47	1.32	0.43	NS
Euryarchaeota	*Methanobrevibacter*	90.63	93.10	93.28	95.32	3.86	NS
	Thermoplasmatales	1.23	1.92	1.22	1.38	0.72	NS
Unclassified	Archaea_unclassified	6.71	3.48	4.30	2.31	3.57	NS

Within a row, the means without a common superscript letter differ at $P < 0.05$, and the means without superscript letter indicate no significance, $P > 0.05$

NS non-significant between groups (i.e., $P > 0.05$)

metabolic efficiency of the diet due to immature rumen development in younger animals.

Investigation of fermentative parameters showed that ammonia-N and VFA concentrations in adult cows were increased to a higher extent than those in the heifer group after mineral salt intake, in particular, a marked increase in ammonia-N and propionate levels ($P < 0.05$). Although the concentrations of acetate and butyrate were also increased, the differences in these parameters between groups were not significant ($P > 0.05$). The acetate to propionate (A:P) ratio in adults fed a mineral salt diet was markedly decreased ($P < 0.05$). Compared with adults, heifers displayed relatively

constant ammonia-N and VFA concentrations after mineral salt intake. The pH was not obviously influenced by mineral salt in both the heifer and adult groups.

Sequencing Depth and Estimation of Alpha Diversity

We investigated both bacterial and archaeal communities using the high-throughput Illumina sequencing technique. Valid reads contained more than 18,000 sequences for each sample. For bacteria, a total of 779,669 high-quality reads remained, with an average of 32,486 reads per sample, ranging from 18,000 to 36,000. These sequences were assigned to 1704 OTUs based on 97% similarity. For archaea, 707,021 high-quality reads remained, with an average of 29,459 reads per sample, ranging from 20,000 to 37,400. These sequences were assigned to 381 OTUs based on 97% similarity. To eliminate uneven distribution of sequencing data, we adopted random subsampling for downstream diversity analysis. The subsampling threshold was set by the lowest number of sequences obtained in the samples, with cutoff values of 20,254 and 18,084 for bacteria and archaea, respectively.

Subsampling sequences yielded sufficient resolution of bacterial and archaeal communities, as evident from rarefaction curve analysis (Fig. S1). Bacterial and archaeal community diversities were measured using both the Shannon-Wiener and Simpson indices. Bacterial and archaeal indices were not significantly different for both heifers and adults fed a mineral salt diet, compared to their counterparts ($P > 0.05$). The OTU numbers and estimated ACE and Chao1 values indicated an inverse trend for heifers and adults fed a mineral salt diet. Specifically, these values were increased for bacteria but decreased for archaea in adults, and the opposite trend was observed in heifers (Table 2).

Microbiota Distribution and Core OTUs Influenced by Mineral Salt

Overall, 25 phyla of bacterial taxa were detected in the samples, among which *Bacteroidetes* and *Firmicutes* were the most dominant, accounting for 92% of the total reads (Fig. S2a). *Proteobacteria*, *Tenericutes*, and *Spirochaetae* constituted 5% of the total reads (each showing 1.5–2% abundance). *Prevotella*_1, *Succiniclasticum*, Rikenellaceae_RC9_gut, and Christensenellaceae_R-7 were more predominantly detected between groups using ANOVA (Table 3; Fig. S2b), which listed the genus taxa accounting for at least 85% abundance in each sample. For archaea, only three phyla were detected, with Euryarchaeota and an unclassified group accounting for more than 98% abundance (Fig. S2c), but no significant differences observed between the groups at the genus level (Table 3). *Methanobrevibacter* contributed to about 93% total abundance of archaea (Fig. S2d).

Non-metric multidimensional scaling analysis was applied to determine the effects of bacterial and archaeal compositions on sample separation, based on the Bray-Curtis similarity metric with 97% sequence identity at the phylotype level (Fig. 1). The ordination plot showed a significant effect of mineral salt on divergence of bacterial composition in samples from adult ($P < 0.05$) group rather than from heifer ($P > 0.05$) group using analysis of similarities (ANOISM). In terms of archaeal composition, samples were not separated according to their own groups ($P > 0.05$), indicating no effects of mineral salt on archaeal composition and shift. The effects of mineral salt on sample separation revealed key roles of bacteria in reducing enteric methane from adult fed a mineral salt diet, suggestive of differential bacterial activities or interactions related to methane mitigation depending on age.

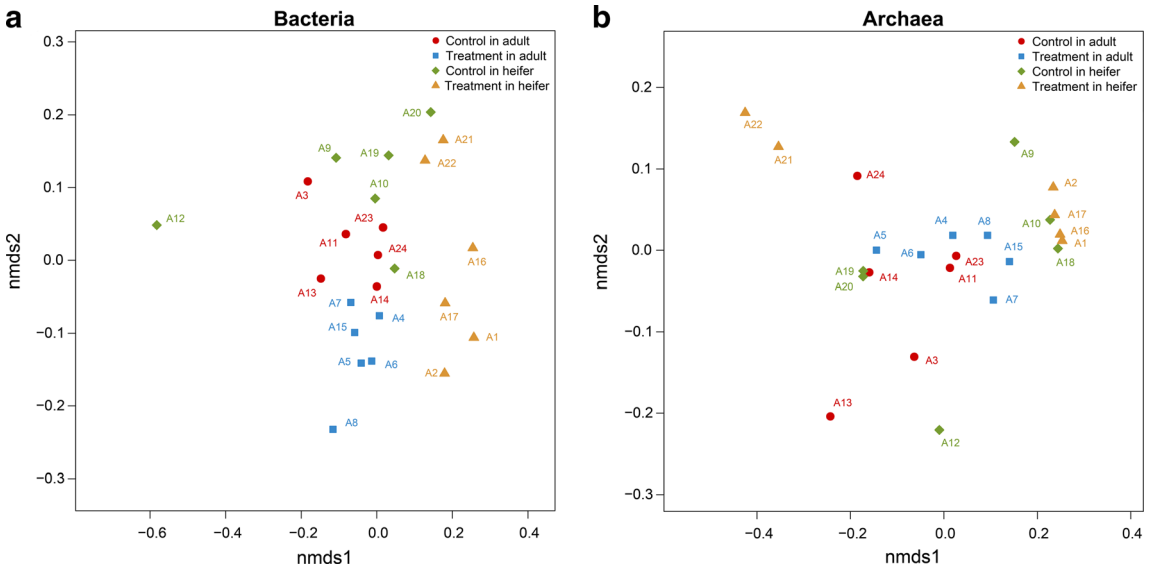

Fig. 1 Ordination plot of the rumen samples. Non-metric multidimensional scaling (NMDS) depicting spatial separation represented by bacterial and archaeal communities. The samples represented by bacteria and archaea are illustrated in **a** and **b**

To identify specific OTUs that were differentially distributed in hosts subjected to different dietary regimes, we used LEfSe, a robust tool that distinguishes not only statistical significance but also biological relevance. Differential OTUs with abundance >0.1% were selected for further analysis. For adults, a total of 30 OTUs were significantly different between the two groups, with 18 being more abundant in the treatment group and 13 in the control group. Two predominant phylotypes of *Succiniclasticum* (OTU449 and OTU1336) were significantly decreased, while numerous phylotypes of *Prevotella* (OTU57, 68, 155, 335, 512, 809, 839, 950, 997, 1045, 1386, and 1612) and *Prevotellaceae* (OTU88 and 307) were increased in the treatment group (Fig. 2a, c). For heifers, 12 OTUs were differentially represented between the two groups to a significant extent, with eight being more abundant in the treatment group and four in the control group. The predominant phylotypes of *Succiniclasticum* (OTU449) and *Prevotella* (OTU1365) were decreased in the treatment group, and other phylotypes occurred in relatively low abundance of <0.5% (Fig. 2b, d). Specific archaea were not differentially distributed among hosts under different dietary regimes, indicating relatively stable composition regardless of mineral salt intake (Fig. S3). We also carried out the correlation analysis of differential abundances for *Methanobrevibacter* and bacteria (e.g., *Succiniclasticum* and *Prevotella*). For adult, *Methanobrevibacter* abundance was weakly correlated with other bacterial phylotypes ($P > 0.05$) and most of *Succiniclasticum* and *Prevotella* phylotypes were significantly negatively correlated in abundance ($P < 0.05$) (Fig. 3a). For heifer, the similar weak correlations of *Methanobrevibacter* were also observed with other bacterial phylotypes, but *Succiniclasticum* was not significantly correlated with other

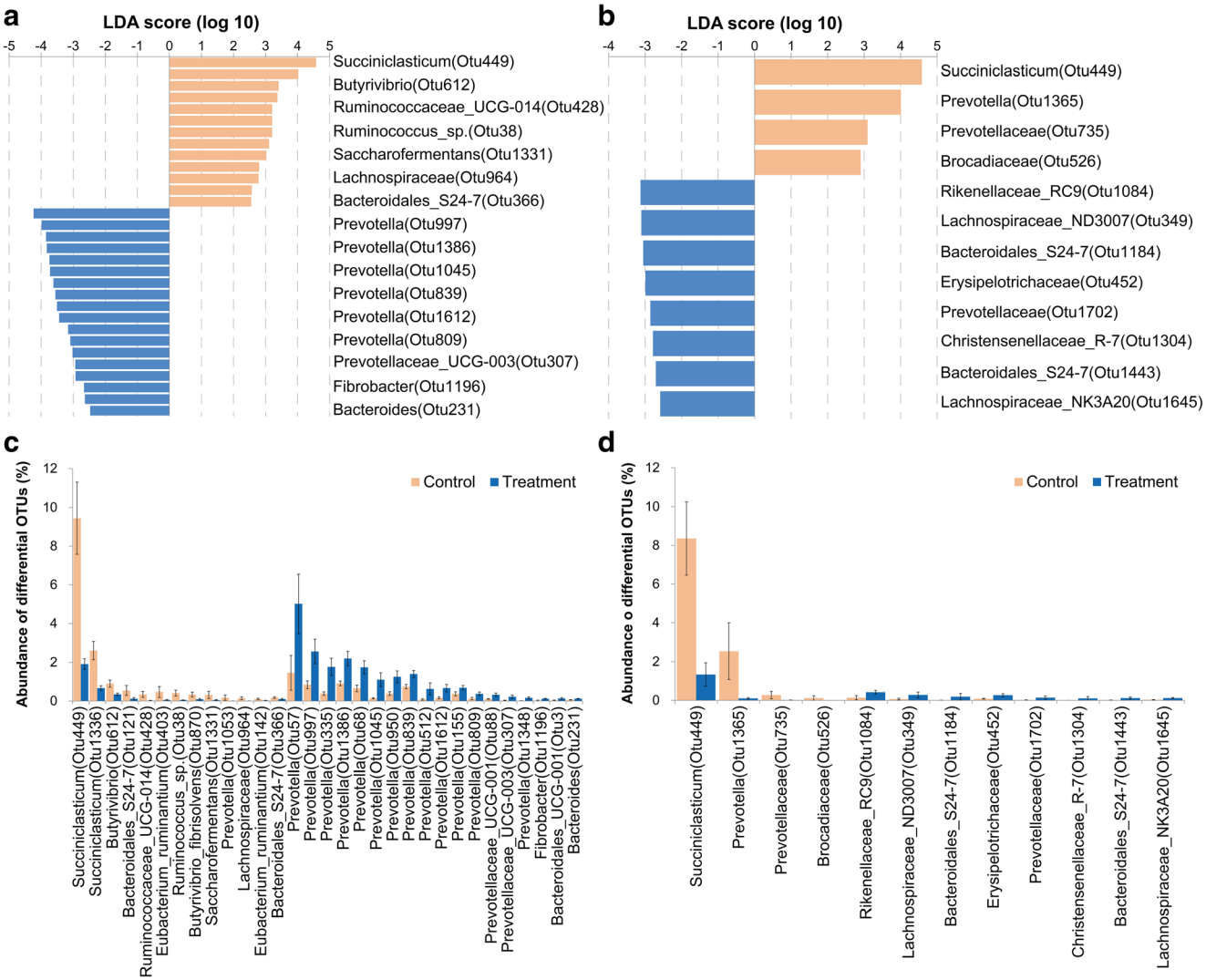

Fig. 2 Differential bacterial OTUs identified by LEfSe between the treatment and control groups. Histogram shows OTUs that are more differential and abundant in the treatment (*blue*) or control (*orange*) group for adult (**a**, **c**) and heifer (**b**, **d**)

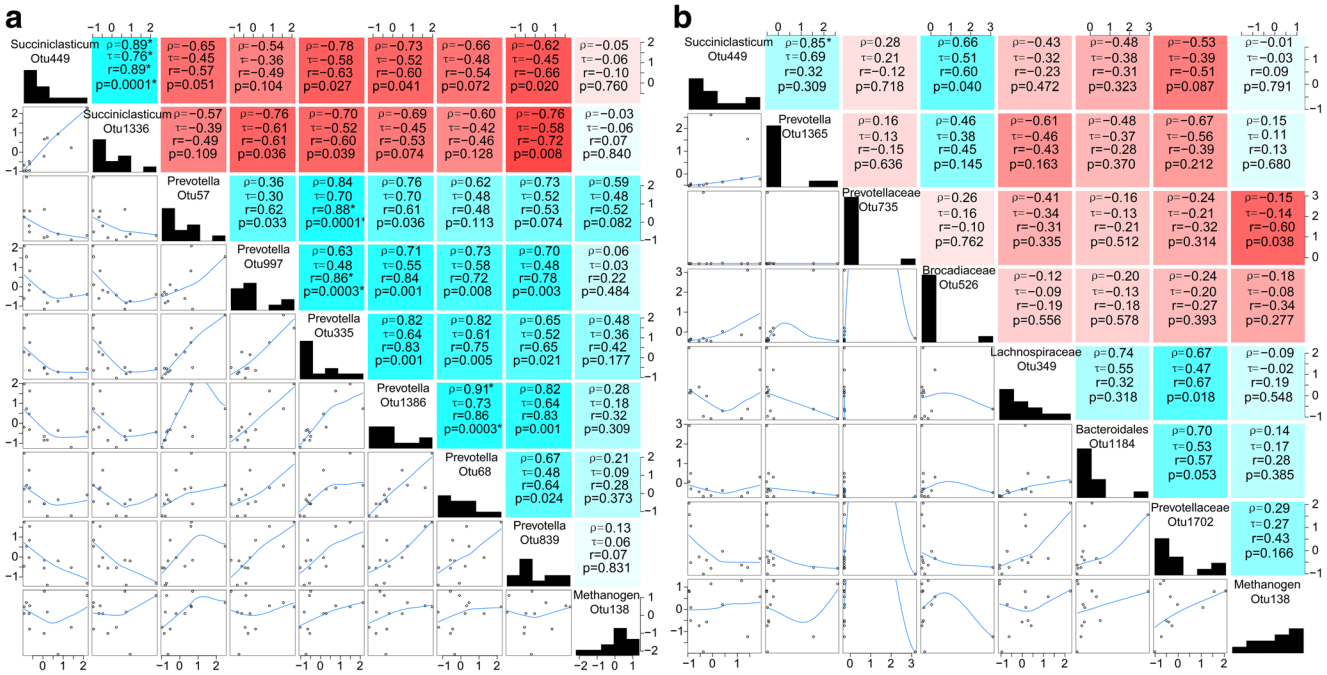

Fig. 3 The correlations of differential bacterial abundance with *Methanobrevibacter* abundance in adult **(a)** and heifer **(b)**

bacterial phylotypes in abundance ($P > 0.05$) (Fig. 3b). Furthermore, the bi-plot of redundancy analysis revealed a positive relationship between propionate production and *Prevotella*_1 abundance, which was dominant in the adult treatment group. *Succiniclasticum* and Succinivibrionaceae_unclassified showed a negative relationship with propionate production and were dominant only in samples of heifer groups (Fig. 4). Our results further confirmed the differential response to mineral salt in adults or heifers and the inhibitory effects of propionate-producing bacteria on methanogenesis.

Network Analysis of Microbiota

For both adult and heifer, intake of mineral salts induced effects on the rumen microbiota and altered patterns of microbial interactions, as revealed by network analysis. *Succiniclasticum* phylotypes (OTU449 and 1336) were key hubs shared by adults and heifers of the control groups. However, different patterns were observed in response to mineral salts, with a specific increase in abundance of other *Prevotella* phylotypes in adults, analogous to LEfSe data, that outcompeted *Succiniclasticum* (OTU449 and 1336) (Fig. 5a, b). In heifers ingesting mineral salts, network analysis disclosed no compensatory effect of *Prevotella*, but some dominant *Bacteroidetes* and unclassified *Bacteroidales* were increased (Fig. 5c, d; Table 3). A further search of unclassified *Bacteroidales* against nucleotide databases using BLASTN revealed ~85% sequence identity with *Barnesiella* involved in succinate production.

Discussion

Ruminants instinctively consume mineral salts in nature. Mineral salts are generally added to the diet as a supplementary nutrient or supplied in the form of bricks for consumption to improve animal performance [26, 27]. Despite their extensive use, the effects of mineral salts on the rumen fermentation and microbiota have not been thoroughly investigated. However, a previous study by our group revealed that mineral salt supplements are effective in methane reduction, suggesting that the microbial activity response to mineral salt is associated with methane production (unpublished).

In the present study, adult cows and heifers showed differential responses to mineral salt intake, mainly in terms of methane emission and fermentation. Adults fed a mineral salt diet displayed reduced methane emission and increased VFA and ammonia concentrations in the rumen while heifers did not show obvious changes. The rumen bacteria integrate ammonia-N into amino acids used for microbial protein synthesis, and their digestion supplies amino acids to the host animals [28]. The increased concentrations of ammonia-N indicate that mineral salts enhance the deamination activity of the rumen microbes in the degradation of food protein in adults. VFAs are extremely important for ruminants, contributing more than 70% of the energy supply. The rumen microbial fermentation produces propionate, butyrate, and acetate as the main VFAs, which are utilized for milk and meat production and supply a major proportion of the daily energy requirement of ruminants [29]. Reduced A:P ratios in the rumen are associated with increased feed efficiency and reduced methane production [30–32]. Therefore, increased propionate

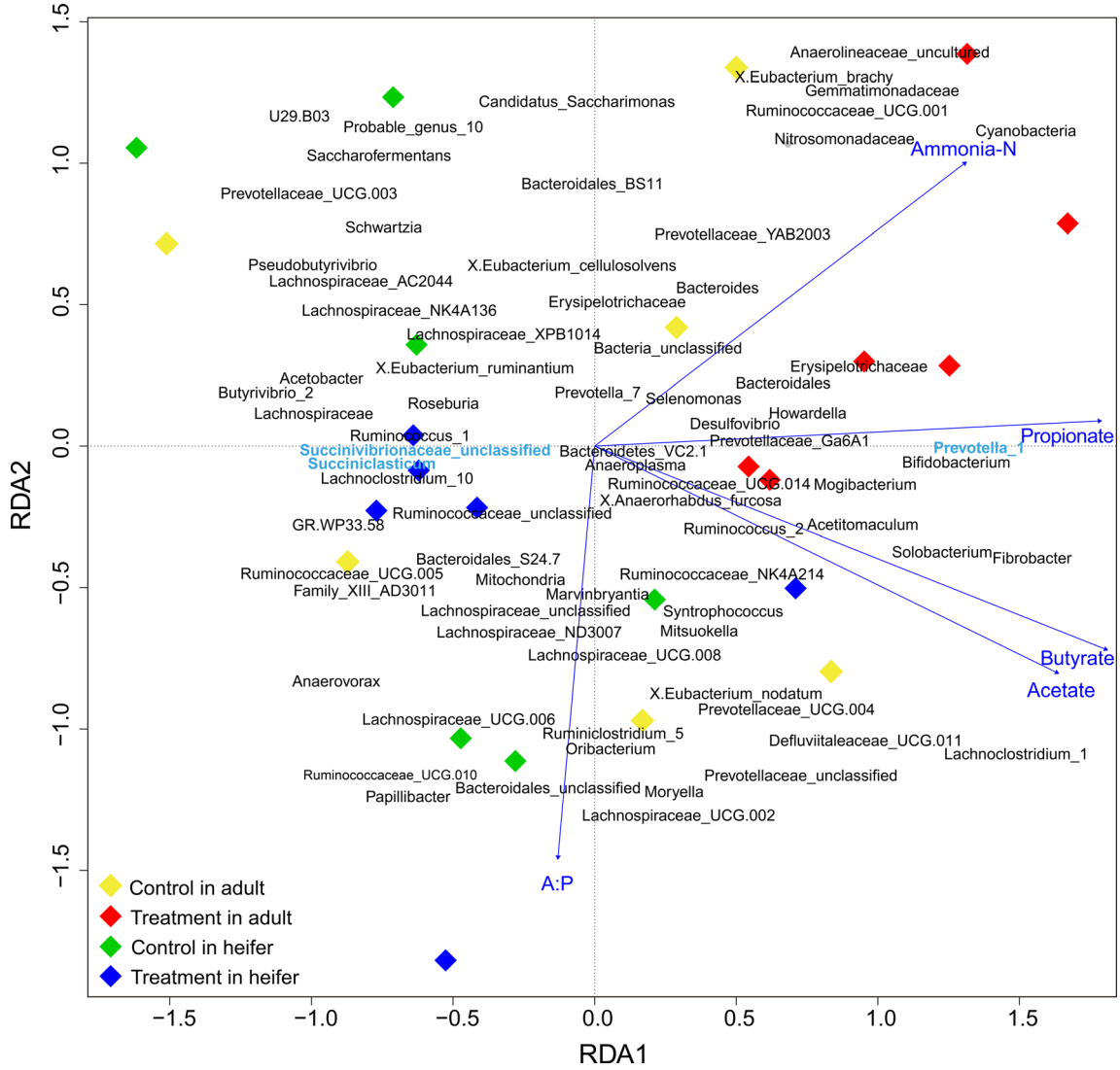

Fig. 4 Effects of mineral salts on ruminal parameters, sample separation, and bacterial taxa using redundancy analysis (RDA). *Dots* are colored *yellow* for adult and *green* for heifer as control; *red* for adult and *blue* for heifer as treatment

concentration and reduced A:P ratio suggest a shift of the metabolic pathways of some microbes related to carbohydrate degradation in adults. A previous report on bovine rumen showed that greater propionate production is accompanied by increased abundance of *Prevotella* sp., at the expense of simultaneous acetate and butyrate production [33]. Similar results were obtained in our study, whereby predominant *Prevotella* exceeded 48% of the total bacterial content in the rumen samples of adults fed a mineral salt diet (Table 3). The above findings suggest that mineral salt reduces enteric methane emissions in adults by facilitating propionate production in the rumen, which could compete with methanogens for hydrogen and contribute to the attenuation of methanogenesis [34].

16S rRNA gene sequencing of archaea revealed *Methanobrevibacter* as the most abundant OTU in the rumen in the present study, consistent with previous reports [35–39], but its abundance was not significantly influenced by mineral salt in either adults or heifers. This finding was not consistent with reduced enteric methane emission in adults, indicating that bacterial communities other than *Methanobrevibacter* are affected by mineral salt to influence methane production. The importance of bacterial community in enteric methane production was reported that different ruminotypes are linked with the low-methane emission trait in sheep [40]. Intergroup comparisons disclosed that differential OTUs are influenced by mineral salt, including phylotypes affiliated with *Prevotella*, *Succiniclasticum*, Ruminococcaceae, *Butyrivibrio*, *Eubacterium*, *Saccharofermentans*, *Lachnospiraceae*, *Prevotellaceae*, *Fibrobacter*, *Bacteroidetes*, unclassified *Bacteroidales*, *Brocadiaceae*, *Rikenellaceae*, and Erysipelotrichaceae (Fig. 2). Among the

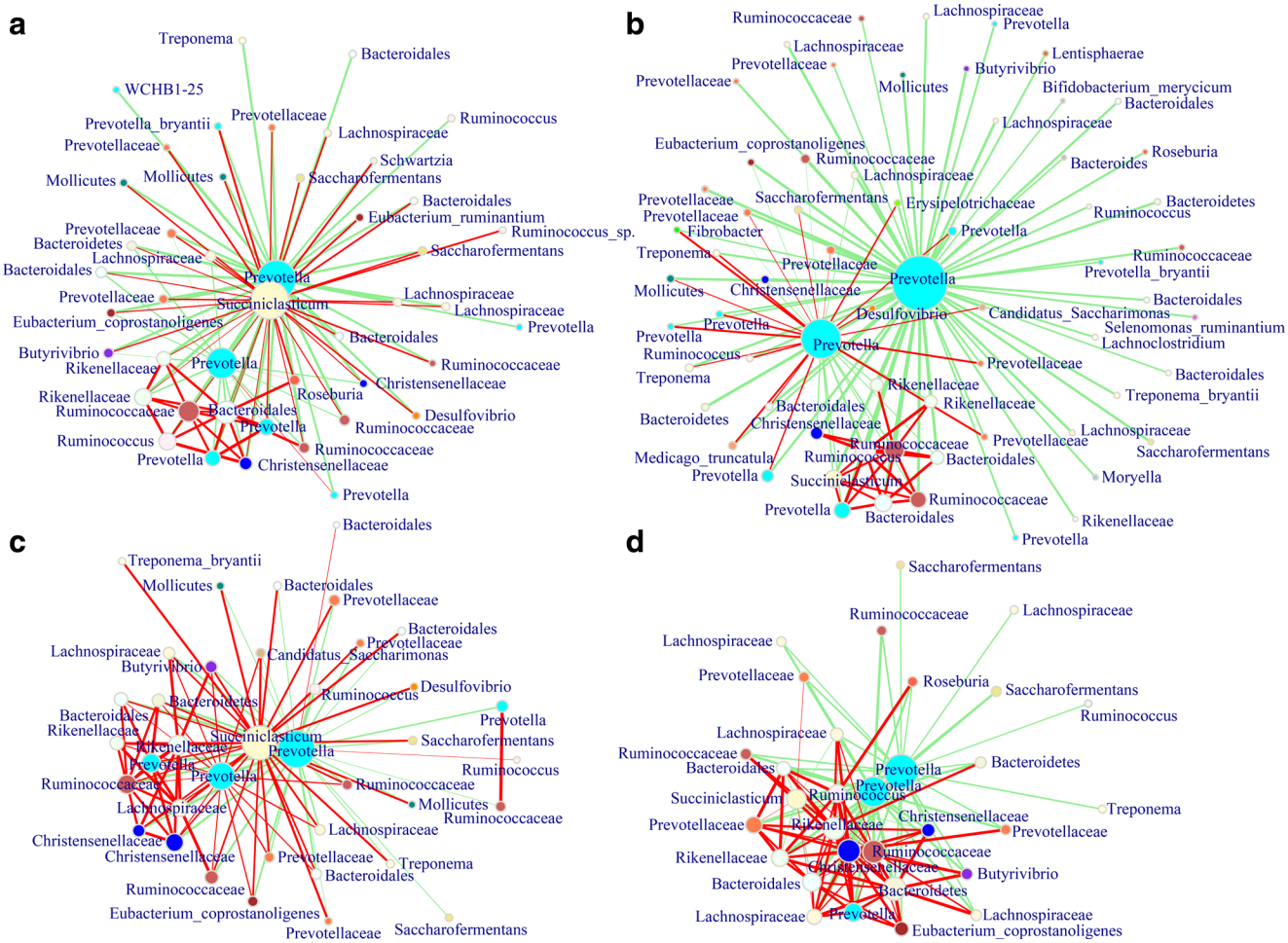

Fig. 5 Relationships of bacterial communities. The networks are based on the adjacency matrix of microbial abundance and correlation analysis at the phylotype level for adult control **(a)**, adult treatment **(b)**, heifer control **(c)**, and heifer treatment **(d)**. The *nodes* represent the bacterial taxa dominant in the samples, with the type and average abundance grouped by color and size. The *edges* represent the correlation between bacterial taxa, with *red* as positive and *green* as negative direction, respectively. The *width of lines* represents the strength of correlation

bacterial taxa, *Prevotella* and *Succiniclasticum* were the most abundant genera, belonging to the two dominant phyla of *Bacteroidetes* and *Firmicutes*, respectively. After an adaptive period to a dietary regime supplemented with mineral salt, the *Succiniclasticum* content was reduced significantly in both adult and heifer cows and succeeded by *Prevotella* in adults. *Prevotella* is involved in propionate production, which is further used for energy by the host [41, 42]. Therefore, the increased abundance of *Prevotella* phylotypes may account for the enhanced propionate concentration in the rumen of adults fed a mineral salt diet, and potentially underlie inhibition of methanogenesis, as reported previously [43, 44]. Network analysis further supported the importance of *Prevotella* in animals fed a mineral salt diet, showing increased abundance of *Prevotella* outcompeting other genera, as reflected by the negative correlations and independent occurrence (Fig. 5). Recently, Denman et al. [45] reported an enteric methane-inhibiting mechanism of bromochloromethane and confirmed its ability to shift rumen fermentation to propionate production, mediated by an increase in the populations of *Prevotella* and *Selenomonas*. It was also reported that the rumen OTUs assigned to *Prevotella* were promoted under chloroform treatment, which reduced enteric methane production [46].

Succiniclasticum is affiliated to the order *Selenomonadales* and specializes in fermenting succinate and converting it to propionate [47]. In the present study, *Succiniclasticum* abundance was decreased following mineral salt intake, and a simultaneous increase in *Prevotella* compensated for the loss of *Succiniclasticum* and propionate in adult cows. However, this compensatory effect was not observed in heifers. LEfSe analysis did not detect a similar boost in *Prevotella* phylotypes or other propionate-producing bacteria, making it difficult to be explained at the phylotype level. Some *Bacteroidetes* and unclassified genera of *Bacteroidales* were increased in heifers fed a mineral salt diet (Table 3). These taxa were related to *Barnesiella*, with 85% sequence similarity, based on a

BLASTN search of nucleotide databases. *Barnesiella* produces succinate and acetate as end products of glucose metabolism [48]. The cumulative abundance of these *Barnesiella*-like taxa was found to be associated with the loss of *Succiniclasticum* abundance. Accordingly, we propose that these potential propionate-producing bacteria compensate for propionate production more or less.

Additionally, based on the same dietary regime, comparison between adults and heifers revealed differential microbial patterns in response to mineral salt, especially for phylotypes of *Succiniclasticum* and *Prevotella*. This phenomenon led to the speculation that bacterial distribution patterns and resource competition among organisms are regulated by age-dependent effects coupled with mineral-salt-induced pathways. As for the reason why high-abundant *Succiniclasticum* is coupled with more methane production in adult rather than in heifer, necessary evidence on metagenomics and metabonomics are still needed.

Conclusion

In summary, the effects of a mineral salt diet on the rumen microbiome were investigated using high-throughput sequencing to explore the mechanisms underlying enteric methane reduction. The response of microbiota in the rumen of adult or heifer cows to mineral salt differed mainly in terms of predominant propionate-producing bacteria. Mineral salt intake triggered a shift from predominant *Succiniclasticum* to *Prevotella* and *Prevotellaceae* in adults, but a similar effect was not observed in heifers, which simply maintained propionate homeostasis. Our data suggest that a mineral-salt-rich diet reduces enteric emissions mainly through enhancing the propionate-producing pathway. Moreover, this inhibitory effect is dependent on animal age. Future study will include metagenomics and metabonomics to relate biological function to the specific taxa found here, providing further insight to the response of key microbes to mineral salts.

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Compliance with Ethical Standards This study was performed on an experimental farm and granted permission by farm administrators. The experimental protocol was approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences, and care of experimental animals was provided in accordance with Chinese standards.

Conflict of Interest The authors declare that they have no competing interests.

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