16S amplicon sequencing and untargeted metabolomics reveal changes in rumen microorganisms and metabolic pathways involved in the reduction of methane by cordycepin[1]

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Abstract

As a major contributor to methane production in agriculture, there is a need for a suitable methane inhibitor to reduce ruminant methane emissions and minimize the impact on the climate. This work aimed to explore the influence of cordycepin on rumen fermentation, gas production, microbiome and their metabolites. A total of 0.00, 0.08, 0.16, 0.32, and 0.64 g L^{-1} cordycepin were added into fermentation bottles containing 2g total mixed ration for *in vitro* ruminal fermentation, and then the gas produced and fermentation parameters were measured for each bottle. Samples from the 0.00 and 0.64 g L^{-1} cordycepin addition were selected for 16S rRNA gene sequencing and metabolome analysis. The result of this experiment indicated that the addition of cordycepin could linearly increase the concentration of total volatile fatty acid, ammonia nitrogen, the proportion of propionate, valerate, and isovalerate, and linearly reduce ruminal pH and methane, carbon dioxide,

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hydrogen and total gas production, as well as the methane proportion, carbon dioxide proportion and proportion of butyrate. In addition, there was a quadratic relationship between hydrogen and cordycepin addition. At the same time, the relative abundance of *Succiniclasticum*, *Prevotella*, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Christensenellaceae_R_7_group*, *unclassified_F082*, *Veillonellaceae_UCG_001*, *Dasytricha*, *Ophryoscolex*, *Isotricha*, *unclassified_Eukaryota*, *Methanobrevibacter,* and *Piromyces* decreased significantly after adding the maximum dose of cordycepin. In contrast, the relative abundance of *Succinivibrio*, *unclassified_Succinivibrionaceae*, *Prevotellaceae_UCG_001*, *unclassified_Lachnospiraceae*, *Lachnospira*, *Succinivibrionaceae_UCG_002*, *Pseudobutyrivibrio*, *Entodinium*, *Polyplastron*, *unclassified_Methanomethylophilaceae*, *Methanosphaera,* and *Candidatus_Methanomethylophilus* increased significantly. Metabolic pathways such as biosynthesis of unsaturated fatty acids and purine metabolism and metabolites such as arachidonic acid, adenine, and 2′-deoxyguanosine were also affected by the addition of cordycepin. Based on this, we conclude that cordycepin is an effective methane emission inhibitor that can change the rumen metabolites and fermentation parameters by influencing the rumen microbiome, thus regulating rumen methane production. This experiment may provide a potential theoretical reference for developing Cordyceps byproduct or additives containing cordycepin as methane inhibitors.

Keywords： cordycepin, *in vitro* rumen fermentation, rumen microbiome, metabolome, methane production

1. Introduction

Methane is a major greenhouse gas. Its greenhouse effect is 25 times greater than that of carbon dioxide (Li *et al.* 2021). It has been established that global warming caused by the increasing greenhouse effect may lead to environmental effects such as rising sea levels and frequent extreme weather, affecting human daily life (Mikhaylov *et al.* 2020). Greenhouse gases from ruminants, deforestation, and fertilization account for a quarter of all emissions from human sources (IPCC 2019). The greenhouse gas emissions caused by ruminants are divided into direct and indirect emissions. Indirect emissions originate from external activities such as deforestation resulting from feed production and forage growth, while direct emissions refer to the enteric fermentation of ruminants (Cottle *et al.* 2011). Hence, lowering methane emissions from ruminants can be achieved through improved management techniques but, more importantly, mitigating

methane produced from enteric fermentation (Cottle *et al*. 2011).

The feed digestion process in ruminants depends on the microorganisms inhabiting the rumen. Rumen microorganisms degrade the feed to produce ammonia-N and volatile fatty acids, which are then converted into protein and energy required by ruminants (Santra *et al.* 2003). However, methane production consumes the carbon source in feed necessary to support milk production and meet ruminants' nutritional needs, consequently reducing the feed conversion rate. Therefore, inhibiting rumen methane production in ruminants can help improve meat and milk production (Mustapha *et al.* 2017). Ruminants produce methane via the interaction of the soluble hydrogen (from volatile fatty acid (VFA)) in rumen fluid and carbon dioxide through the rumen's methanogens (Wang *et al.* 2022). As a result, inhibiting the activity of methanogens or reducing their reaction substrates directly or indirectly is the key to reduce methane emissions from ruminants (Baker 1999).

Research of reducing methane emissions from ruminants is progressing rapidly, and several strategies have been and are currently being explored. It has been reported that vaccines have been developed to stimulate the immune system of ruminants to produce antibodies against methanogens and to reduce methane production (Hook *et al.* 2010). Still, in different studies, the vaccine's effectiveness in reducing methane emissions varied (Hook *et al*. 2010). Screening breeding populations with low methane emission genetic traits has also been used to control the methane emission of ruminants (Ghavi Hossein-Zadeh 2023). Feed additives or dietary strategies were also be used to decrease methane (Hristov *et al.* 2013). Previous studies have shown that reducing the forage-to-concentrate ratio can reduce methane emissions; however, concentrates are expensive and, particularly in high levels, often lead to rumen acidosis in cattle (Nampoothiri *et al.* 2020; Thakur *et al.* 2021; Barbosa *et al.* 2018). Therefore, there remains a need to find a forage-to-concentrate ratio that maintains production quantity and quality, animal health and economic costs while reducing methane emissions. Methane emissions are also influenced by the quality and maturity of pastures (Thompson *et al.* 2020; Dini *et al.* 2018). In the reviews from Palangi *et al.* (2022) and Hook *et al*. (2010), the authors suggest that methane production in ruminants can be effectively inhibited by dietary oils or lipids, tannin-rich diets, microalgae, macroalgae, probiotics and monensin. Previous studies have found that adding monensin to feed can increase the daily weight gain of dairy cows, improve dry matter digestibility (Goodrich *et al.*

1984), and reduce methane output (Odongo *et al.* 2007). However, as monensin is an antibiotic, its use in livestock has been banned in many countries. Hence, it is necessary to develop an economical and harmless feed additive to control methane emissions from ruminants. *Cordyceps militaris* has achieved decent results in inhibiting methane emission *in vitro* as an additive, which contains a variety of active substances, such as cordycepin, cordyceps polysaccharide, and cordyceps superoxide dismutase, and so on (Kim *et al.* 2014). However, it is still unclear which specific substance in *Cordyceps militaris* inhibit methane production. It has been reported that adding Chinese herbal extracts to the feed of dairy cows can reduce the output of methane in the rumen (Vargas *et al.* 2020; Lee *et al.* 2020; Wang *et al.* 2012). Therefore, the aim of this study is to investigate further the effect of cordycepin, the active component in *Cordyceps militaris*, on methane emissions from the rumen.

Cordycepin, known as 3′-deoxyadenosine, was initially separated from *Cordyceps militaris* (Cunningham *et al.* 1950). With the development of modern medicine, cordycepin has been proven to enhance immunity (Tuli *et al.* 2013; Nakamura *et al.* 2015; Shin *et al.* 2009) and regulate microbiota. Jiang *et al.* (2019) has proven that cordycepin can effectively inhibit the growth of seven bacteria types, including both gram-positive and gram-negative strains, by destroying bacterial cell membranes and then binding to bacterial genomic deoxyribonucleic acid (DNA) to disrupt bacterial functions. The experiment of Ahn *et al.* (2000) confirms that the activities of *Clostridium paratrificum* and *Clostridium perfringens* (gram-positive) could be inhibited by cordycepin. Huang *et al.* (2019) also found that cordycepin inhibited *Mycobacterium bovis* and *Mycobacterium tuberculosis* (gram-positive) by hijacking bacterial adenosine kinase. It can be seen that cordycepin has an effective regulatory effect on the activity of gram-positive bacteria. The rumen's main methanogen is *Methanobrevibacter*, a gram-positive bacterium (Miller 2015; Newbold *et al.* 2020). Therefore, it is also worth investigating whether cordycepin specifically inhibits the activity and amounts of methanogens.

At the same time, we also understand that cordycepin can affect the metabolic pathways of cells. For example, because of the similarity in its structure to adenosine, cordycepin can participate in the biosynthesis of DNA and ribonucleic acid (RNA) after entering cells. During transcription, many enzymes are unable to differentiate adenosine from cordycepin, resulting in the substitution of cordycepin for normal nucleosides, thereby preventing the further incorporation of

nitrogenous bases (guanosine, adenosine, cytidine, and thymosine), resulting in early transcription termination (Tuli *et al*. 2013). Based on the above, we hypothesize that cordycepin may affect the expression of metabolic products by inhibiting *de novo* biosynthesis of purines in the rumen and affecting the activity and quantity of rumen methanogens by early termination of their transcription, ultimately reducing methane production. Therefore, in this experiment, 16S amplicon sequencing and untargeted metabolomics were adopted to explore the impact of cordycepin on gas production, fermentation indexes, the relative abundance of rumen microorganisms, metabolic pathways, and differential metabolites *in vitro*. This experiment provides references for developing a series of Cordyceps byproducts or additives containing cordycepin to play its role in reducing methane emissions in ruminants.

2. Materials and methods

2.2. Animals, diet, and experimental design

The animal use protocol was approved following the Animal Care and Use Committee of Northeast Agricultural University (protocol number: NEAUEC20230267). Three lactating Chinese Holstein cows (days in milk = 120±11 d, milk yield = 28.9±2.3 kg d^{-1}, parity = 2) were housed individually in a tethered barn stall, and free access to water. All cows were fed the same amount of TMR and milked twice daily at 0630 and 1830. Table 1 details the ingredients and chemical composition of the experimental diets which meets NRC nutrient requirements of lactating cows (NRC 2001).

The techniques for anaerobic culture and experimental procedures were similar to that described in Xin *et al.* (2021). Rumen fluid, sampled from the three fistulated cows before morning feeding, was flushed with oxygen-free carbon dioxide (CO_2) in a vacuum flask, sieved through a four-layered cheesecloth before mixing with pre-warmed (39°C) buffer, and prepared according to Soliva *et al.* (2007), at a proportion of 2:1 (buffer: ruminal fluid, v:v) under a continual CO_2 flow to maintain an anaerobic environment. After mixing, 150 ml was transferred to a 200 ml glass bottle containing 2 g total mixed ration (TMR) substrates. Cordycepin with 98% purity (CAS code: 73-03-0) from Shanghai Macklin Biochemical Co., Ltd. was purchased. The cordycepin were added to the rumen fluid to make a final concentration of 0.00, 0.08, 0.16, 0.32 and 0.64 g L^{-1}. Silicone stoppers with gas-tight collection bags were used to seal the sample bottles (six replicates per treatment) before incubating them in water bath with shaker at 39°C and 40–50 rpm for 24 h. The incubations

were conducted in triplicate.

2.3. Total Gas, Hydrogen, Carbon Dioxide, Methane Production, and Rumen Fermentation Analysis

After inoculation, the gas was collected into a bag, and the gas volume was measured by using a syringe. A total of 0.5-mL subsample of gas was analyzed for hydrogen, carbon dioxide, and methane content following the procedure of Kim *et al*. (2014) with a Gas chromatograph (GC-8A; Shimadzu Co. Ltd., Tokyo, Japan). The proportion of methane, carbon dioxide and hydrogen is obtained by dividing the methane, carbon dioxide and hydrogen production by the total gas production. After gas production determination, the pH of the culture fluid was measured with a pH meter (Sartorius basic pH meter, Göttingen, Germany). The rumen culture liquor was strained through a 4-layered cheesecloth, and 2 mL per 10 mL of metaphosphoric acid (25%, wt vol^{-1}) was added before storing at -20°C until the VFA and ammonia-nitrogen levels were determined. VFA concentration was determined on a gas chromatograph (GC-8A; Shimadzu Corp., Kyoto, Japan) according to the method of Hu *et al.* (2005) and ammonia-nitrogen levels were determined following the phenol-hypochlorite method (Broderick *et al.* 1980).

2.4. DNA Extraction, Sequencing, and Diversity Analysis

At the end of incubation, 20 ml of culture liquor with a maximum dose of 0.64 g L^{-1} of cordycepin was allocated into two equal portions before storing at -80°C for 16S amplicon sequencing and untargeted metabolomics analysis.

The procedure involved the extraction of total DNA from the culture liquor using QIAamp Fast DNA stool Mini Kit (Qiagen, Cat# 51604) followed by the amplification of PCR conducted with barcoded specific bacteria primers that targeted the variable region 3-4 (V3-V4) of the 16S rRNA gene: forward primer 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and reverse primer 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ (Wang *et al.* 2023). Primers F341 (5′-GYGCASCAGKCGMGAAW-3′) and R806 (5′-GGACTACVSGGGTATCTAAT-3′) were used to amplify the V3-V4 region of the 16S rRNA gene for archaeal analysis. Protozoa analysis involved amplifying the V3-V4 region of the 18S rRNA gene using TAReuk454FWD1 F (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3 R (5′-ACTTTCGTTCTTGATYRA-3′) primers. And for fungi analysis, the ITS region of the 16S rRNA gene for fungi was amplified using primers F (CTTGGTCATTTAGAGGAAGTAA) and R (GCTGCGTTCTTCATCGATGC). According to standard

protocols, sequencing libraries and paired-end sequencing was constructed on an Illumina NovaSeq6000 platform from Biomarker Technologies Co, Ltd. (Beijing, China). Following Yang *et al.* (2020), paired-end reads were merged using FLASH v1.2.7, and tags with over six mismatches were ignored. Merged tags with an average quality score of <20 in a 50 bp sliding window were identified using Trimmomatic (Sheng *et al.* 2019), and tags shorter than 350 bps were eliminated. Using USEARCH, potential chimeras were eliminated, and the denoised sequences were clustered into operational taxonomic units (OTUs) with a similarity of 97% (version 10.0). All OTUs were given a taxonomy via a QIIME search against the Silva databases (Release 128).

The microbiome was compared as beta diversity using principal component analysis (PCA). For ruminal microbiome abundance, taxa abundance at the genus level was contrasted statistically between the control and treatment groups.

2.5. Non-Targeted Metabolomics Analysis

A total of 10 μL per sample of the prepared rumen fluid was pooled for quality control. The LC/MS system for metabolomics analysis comprised Waters Acquity I-Class PLUS ultra-high performance liquid tandem and Waters Xevo G2-XS QTOF high-resolution mass spectrometer with a Waters Acquity UPLC HSS T3 (1.8 um, 2.1 mm×100mm) column (Zhang *et al.* 2021). The injection volume was 1 μL for both the positive and negative, having a mobile phase A (0.1% formate aqueous solution) and mobile phase B (0.1% formate acetonitrile), respectively.

Following the software acquisition control (MassLynx V4.2, Waters), primary and secondary mass spectrometry data were extracted using Waters Xevo G2-XS QTOF high-resolution mass spectrometer in MSe mode. Dual-channel data acquisition was performed simultaneously with a low collision energy (2 V) and a high collision energy (10-40 V) per cycle and 0.2s scanning frequency for a mass spectrum. As described in Feng *et al.* (2022), the ESI ion source had a capillary voltage of 2000 V (positive ion mode) or -1500V (negative ion mode), a 30V cone voltage, a 150°C ion source temperature, 500°C desolvent gas temperature, 50 L h^{-1} backflush gas flow rate, and 800 L h^{-1} desolventizing gas flow rate. All MassLynx V4.2 data were analyzed using Progenesis QI software online METLIN database for extracting peaks, aligning and other data processing operations. Biomarks' self-built library was used for identification, theoretical fragment identification, and mass deviation under 100 ppm. Progenesis QI software was used to analyze all of the MassLynx V4.2 data in order to perform peak extraction, peak alignment, and other data

processing operations based on the Biomark's custom identification library and the online METLIN database, as well as theoretical fragment identification and mass deviation calculations. They are all under 100 ppm.

Positive and negative data were pooled. The dependability of the model was tested using 200 times permutations after normalizing the original peak area data with the total peak area using PCA and orthogonal projections to latent structures-discriminant analysis (OPLS-DA). The difference multiples were calculated and then compared following the grouping information. Further, compounds that had been identified were searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases for classification and pathway information. A calculation and comparison between the difference multiples were then estimated following the grouping information and the differential significance *P*-value for each compound was determined by using the T-test. Multiple cross-validations were used to calculate the model's variable importance in projection (VIP) value, while a technique that integrates the difference multiple, *P*-value, and VIP value of the OPLS-DA model was used for filtering the differential metabolites at fold change (FC)>1, or <-1, *P*-value<0.05, and VIP>1. A hypergeometric distribution test was used to determine the different metabolites for KEGG pathway enrichment significance. According to the -log10(*P*-value) and log2(FC) and of the metabolites and using the ggplot2 in the R language, the metabolites of interest were filtered using volcano plots.

2.6. Statistical analysis

All data from the experiment were analyzed using SAS software (Version 9.4; SAS Institute Inc., Cary, NC). Data about gas production and proportion and rumen fermentation parameters were finally tested for normal distribution and homogeneity of variance using histograms and formal statistical tests as part of the UNIVARIATE procedure of SAS, and then data were analyzed using the one-way ANOVA. Orthogonal polynomial contrasts were also used to analyze cordycepin's linear, quadratic and cubic effects among the different concentrations. The non-parametric Kruskal-Wallis Test was used to examine the effects of cordycepin on relative abundances (%) of rumen microbiome *in vitro* after 24 h incubation, and results were presented as mean and standard deviation. Means are declared significantly different when $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

3. Results

3.1 Effects of Cordycepin on ruminal gas production, proportion, and fermentation parameters

In Table 2, total gas production, hydrogen production, methane proportion, methane production, carbon dioxide production, and carbon dioxide proportion of fermentation liquid containing different doses of cordycepin showed a linear decline (P<0.05). Hydrogen proportion also has a quadric relation with the concentration of cordycepin (P<0.05). The proportion of carbon dioxide was also reduced cubically (P<0.05). Cordycepin concentration did not show a significant dose effect on hydrogen proportion (P>0.10).

As shown in Table 3, we used five groups of fermentation liquid containing different doses of cordycepin in rumen fermentation *in vitro* to indicate a linear increase in ammonia-N, total volatile acid, propionate, isovalerate, and valerate (P<0.05), while pH, butyrate, and the ratio of acetate to propionate showed a linear decrease (P<0.05). The concentration of cordycepin had no effect on the proportion of acetate and isobutyrate (P>0.10). The fluid pH also decreased quadratically and cubically (P<0.05), whereas ammonia-N and butyrate showed a quadratic increase (P<0.05). In addition to the proportion of acetate, isobutyrate, and hydrogen, the effect of adding cordycepin on other indicators showed a dose effect. When the concentration of cordycepin is 0.64 g L^{-1}, it has the greatest impact on all indexes of fermentation liquid, except the proportion of acetate, isobutyrate, and hydrogen proportion. Therefore, we selected fermentation liquid with a concentration of 0.64 g L^{-1} for 16S amplicon sequencing and untargeted metabolomics analysis.

3.2 Effects of Cordycepin on the relative abundances and structure of the rumen microbiome

Table 4 shows the bacteria, archaea, protozoa, and fungi in the fermentation fluid of the control group (CON) and the maximum dose group of cordycepin (CORD) alpha diversity index. The addition of cordycepin significantly decreased the Simpson, Shannon, abundance-based coverage estimator (ACE) and Chao1 of bacteria (P<0.05), significantly increased the Simpson, Shannon, ACE and Chao1 of protozoa (P<0.05), and significantly increased the ACE and Chao1 of archaea (P<0.05), while the Simpson and Shannon of archaea, ACE, Chao1, Simpson, and Shannon of fungi did not change significantly (P>0.10). Table 5 and Figure 1 show the top 15 microbial genera with the relative abundance of bacteria, protozoa, archaea, and fungi at the genus level. The relative abundance of *Succiniclasticum*, *Prevotella*, *Rikenellaceae_RC9_gut_group*, *NK4A214_group, Christensenellaceae_R_7_group*, *unclassified_F082*, *Veillonellaceae_UCG_001*, *Dasytricha, Ophryoscolex*, *Isotricha*,

unclassified_Eukaryota, *Methanobrevibacter* and *Piromyces* in the CORD group was significantly lower than that in the control group (P<0.05), while the relative abundance of *Succinivibrio*, *unclassified_Succinivibrionaceae*, *Prevotellaceae_UCG_001*, *unclassified_Lachnospiraceae*, *Lachnospira*, *Succinivibrionaceae_UCG_002*, *Pseudobutyrivibrio*, *Entodinium*, *Polyplastron*, *unclassified_Methanomethylophilaceae*, *Methanosphaera* and *Candidatus_Methanomethylophilus* was significantly higher than that in the control group (P<0.05). PCA indicated differences in bacteria, protozoa, and archaea between the control group and the CORD group, and the distance between the two groups was relatively large (Figure 2).

3.3 Effects of Cordycepin on the rumen metabolites and KEGG Pathway

In this experiment, a total of 2414 metabolites were detected, and 823 differential metabolites were detected between the control group and the CORD group, including 392 up-regulated and 431 down-regulated metabolites. The differential metabolites were labeled according to the KEGG database, and KEGG enrichment analysis was conducted on the control group and the CORD group, showing the annotated differential metabolite quantity, metabolite ratio, and P-value in 20 pathways (Figure 3).

As shown in Figure 4, in order to distinguish the differences between the two groups of metabolites, we used a volcano distribution map. Up-regulated differently expressed metabolites, down-regulated differentially expressed metabolites, and the metabolites detected but not substantially different are represented by the red, blue, and gray solid dots, respectively.

In addition, metabolomic PCA revealed significant metabolite variations between the control group and the CORD group. We used the OPLS-DA analysis to assess the control and CORD group's differences; the stability and reliability of the model depend on the closeness of the R^2Y and Q^2Y values to 1, i.e., the OPLS-DA can be used to filter differential metabolites (R^2Y=0.999, Q^2Y=0.956). Blue dots and red dots in Figure 5c represent R^2Y and Q^2Y of the permutation model, respectively. The two dashed lines represent the regression lines of R^2Y and Q^2Y fits. If the Q^2Y fitted regression line slope is positive, the model is meaningful, and the blue dots are generally above the red dots, the independence of the modeled training and test sets is good. The OPLS-DA score plots between the control group and the CORD group showed clear and noticeable separation, implying that their ruminal fluid metabolic profiles significantly differed from the control group during the experiment.

Among these different metabolic pathways, we selected a subset of metabolic pathways and metabolites that are significantly related to rumen fermentation (Table 5 and Table 6). Table 5 shows the significantly different metabolic pathways (P<0.05) that are significantly related to rumen fermentation and analyzed for KEGG enrichment, including biosynthesis of unsaturated fatty acids, purine metabolism, and linoleic acid metabolism. Table 6 is the differential metabolite noted in the differential metabolic pathway in Table 5. (13Z, 16Z)-docosadienoic acid, dihomo gamma linolenate, arachidonic acid, (6Z, 9Z, 12Z)-octadecadienoic acid, arachidonic acid, and (5Z, 8Z, 11Z, 14Z, 17Z)-icosapentenoic acid in the biosynthesis of unsaturated fatty acids pathway were significantly different from the control group (P<0.05). In the pathway of purine metabolism, there were significant differences between allantoic acid, adenine, xanthine, uric acid, adenosine 3'-monophosphate, photosporosylformylglycinamine, guanosine-5′-diphosphate (GDP), guanosine diphosphate, 2′-deoxyadenosine, xanthosine, deoxyguanosine monophosphate (dGMP), 2′-deoxyguanosine-5′-monophosphate, 2′-deoxyguanosine, deoxysinosine, and hypoxanthine, and the control group (P<0.05). In linoleic acid metabolism, there were significant differences on 9,12,13-TriHOME, (10E,12Z)-9-Oxooctadeca-10,12-dienoic acid (9-OxoODE), dihomo-gamma-linolenate, arachidonic acid, (6Z, 9Z, 12Z)-octadecadienoic acid, arachidonic acid between the CORD group and the control group (P<0.05).

4. Discussion

In vitro gas production technique simulates the fermentation of substrates in the rumen by using the mixture of rumen fluid and buffer and can be used to evaluate the nutritional value of ruminant feed and the relief effect of feed or additives on methane emissions (Hatew *et al*. 2015). *In vitro* gas production technique has advantages such as easy reproducibility, the possibility to assay a large number of samples within the same incubation run, to allow for measuring successive readings in the same repetition (Amanzougarene *et al.* 2020), and the selection of appropriate additive concentrations through *in vitro* gas production technique has a certain reference value for future *in vivo* gas production experiments (Zhou *et al.* 2020; Elghandour *et al.* 2016). Therefore, we used *in vitro* gas production technique to investigate the effects of different concentrations of cordycepin on rumen methane production.

Cordyceps is a traditional Chinese herbal medicine with high medical value (Paterson 2008). As a functional component of cordyceps, cordycepin has antibacterial and anti-inflammatory effects

(Tuli *et al*. 2013), but there are no studies on its potential to enhance rumen fermentation in ruminants and affect rumen gas production.

4.1 Effects of Cordycepin on ruminal gas production, proportion, and fermentation parameters

Feed, as the substrate, can be used by rumen microorganisms to produce gases such as carbon dioxide, methane, and hydrogen through fermentation, so gas production can be used to reflect substrate utilization by rumen microorganisms (Macome *et al.* 2018). In this experiment, as the concentration of cordycepin increased, total gas production, methane, carbon dioxide, and hydrogen emissions during 24h *in vitro* fermentation significantly decreased, which directly demonstrated the ability of cordycepin to lower methane production in ruminants. The pH of fermentation fluid decreased with the increase of cordycepin concentration, which may have a relationship with the increase in total VFA. VFA and ammonia-nitrogen are rumen microbial fermentation products, and VFA production is related to gas production to a certain extent (Wang *et al*. 2022). In this study, the proportion of propionate correlated positively with the concentration of cordycepin, which may indicate that some VFA metabolic pathways in the rumen have shifted to propionate formation (Wang *et al*. 2022). In addition, the concentration of ammonia- nitrogen also increases with the improvement in the concentration of cordycepin. The results of gas production and rumen fermentation parameters show that cordycepin has a dose effect on regulating gas production and rumen fermentation parameters. Therefore, we selected the control and maximum dose (0.64 g L^{-1}) groups for 16S rRNA sequencing and metabonomic analysis to explore the mechanism of cordycepin inhibiting rumen methane production.

4.2 Effects of Cordycepin on the relative abundances and structure of the rumen microbiome

The diversity and richness of rumen microbiota can reflect the regulatory effect of cordycepin on rumen microbiota. Using the alpha diversity index, which included ACE, Chao1, Shannon, and Simpson, evaluated the microbial richness and diversity. In the alpha diversity analysis, significant changes occurred in the ACE, Chao1, Simpson, and Shannon of bacteria and protozoa and the ACE and Chao1 of archaea, indicating that cordycepin partly affected the abundance and diversity of rumen microorganisms.

Methane is generated in the rumen in three main ways: hydrogenotrophic, acetoclastic, and methyl (Wang *et al*. 2022). The hydrogenotrophic pathway refers to the process in which hydrogen is oxidized to H^+ and carbon dioxide is reduced to methane. In the acetoclastic pathway, acetic acid

is activated by ATP to produce methane. The methyl pathway involves the reduction of a methyl compound (CH_3-R) to methane in the presence of hydrogen and its oxidation to carbon dioxide in the absence of hydrogen. Still, the oxidation of one methyl group provides enough electrons to reduce three methyl groups to methane to continue producing methane (Lyu *et al.* 2018). Approximately 82% of methane in the rumen is synthesized via the hydrogenotrophic pathway (Liang *et al.* 2020). A significant change in *Methanobrevibacter*, *unclassified_Methanomethylophilaceae* and *Methanosphaera* were found in this experiment (Figure 1 and Table 5). In most studies, *Methanobrevibacter* is the most abundant methanogen in the rumen (Newbold *et al*. 2020), and similarly revealed in our study (Figure 1 and Table 5). Most methanogens, such as *Methanobrevibacter,* generate methane by using hydrogen and carbon dioxide (hydrogenotrophic pathway) (Hook *et al*. 2010), but *unclassified_Methanomethylophilaceae* and *Methanosphaera* produce methane by reduction of methanol or methylamine, which means that these two methanogens produce methane through the methyl pathway (Zhao *et al.* 2020; Garcia *et al.* 2000).

In the genus level, the top 15 of bacteria in the relative abundance of fermentation fluid, *Succinivibrio*， *unclassified_Succinivibrionaceae,* and *Succinivibrionaceae_UCG_002* can utilize the carbohydrates of the substrate to generate succinate and convert it to propionate. And there is a significant negative correlation between the relative abundance of the genus *Succinivibrionaceae_UCG_002* and gas production (McCabe *et al.* 2015; Wei *et al.* 2022; Ren *et al.* 2019). Compared to the control group, the CORD group showed a significantly higher relative abundance of these three genera. This may be the reason for the significantly improved proportion of propionate with increased cordycepin addition. There was a significant decrease in the ratio of acetate to propionate because of no significant change in the proportion of acetate. The production of propionate can consume 4 [H], reducing the production of hydrogen, while the reduction of [H] also prevents methane formation by methanogens, which leads to reduction of methane production (Wang *et al*. 2022). And decreasing [H] may account for the significant decrease in hydrogen (Table 2). In addition, the development of VFA towards propionate production will reduce carbon dioxide and hydrogen production (Wang *et al*. 2022), which is in line with the results of our experiment (Tables 2 and 3).

The genus *Prevotelaceae UCG-001* can produce succinate and acetate (Fan *et al.* 2021).

Succinivibrio, *unclassified_Succinivibrionaceae,* and *Succinivibrionaceae_UCG_002* may utilize the succinate to generate propionate. Although there was no significant change in the proportion of acetate, the increase of total VFA in the cordycepin group also meant an increase in acetate content, which may be related to the significantly increased relative abundance of the *Prevotelaceae UCG-001* in the CORD group. Further, a previous study found a negative association between the genus *Prevotella* and methane emissions (Aguilar-Marin *et al.* 2020). However, in this experiment, we observed a significantly lower relative abundance of *Prevotella* in the CORD group compared to the control group. It has been reported that *Prevotella* in the gut of rats fed with cordycepin significantly decreased compared to the control group (An *et al.* 2018). It is possible that the intake of cordycepin had an effect on *Prevotella,* causing it to lose its advantage in competition with other bacteria genera, and that other genera of bacteria may have a stronger ability (directly or indirectly) to inhibit methane emissions, resulting in a reduction in methane emissions.

Rikenellaceae RC9 gut group is linked with rumen fermentation patterns, rumen epithelial development and fiber digestion (Qiu *et al.* 2022). The relative abundance of the genus *Rikenellaceae RC9 gut group* decreased significantly when tucumã oil was added to the diet (Ramos *et al.* 2018). Tucumã oil and cordycepin also have antibacterial effects. Therefore, the decrease in the relative abundance of the genus *Rikenellaceae RC9 gut group* in the CORD group may also be caused by the antibacterial effect of cordycepin. *NK4A214* group is a bacterium belonging to the *Ruminococcacaceae* family, which has strong fiber decomposing activity (Pacífico *et al.* 2021); the decrease of relative abundance in the *NK4A214* group may lead to a decline in the ability of fiber degradation, resulting in a decline in the amount of [H] produced during fiber decomposition, thereby affecting the production of methane (Li *et al.* 2022). As the cordycepin dosage increased, there was a significant increment in the concentration of ammonia nitrogen; possibly *unclassified_Lachnospiraceae,* and *Lachnospira* can produce ammonium (Amaretti *et al.* 2019), and the relative abundance of these two genera increased, leading to a higher concentration of ammonia nitrogen.

In addition, the presence of protozoa can also increase ammonia concentration (Ranilla *et al.* 2007). Ellis *et al.* (1991) had previously noted that *Dasytricha* could ferment carbohydrates to produce butyrate, and the relative abundance of *Dasytricha* in the CORD group of this study was

significantly lower than the control group. As indicated in Table 2, the significant decrease in butyrate proportion in the highest dose group in comparison to the control group also confirmed this. In this experiment, the relative abundance of *Entodinium* in the CORD group was significantly higher than that in the control group. Rumen protozoa and methanogens are closely related, and their associated methanogens exist in the cytoplasm or outer surface of protozoa by utilizing the abundant hydrogen production of rumen protozoa (Tapio *et al.* 2017). Although *Entodinium* has been shown to be positively correlated with methane emissions, the increase in methane emissions may be related to methanogens parasitic on *Entodinium*.

Only *Piromyces* in the top 15 fungi had significant changes in relative abundance, and their relative abundance decreased with the addition of cordycepin. *Piromyces* is a fiber-decomposing anaerobic fungus that provides substrates such as hydrogen, carbon dioxide, acetate and formate for methane production of methanogens (Cheng *et al.* 2018; Li *et al*. 2017). Jin *et al.* (2011) found a correlation between *Pyromyces* and *Methanobrevibacter*, and a reduction in the relative abundance of *Pyromyces* may also be one of the reasons for the decrease in the relative abundance of *Methanobrevibacter*. Some microorganisms can produce CO_2 by utilizing the fiber or starch of feed, such as *Pyromyces*, and cordycepin may cause a significant decrease in CO_2 production by inhibiting its activity (Liang *et al*. 2020; Li *et al*. 2017). Therefore, cordycepin may inhibit the hydrogenotrophic pathway to low methane production by decreasing [H] and CO_2. The decrease in [H] may reduce the relative abundance of *Methanobrevibacter* as a hydrogenotrophic methanogen (Siegert *et al.* 2014) but has no impact on *unclassified_Methanomethylophilaceae* and *Methanosphaera*, which may account for the significant increase in the relative abundance of *unclassified_Methanomethylophilaceae* and *Methanosphaera*.

4.3 Effects of Cordycepin on the rumen metabolites and KEGG Pathway

In order to explore the role of cordycepin in regulating methane production through its effects on ruminal metabolic pathways, metabolomics techniques were used to analyze ruminal metabolites. There are partial differences in metabolites between the CORD group and the control group, as shown in Figure 4. The biosynthesis of unsaturated fatty acids and linoleic acid metabolism pathways were enriched by KEGG enrichment analysis. In these two pathways, 8 kinds of unsaturated fatty acid content, including (13Z,16Z)-docosadienoic acid, was significantly increased after the addition of cordycepin, while the content of (5Z,8Z,11Z,14Z,17Z)-

icosapentaenoic acid was significantly decreased (Table 7). This indicates that cordycepin has the ability to regulate the synthesis of unsaturated fatty acids by rumen microorganisms. Unsaturated fatty acids (UFA) are toxic to rumen microbes, which removed [H] during the conversion of UFA into saturated fatty acids (SFA), reducing the concentration of [H] in rumen fluid and resulting in lower methane emissions (Li *et al.* 2023). Purine metabolism was also enriched in this experiment, which contains 15 differential metabolites (Table 7). It has been reported that cordycepin can replace adenosine triphosphate (ATP) to cause abnormal purine metabolism, and cordycepin can increase the ratio of adenosine monophosphate (AMP) to ATP (Qin *et al.* 2019), which may be the reason for the significant upregulation of adenosine 3′-monophosphate content in CORD group. Cordycepin can also reduce the concentration of guanosine triphosphate (GTP), GDP, and guanosine monophosphate (GMP) in bacteria, affecting the process of mRNA synthesis in bacteria (Zain-ul-abedin *et al.* 1983), consistent with our experimental results (Table 7). This may also be the reason for the relative abundance reduction of some bacteria (Table 5), such as *Prevotella* and *Methanobrevibacter*, resulting in decreased methane production.

To sum up, the mechanism by which cordycepin affects methane emission *in vitro* rumen fermentation may be divided into direct and indirect effects. The direct effect is that cordycepin interferes with the mRNA synthesis process by reducing the concentrations of GMP, GDP and GTP in methanogens, affects the normal reproduction of methanogens, reduces the relative abundance of *Methanobrevibacter*, and leads to a decreased methane production. As for the indirect effect, on the one hand, cordycepin affects the hydrogenotrophic pathway that produces methane by reducing [H] required for the production of hydrogenotrophic methanogens. The decrease in the concentration of [H] that can be utilized by *Methanobrevibacter*, a hydrogenotrophic methanogen and the most abundant methanogen in this experiment, results in the relative abundance of *Methanobrevibacter* and subsequently leads to a reduction in methane production. On the other hand, cordycepin reduces methane production by reducing the relative abundance of some protozoa, the host of some methanogens.

5. Conclusion

In this study, methane emissions decreased linearly with increasing cordycepin concentration, and methane production were lowest at the highest concentration. The suppression of methane emissions may be due to the higher relative abundance of succinic acid-producing bacteria such

as *Succinivibrio*， *unclassified_Succinivibrionaceae,* and *Succinivibrionaceae_UCG_002* caused by cordycepin, leading to a shift in VFA metabolic pathway towards propionate formation, thereby reducing the content of [H] in the fermentation fluid and reducing the production of carbon dioxide and hydrogen. The lack of [H] also directly inhibits the activity of *Methanobrevibacter*, reducing methane production. In addition, cordycepin influenced the metabolic pathway to produce unsaturated fatty acids and also affected the purine metabolic pathway, which may be one of the reasons for the lower relative abundance of *Methanobrevibacter* in the CORD group, leading to lower methane production. Therefore, cordycepin is a potent methane inhibitor that can reduce methane emissions while improving rumen fermentation. And this study provided evidence that byproducts or additives containing cordycepin can be used as methane inhibitors for feeding ruminants.

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Declaration of competing interest

The authors declare no competing interests.

Data availability

The Illumina sequencing raw data for our samples have been deposited in the NCBI Sequence Read Archive (SRA) under accession number: PRJNA976063.

Ethical Approval

The animal use protocol was approved following the Animal Care and Use Committee of Northeast Agricultural University (protocol number: NEAUEC20230267).

References

Aguilar-Marin S B, Betancur-Murillo C L, Isaza G A, Mesa H, Jovel J. 2020. Lower methane emissions were associated with higher abundance of ruminal *Prevotella* in a cohort of Colombian buffalos. *BMC Microbiology*, **20**, 1-13.

Ahn Y-J, Park S-J, Lee S-G, Shin S-C, Choi D-H. 2000. Cordycepin: selective growth inhibitor derived

from liquid culture of *Cordyceps militaris* against *Clostridium* spp. *Journal of Agricultural and Food Chemistry*, **48**, 2744-2748.

Amanzougarene Z, Fondevila M. 2020. Fitting of the *in vitro* gas production technique to the study of high concentrate diets. *Animals*, **10**, 1935.

Amaretti A, Gozzoli C, Simone M, Raimondi S, Righini L, Pérez-Brocal V, García-López R, Moya A, Rossi M. 2019. Profiling of protein degraders in cultures of human gut microbiota. *Frontiers in Microbiology*, **10**, 2614.

An Y, Li Y, Wang X, Chen Z, Xu H, Wu L, Li S, Wang C, Luan W, Wang X. 2018. Cordycepin reduces weight through regulating gut microbiota in high-fat diet-induced obese rats. *Lipids in Health and Disease*, **17**, 1-10.

Baker S. 1999. Rumen methanogens, and inhibition of methanogenesis. *Australian Journal of Agricultural Research*, **50**, 1293-1298.

Barbosa A L, Voltolini T V, Menezes D R, De Moraes S A, Nascimento J C S, De Souza Rodrigues R T. 2018. Intake, digestibility, growth performance, and enteric methane emission of Brazilian semiarid non-descript breed goats fed diets with different forage to concentrate ratios. *Tropical Animal Health and Production*, **50**, 283-289.

Broderick G A, Kang J H. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *Journal of Dairy Science*, **63**, 64-75.

Cheng Y, Shi Q, Sun R, Liang D, Li Y, Li Y, Jin W, Zhu W. 2018. The biotechnological potential of anaerobic fungi on fiber degradation and methane production. *World Journal of Microbiology and Biotechnology*, **34**, 155.

Cottle D J, Nolan J V, Wiedemann S G. 2011. Ruminant enteric methane mitigation: a review. *Animal Production Science*, **51**, 491-514.

Cunningham K, Manson W, Spring F, Hutchinson S. 1950. Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) Link. *Nature*, **166**, 949-949.

Dini Y, Gere J I, Cajarville C, Ciganda V S. 2018. Using highly nutritious pastures to mitigate enteric methane emissions from cattle grazing systems in South America. *Animal Production Science*, **58**, 2329-2334.

Elghandour M M Y, Kholif A E, Salem A Z M, Olafadehan O A, Kholif A M. 2016. Sustainable anaerobic rumen methane and carbon dioxide productions from prickly pear cactus flour by organic acid salts

addition. *Journal of Cleaner Production*, **139**, 1362-1369.

Ellis J E, Mcintyre P S, Saleh M, Williams A G, Lloyd D. 1991. Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the ruminal ciliate *Polyplastron multivesiculatum*. *Applied and Environmental Microbiology*, **57**, 1400-1407.

Fan Q, Wanapat M, Hou F. 2021. Rumen bacteria influence milk protein yield of yak grazing on the Qinghai-Tibet plateau. *Animal Bioscience*, **34**, 1466–1478.

Feng Z D, Zhong Y F, He G L, Sun H, Chen Y J, Zhou W H, Lin S M. 2022. Yeast culture improved the growth performance, liver function, intestinal barrier and microbiota of juvenile largemouth bass (*Micropterus salmoides*) fed high-starch diet. *Fish & Shellfish Immunology*, **120**, 706-715.

Garcia J-L, Patel B K C, Ollivier B. 2000. Taxonomic, phylogenetic, and ecological diversity of methanogenic archaea. *Anaerobe*, **6**, 205-226.

Ghavi Hossein-Zadeh N. 2023. A meta-analysis of the genetic contribution to greenhouse gas emission in sheep. *Journal of Animal Breeding Genetics*, **140**, 49-59.

Goodrich R, Garrett J, Gast D, Kirick M, Larson D, Meiske J. 1984. Influence of monensin on the performance of cattle. *Journal of Animal Science*, **58**, 1484-1498.

Hook S E, Wright A D, Mcbride B W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea*, **2010**, 945785.

Hristov A N, Ott T, Tricarico J, Rotz A, Waghorn G, Adesogan A, Dijkstra J, Montes F, Oh J, Kebreab E. 2013. Special topics—Mitigation of methane and nitrous oxide emissions from animal operations: III. A review of animal management mitigation options. *Journal of Animal Science*, **91**, 5095-5113.

Hu W-L, Liu J-X, Ye J-A, Wu Y-M, Guo Y-Q. 2005. Effect of tea saponin on rumen fermentation *in vitro*. *Animal Feed Science and Technology*, **120**, 333-339.

Huang F, Li W, Xu H, Qin H, He Z-G. 2019. Cordycepin kills *Mycobacterium tuberculosis* through hijacking the bacterial adenosine kinase. *PloS one*, **14**, e0218449.

IPCC. 2019. Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems.

Jiang Q, Lou Z, Wang H, Chen C. 2019. Antimicrobial effect and proposed action mechanism of cordycepin against *Escherichia coli* and *Bacillus subtilis*. *Journal of Microbiology*, **57**, 288-297.

Jin W, Cheng Y F, Mao S Y, Zhu W Y. 2011. Isolation of natural cultures of anaerobic fungi and

indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresource Technology*, **102**, 7925-7931.

Kim W, Hanigan M, Lee S, Lee S, Kim D, Hyun J, Yeo J, Lee S. 2014. Effects of *Cordyceps militaris* on the growth of rumen microorganisms and *in vitro* rumen fermentation with respect to methane emissions. *Journal of Dairy Science*, **97**, 7065-7075.

Lee S J, Lee Y J, Eom J S, Kim H S, Choi Y Y, Jo S U, Kang S N, Park H Y, Kim D H, Lee S S. 2020. Effects of the appropriate addition of antioxidants from *Pinus densiflora* and *Mentha canadensis* extracts on methane emission and rumen fermentation. *Animals*, **10**, 1888.

Li Q S, Wang R, Ma Z Y, Zhang X M, Jiao J Z, Zhang Z G, Ungerfeld E M, Yi K L, Zhang B Z, Long L, Long Y, Tao Y, Huang T, Greening C, Tan Z L, Wang M. 2022. Dietary selection of metabolically distinct microorganisms drives hydrogen metabolism in ruminants. *ISME Journal*, **16**, 2535-2546.

Li Y, Gao J, Lv J, Lambo M T, Wang Y, Wang L, Zhang Y. 2023. Replacing soybean meal with high-oil pumpkin seed cake in the diet of lactating Holstein dairy cows modulated rumen bacteria and milk fatty acid profile. *Journal of Dairy Science*, **106**, 1803-1814.

Li Y, Jin W, Mu C, Cheng Y, Zhu W. 2017. Indigenously associated methanogens intensified the metabolism in hydrogenosomes of anaerobic fungi with xylose as substrate. *Journal of Basic Microbiology*, **57**, 933-940.

Li Y, Lv J, Wang J, Zhou S, Zhang G, Wei B, Sun Y, Lan Y, Dou X, Zhang Y. 2021. Changes in carbohydrate composition in fermented total mixed ration and its effects on *in vitro* methane production and microbiome. *Frontiers in Microbiology*, **12**, 738334.

Liang J, Nabi M, Zhang P, Zhang G, Cai Y, Wang Q, Zhou Z, Ding Y. 2020. Promising biological conversion of lignocellulosic biomass to renewable energy with rumen microorganisms: A comprehensive review. *Renewable and Sustainable Energy Reviews*, **134**, 110335.

Lyu Z, Shao N, Akinyemi T, Whitman W B. 2018. Methanogenesis. *Current Biology*, **28**, R727-R732.

Macome F M, Pellikaan W F, Hendriks W H, Warner D, Schonewille J T, Cone J W. 2018. *In vitro* gas and methane production in rumen fluid from dairy cows fed grass silages differing in plant maturity, compared to *in vivo* data. *Journal of Animal Physiology and Animal Nutrition*, **102**, 843-852.

Mccabe M S, Cormican P, Keogh K, O'connor A, O'hara E, Palladino R A, Kenny D A, Waters S M. 2015. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised *Succinivibrionaceae* and an increase of the *Methanobrevibacter gottschalkii* clade in feed restricted

cattle. *PloS one*, **10**, e0133234.

Mikhaylov A, Moiseev N, Aleshin K, Burkhardt T. 2020. Global climate change and greenhouse effect. *Entrepreneurship and Sustainability Issues*, **7**, 2897-2913.

Miller T L. 2015. Methanobrevibacter. Bergey's Manual of Systematics of Archaea and Bacteria, 1-14.

Mustapha N A, Sharuddin S S, Zainudin M H M, Ramli N, Shirai Y, Maeda T. 2017. Inhibition of methane production by the palm oil industrial waste phospholine gum in a mimic enteric fermentation. *Journal of Cleaner Production*, **165**, 621-629.

Nakamura K, Shinozuka K, Yoshikawa N. 2015. Anticancer and antimetastatic effects of cordycepin, an active component of *Cordyceps sinensis*. *Journal of Pharmacological Sciences*, **127**, 53-56.

Nampoothiri V M, Mohini M, Malla B A, Mondal G, Pandita S. 2020. Animal performance, and enteric methane, manure methane and nitrous oxide emissions from Murrah buffalo calves fed diets with different forage-to-concentrate ratios. *Animal Production Science*, **60**, 780-789.

Newbold C J, Ramos-Morales E. 2020. Review: ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal*, **14**, s78-s86.

NRC. 2001. Nutrient Requirements of Dairy Cattle: 2001. National Academies Press.

Odongo N, Bagg R, Vessie G, Dick P, Or-Rashid M, Hook S, Gray J, Kebreab E, France J, Mcbride B. 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. *Journal of Dairy Science*, **90**, 1781-1788.

Pacífico C, Petri R M, Ricci S, Mickdam E, Wetzels S U, Neubauer V, Zebeli Q. 2021. Unveiling the bovine epimural microbiota composition and putative function. *Microorganisms*, **9**, 342.

Palangi V, Taghizadeh A, Abachi S, Lackner M. 2022. Strategies to mitigate enteric methane emissions in ruminants: A review. *Sustainability*, **14**, 13229.

Paterson R R. 2008. Cordyceps: a traditional Chinese medicine and another fungal therapeutic biofactory? *Phytochemistry*, **69**, 1469-1495.

Qin P, Li X, Yang H, Wang Z Y, Lu D. 2019. Therapeutic potential and biological applications of cordycepin and metabolic mechanisms in cordycepin-producing fungi. *Molecules*, **24**, 2231.

Qiu X, Qin X, Chen L, Chen Z, Hao R, Zhang S, Yang S, Wang L, Cui Y, Li Y. 2022. Serum biochemical parameters, rumen fermentation, and rumen bacterial communities are partly driven by the breed and sex of cattle when fed high-grain diet. *Microorganisms*, **10**, 323.

Ramos A F, Terry S A, Holman D B, Breves G, Pereira L G, Silva A G, Chaves A V. 2018. Tucumã oil

shifted ruminal fermentation, reducing methane production and altering the microbiome but decreased substrate digestibility within a RUSITEC fed a mixed hay–concentrate diet. *Frontiers in Microbiology*, **9**, 1647.

Ranilla M J, Jouany J P, Morgavi D P. 2007. Methane production and substrate degradation by rumen microbial communities containing single protozoal species *in vitro*. *Letters in Applied Microbiology*, **45**, 675-680.

Ren H, Su X, Bai H, Yang Y, Wang H, Dan Z, Lu J, Wu S, Cai C, Cao Y. 2019. Specific enrichment of microbes and increased ruminal propionate production: The potential mechanism underlying the high energy efficiency of Holstein heifers fed steam-flaked corn. *AMB Express*, **9**, 1-11.

Santra A, Karim S. 2003. Rumen manipulation to improve animal productivity. *Asian-australasian Journal of Animal Sciences*, **16**, 748-763.

Sheng D D, Zhao S M, Gao L, Zheng H F, Liu W T, Hou J, Jin Y X, Ye F, Zhao Q D, Li R, Zhao N P, Zhang L, Han Z P, Wei L X. 2019. BabaoDan attenuates high-fat diet-induced non-alcoholic fatty liver disease via activation of AMPK signaling. *Cell and Bioscience*, **9**, 77.

Shin S, Moon S, Park Y, Kwon J, Lee S, Lee C-K, Cho K, Ha N-J, Kim K. 2009. Role of cordycepin and adenosine on the phenotypic switch of macrophages via induced anti-inflammatory cytokines. *Immune Network*, **9**, 255-264.

Siegert M, Li X F, Yates M D, Logan B E. 2014. The presence of hydrogenotrophic methanogens in the inoculum improves methane gas production in microbial electrolysis cells. *Frontiers in Microbiology*, **5**, 778.

Soliva C, Hess H.2007. Measuring methane emission of ruminants by *in vitro* and *in vivo* techniques. *Measuring Methane Production from Ruminants*, 15-31.

Tapio I, Snelling T J, Strozzi F, Wallace R J. 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of Animal Science and Biotechnology*, **8**, 7.

Thakur S, Mohini M, Malik T A, Howal S, Varun T K, Madavi A, Yadev R D, Mondal G, Datt C. 2021. Performance of crossbred goat kids fed with diets varying in concentrate-to-forage ratio: intake, nutrient utilization, enteric methane emission and body weight changes. *Biological Rhythm Research*, **52**, 1334-1341.

Thompson L R, Rowntree J E. 2020. Invited review: methane sources, quantification, and mitigation in grazing beef systems. *Applied Animal Science*, **36**, 556-573.

Tuli H S, Sharma A K, Sandhu S S, Kashyap D. 2013. Cordycepin: a bioactive metabolite with therapeutic potential. *Life Sciences*, **93**, 863-869.

Vargas J E, Andrés S, López-Ferreras L, Snelling T J, Yáñez-Ruíz D R, García-Estrada C, López S. 2020. Dietary supplemental plant oils reduce methanogenesis from anaerobic microbial fermentation in the rumen. *Scientific Reports*, **10**, 1-9.

Wang J-K, Ye J-A, Liu J-X. 2012. Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance—a review. *Tropical Animal Health and Production*, **44**, 697-706.

Wang K, Xiong B, Zhao X. 2022. Could propionate formation be used to reduce enteric methane emission in ruminants? *Science of the Total Environment*, **855**, 158867.

Wang Y, Yu Q, Wang X, Song J, Lambo M T, Huang J, He P, Li Y, Zhang Y. 2023. Replacing alfalfa hay with industrial hemp ethanol extraction byproduct and Chinese wildrye hay: Effects on lactation performance, plasma metabolites, and bacterial communities in Holstein cows. *Frontiers in Veterinary Science*, **10**, 1061219.

Wei X, Ouyang K, Long T, Liu Z, Li Y, Qiu Q. 2022. Dynamic variations in rumen fermentation characteristics and bacterial community composition during *in vitro* fermentation. *Fermentation*, **8**, 276.

Wei Z, Zhang B, Liu J. 2018. Effects of the dietary nonfiber carbohydrate content on lactation performance, rumen fermentation, and nitrogen utilization in mid-lactation dairy cows receiving corn stover. *Journal of Animal Science and Biotechnology*, **9**, 20.

Xin H S, Khan N A, Liu X, Jiang X, Sun F, Zhang S Z, Sun Y K, Zhang Y G, Li X. 2021. Profiles of odd- and branched-chain fatty acids and their correlations with rumen fermentation parameters, microbial protein synthesis, and bacterial populations based on pure carbohydrate incubation *in vitro*. *Frontiers in Nutrition*, **8**, 733352.

Yang C, Xu Z, Deng Q, Huang Q, Wang X, Huang F. 2020. Beneficial effects of flaxseed polysaccharides on metabolic syndrome via gut microbiota in high-fat diet fed mice. *Food Research International*, **131**, 108994.

Zain-Ul-Abedin, Lopez J M, Freese E. 1983. Induction of bacterial differentiation by adenine-and adenosine-analogs and inhibitors of nucleic acid synthesis. *Nucleosides and Nucleotides*, **2**, 257-274.

Zhang N N, Hu G J, Guo K, Fu T, Lian H X, Wang L F, Gao T Y. 2021. Rumen bacteria and epithelial metabolism contribute to improving N utilization efficiency of calves. *Animal Biotechnology*, **33**, 1480–1491.

Zhao L, Caro E, Holman D B, Gzyl K E, Moate P J, Chaves A V. 2020. Ozone decreased enteric methane production by 20% in an *in vitro* rumen fermentation system. *Frontiers in Microbiology*, **11**, 571537.

Zhou R, Wu J, Lang X, Liu L, Casper D P, Wang C, Zhang L, Wei S. 2020. Effects of oregano essential oil on *in vitro* ruminal fermentation, methane production, and ruminal microbial community. *Journal of Dairy Science*, **103**, 2303-2314.

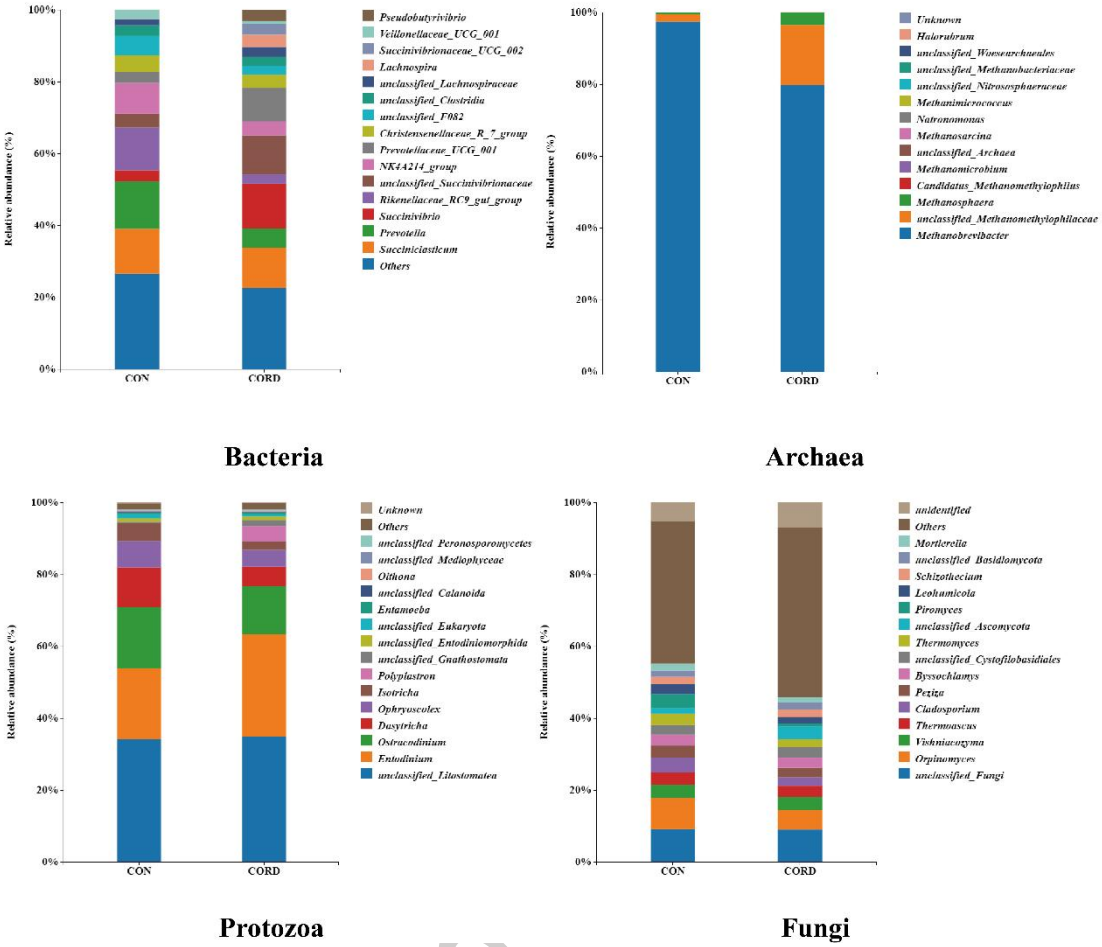

Figure 1 Relative abundance of bacteria, protozoa, archaea and fungi at the genus level in control (CON) group and the group with addition of 0.64 g/L cordycepin group (CORD)

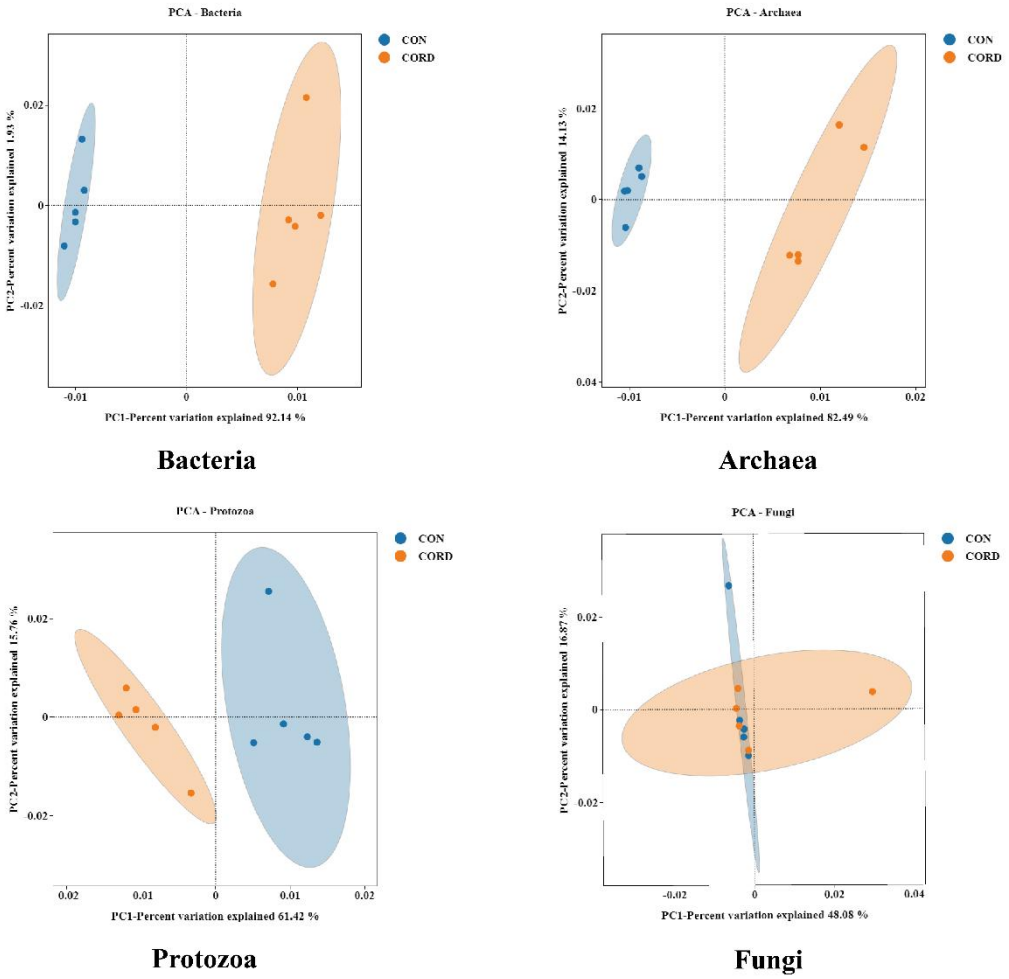

Figure 2 Principal components analysis (PCA) of bacteria, protozoa, archaea and fungi in control (CON) group and the group with addition of 0.64 g/L cordycepin group (CORD)

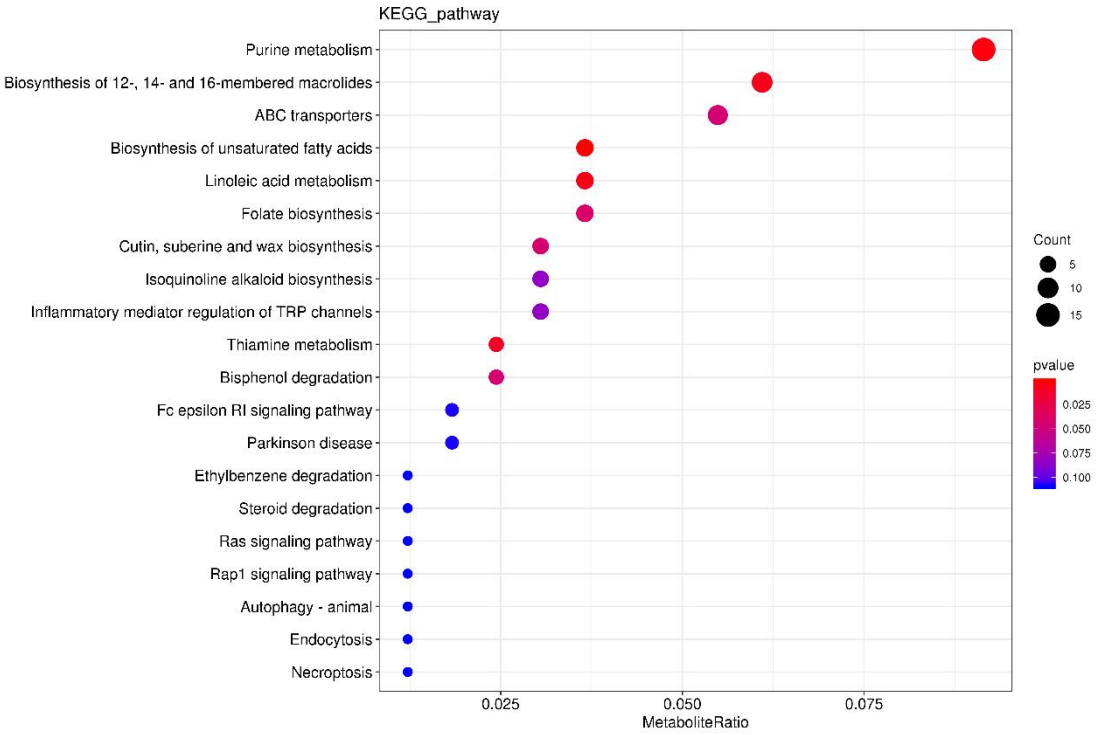

Figure 3 KEGG pathway enrichment analysis performed using the significantly different rumen metabolites between control (CON) group and the group with addition of 0.64 g/L cordycepin group (CORD)

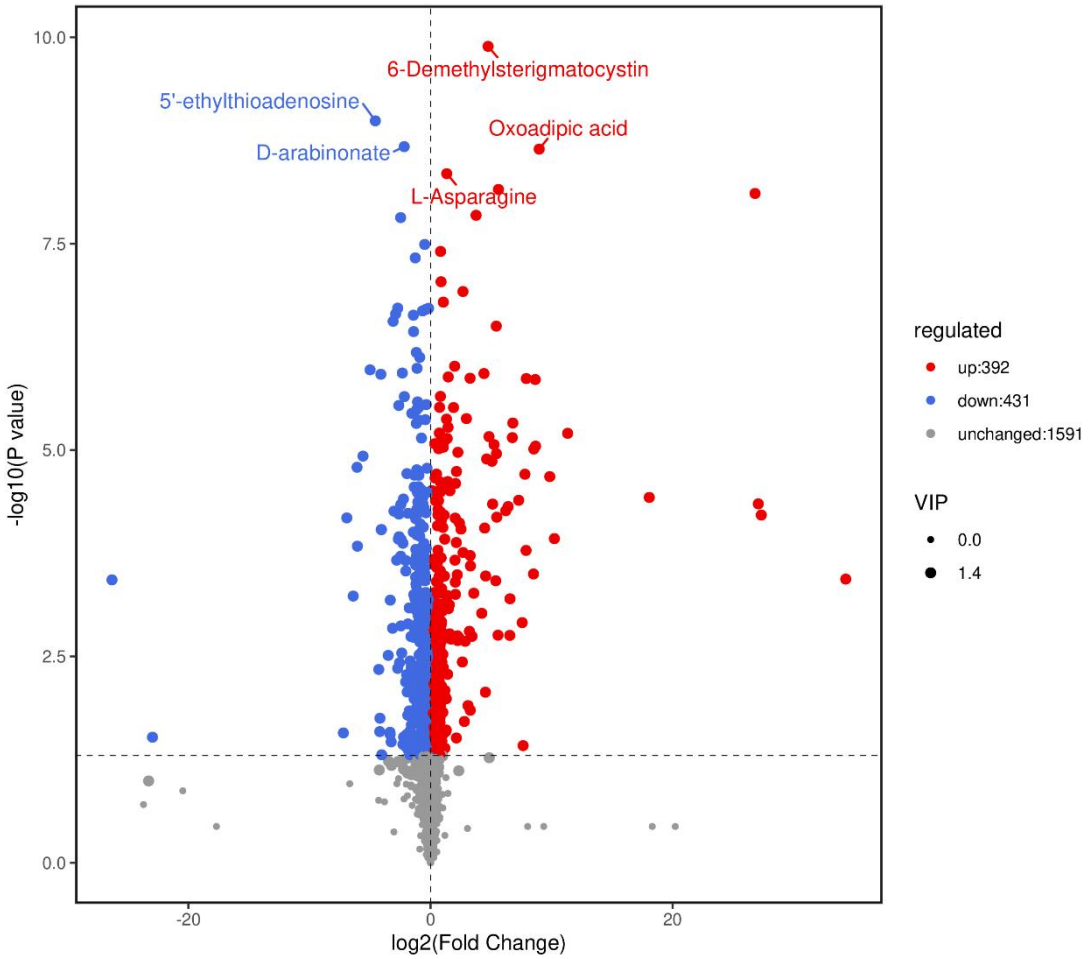

Figure 4 A volcano plot displaying the overall trend of differences in rumen metabolites content between control (CON) group and the group with addition of 0.64 g/L cordycepin group (CORD)

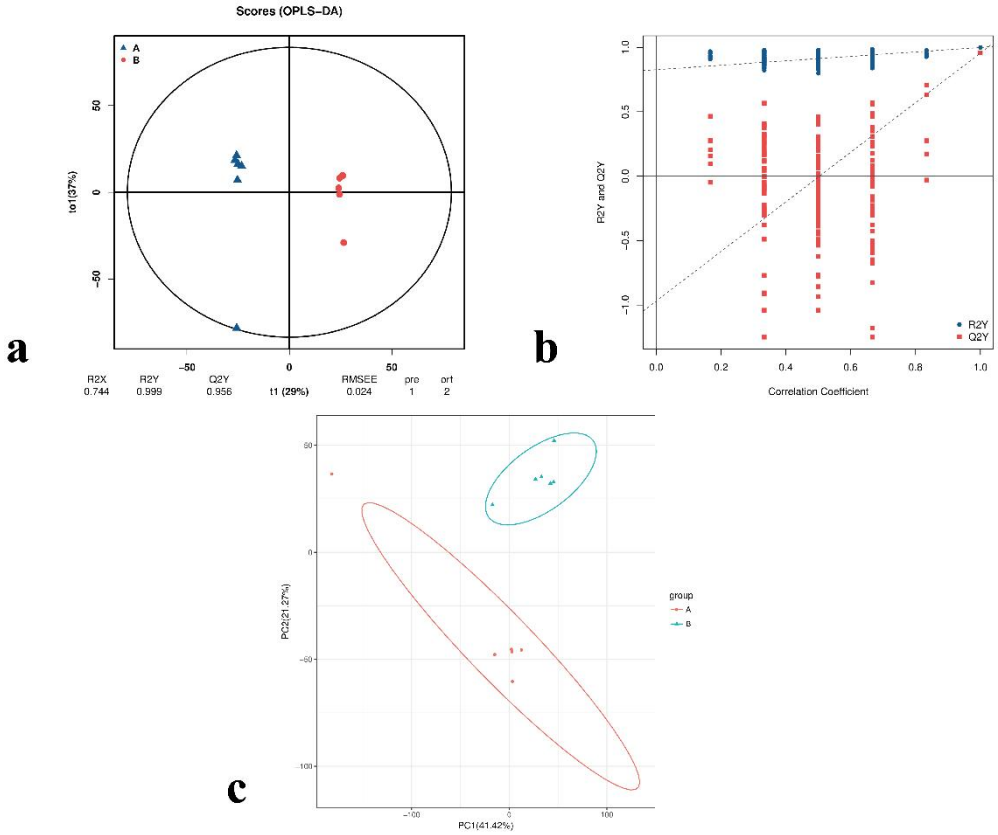

Figure 5 (a) The orthogonal projections to latent structures-discriminant analysis (OPLS-DA) plot, (b) permutation test of OPLS-DA, and (c) principal component analysis (PCA) *in vitro* among different groups. R2Y (cum) and Q2Y (cum) represent the interpretability and predictability of models, respectively. (A: control (CON) group; B: the group with addition of 0.64 g/L cordycepin group (CORD))

Table 1 Ingredient and nutrient content of total mixed ration in the experimental diet (DM basis)

Ingredient	Content, %
Alfalfa hay	15.59
Corn silage	30.28
Soybean meal	11.14
Steam-flaked corn	19.15
Dry corn gluten feed	5.35
DDGS	5.35
Sugar beet pulp pellets	12.03
Premix[1]	1.11
Nutritive values	
NE_L(mCal kg^{-1} of DM)[2]	1.72
CP(%)	16.67
RDP(%CP)	60.35
ADF(%)	21.11
NDF(%)	32.75
peNDF(%)	22.95
Starch(%)	27.12
Fat(%)	3.89
Ash(%)	7.14
Ca(%)	0.61
P(%)	0.59

ADF, acid detergent fiber; CP, crude protein; DDGS, distillers dried grains with solubles; NDF, neutral detergent fiber; NEL, net energy for lactating cow; peNDF, physical effectiveness neutral detergent fiber; RDP, rumen degradable protein.

[1]The premix contained (on a DM basis): 99.17% ash, 14.25% Ca, 5.40% P, 4.93% Mg, 10.64% Na, 2.95% Cl, 12 mg kg^{-1} Co, 1750 mg kg^{-1} Cu, 125 mg kg^{-1} I, 4000 mg kg^{-1} Mn,50 mg kg^{-1} Se, 6 500 mg kg^{-1} Zn, 1000 000 IU kg^{-1} vitamin A, 25 0000 IU kg^{-1} vitamin D3 and 4000 IU kg^{-1} vitamin E.

[2]NE_L was estimated according to Cornell-Penn-Miner dairy (CPM Dairy, version 3.0.10) (Wei *et al*. 2018).

Table 2 Cordycepin supplementation affects the gas production of methane, carbon dioxide, and hydrogen after 24 h *in vitro* culture

Items	Dose (g L^{-1})					SEM	Contrast[1]		
	0.00	0.08	0.16	0.32	0.64		L	Q	C
Total gas production (mL)	158.18[a]	141.64[b]	130.27[c]	114.73[d]	100.82[e]	2.021	<.0001	0.81	0.31
Methane production (mL)	34.28[a]	29.23[b]	26.34[c]	22.74[d]	19.56[e]	0.413	<.0001	0.087	0.13
Carbon dioxide production (mL)	96.32[a]	86.41[b]	76.24[c]	68.65[d]	58.76[e]	1.209	<.0001	0.34	0.60
Hydrogen production (mL)	1.25[a]	1.16[b]	1.06[c]	0.90[d]	0.83[e]	0.0163	<.0001	0.031	0.62
Methane proportion (%)	21.67[a]	20.64[b]	20.22[bc]	19.82[cd]	19.40[d]	0.161	<.0001	0.053	0.43
Carbon dioxide proportion (%)	60.89[a]	61.01[a]	58.52[c]	59.84[b]	58.28[c]	0.250	<.0001	0.020	<.0001
Hydrogen proportion (%)	0.79	0.82	0.81	0.78	0.82	0.0142	0.68	0.014	0.70

SEM, standard error of mean.

[a–e]Means within a row with unlike superscripts differ (P<0.05).

[1]L, linear; Q, quadratic; C, cubic.

Table 3 Influence of cordycepin addition on the fermentation parameters at 24 h incubation *in vitro*

Items	Dose (g L^{-1})					SEM[1]	Contrast[1]		
	0.00	0.08	0.16	0.32	0.64		L	Q	C
pH	6.45[a]	6.41[b]	6.39[c]	6.39[c]	6.32[d]	0.00504	<.0001	0.0002	0.8096
NH_3-N (mg dL^{-1})	32.13[d]	36.80[c]	39.62[b]	39.68[b]	44.98[a]	0.457	<.0001	<.0001	0.66
Total VFA (mmol L^{-1})	79.33[b]	80.62[b]	83.13[a]	83.31[a]	83.81[a]	0.698	<.0001	0.43	0.26
Molar proportion, mmol 100mmol^{-1}									
Acetate	54.37	53.73	54.10	54.38	54.27	0.290	0.77	0.12	0.41
Propionate	24.29[c]	24.77[b]	24.79[b]	25.06[b]	26.07[a]	0.133	0.0002	0.45	0.23
Isobutyrate	1.34	1.34	1.32	1.35	1.34	0.0177	0.97	0.50	0.41
Butyrate	15.98[a]	15.99[a]	15.63[a]	14.87[b]	13.87[c]	0.132	<.0001	0.0057	0.95
Isovalerate	2.14[c]	2.18[c]	2.19[c]	2.30[b]	2.36[a]	0.0219	<.0001	0.16	0.14
Valerate	1.89[c]	1.99[ab]	1.98[b]	2.05[a]	2.09[a]	0.0182	<.0001	0.28	0.02
Acetate/Propionate	2.24[a]	2.17[b]	2.18[ab]	2.17[b]	2.08[c]	0.0227	0.066	0.24	0.30

NH_3-N, ammonia nitrogen; SEM, standard error of mean; Total VFA, total volatile fatty acid.

[a–d]Means within a row with unlike superscripts differ (P<0.05).

[1]L, linear; Q, quadratic; C, cubic.

Table 4 The richness and diversity of the rumen microbiome fermented by the control (CON) diet and supplemented with the most effective dose (0.64 g L^{-1} cordycepin)

Item	CON[1]	CORD[2]	SEM	*P*-value[3]
Bacteria				
ACE	1222.79	1072.90	15.542	0.0001
Chao1	1218.6	1068.51	15.597	0.0001
Simpson	0.99	0.98	0.00085	<.0001
Shannon	8.51	7.51	0.0555	<.0001
Protozoa				
ACE	119.14	145.56	15.389	<.0001
Chao1	118.42	144.07	15.485	<.0001
Simpson	0.92	0.93	0.00324	<.0001
Shannon	4.40	4.57	0.0928	<.0001
Archaea				
ACE	31.4	38.17	1.091	0.0023
Chao1	31.4	38.0	1.039	0.0020
Simpson	0.79	0.80	0.00993	0.94
Shannon	2.84	2.83	0.0440	0.97
Fungi				
ACE	297.13	349.18	42.706	0.41
Chao1	295.26	347.64	42.837	0.41
Simpson	0.98	0.98	0.00484	0.58
Shannon	7.10	6.90	0.172	0.44

ACE, abundance-based coverage estimator; SEM, standard error of mean.

[1]CON = Control group.

[2]CORD = the group supplemented with Cordycepin.

[3]Means are declared significantly different when $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

Table 5 Relative abundances of 15 predominant microbial genera (% of total sequences) of the microbial community in fermenters incubated with the control (CON) diet and supplemented with the most effective dose (0.64 g L^{-1} cordycepin)

Rumen microbiome	CON[1]	CORD[2]	*P*-value[3]
Bacteria			
Succiniclasticum	11.25 ± 0.32	9.75 ± 0.59	0.009
Prevotella	11.93 ± 1.76	4.85 ± 0.50	0.009
Succinivibrio	2.71 ± 0.38	10.83 ± 0.62	0.009
Rikenellaceae_RC9_gut_group	10.77 ± 0.55	2.28 ± 0.18	0.009
unclassified_Succinivibrionaceae	3.42 ± 0.45	9.50 ± 1.16	0.009
NK4A214_group	7.63 ± 0.27	3.46 ± 0.35	0.009
Prevotellaceae_UCG_001	2.74 ± 0.43	8.26 ± 0.77	0.009
Christensenellaceae_R_7_group	4.18 ± 0.25	3.23 ± 0.45	0.009
unclassified_F082	4.86 ± 0.23	2.05 ± 0.13	0.009
unclassified_Clostridia	2.74 ± 0.35	2.31 ± 0.31	0.076
unclassified_Lachnospiraceae	1.36 ± 0.07	2.36 ± 0.11	0.009
Lachnospira	0.0089 ± 0.0082	2.99 ± 0.11	0.0088
Succinivibrionaceae_UCG_002	0.17 ± 0.07	2.73 ± 0.93	0.009
Veillonellaceae_UCG_001	2.26 ± 0.12	0.64 ± 0.12	0.009
Pseudobutyrivibrio	0.025 ± 0.006	2.76 ± 0.36	0.009
Protozoa			
unclassified_Litostomatea	34.10 ± 5.66	34.93 ± 2.99	0.38
Entodinium	19.73 ± 1.27	28.49 ± 3.62	0.0011
Ostracodinium	16.94 ± 2.91	13.36 ± 2.53	0.12
Dasytricha	11.15 ± 4.35	5.40 ± 3.44	0.0372
Ophryoscolex	7.37 ± 1.81	4.62 ± 0.58	0.0054

Isotricha	4.88 ± 1.79	2.35 ± 0.81	0.0054
Polyplastron	0.012 ± 0.019	4.19 ± 0.87	0.0008
unclassified_Gnathostomata	0.40 ± 0.44	1.76 ± 3.68	0.38
unclassified_Entodiniomorphida	1.02 ± 0.24	1.01 ± 0.21	0.92
unclassified_Eukaryota	1.09 ± 0.42	0.52 ± 0.27	0.0163
Entamoeba	0.58 ± 0.35	0.65 ± 0.37	0.63
unclassified_Calanoida	0.22 ± 0.21	0.16 ± 0.18	0.85
Oithona	0.26 ± 0.40	0.091 ± 0.14	0.63
unclassified_Mediophyceae	0.10 ± 0.14	0.24 ± 0.27	0.49
unclassified_Peronosporomycetes	0.11 ± 0.16	0.15 ± 0.19	0.27
Archaea			
Methanobrevibacter	97.32 ± 0.40	79.76 ± 1.63	0.009
unclassified_Methanomethylophilaceae	2.09 ± 0.36	16.65 ± 2.20	0.009
Methanosphaera	0.58 ± 0.082	3.52 ± 1.11	0.009
Candidatus_Methanomethylophilus	0.0025 ± 0.0057	0.036 ± 0.031	0.0132
Methanomicrobium	0.0069 ± 0.0048	0.0064 ± 0.0039	0.60
unclassified_Archaea	0.0019 ± 0.0028	0.0027 ± 0.0031	0.74
Methanosarcina	0.00 ± 0.00	0.0030 ± 0.0053	0.14
Natronomonas	0.0012 ± 0.0017	0.0012 ± 0.0020	1.00
Methanimicrococcus	0.00 ± 0.00	0.0024 ± 0.0054	0.32
unclassified_Nitrososphaeraceae	0.00 ± 0.00	0.0018 ± 0.0027	0.14
unclassified_Methanobacteriaceae	0.00 ± 0.00	0.0012 ± 0.0027	0.32
unclassified_Woesearchaeales	0.00 ± 0.00	0.00061 ± 0.0014	0.32
Halorubrum	0.00 ± 0.00	0.00061 ± 0.0014	0.32
Fungi			
Orpinomyces	8.65 ± 8.13	5.33 ± 3.90	0.60

Vishniacozyma	3.63 ± 0.91	3.74 ± 2.34	0.60
Thermoascus	3.53 ± 0.8	3.04 ± 1.6	0.60
Cladosporium	4.09 ± 2.35	2.28 ± 0.97	0.17
Peziza	3.27 ± 1.33	2.77 ± 1.58	0.92
Byssochlamys	3.12 ± 1.46	2.7 ± 1.72	0.75
unclassified_Cystofilobasidiales	2.61 ± 0.85	3.05 ± 2.04	0.46
Thermomyces	3.23 ± 1.34	2.20 ± 1.93	0.25
unclassified_Ascomycota	1.49 ± 0.54	3.49 ± 3.50	0.17
Piromyces	3.99 ± 1.92	0.94 ± 0.55	0.009
Leohumicola	2.70 ± 1.26	1.71 ± 1.18	0.17
Schizothecium	2.04 ± 0.53	2.14 ± 1.28	0.60
unclassified_Basidiomycota	1.69 ± 0.73	1.91 ± 0.35	0.46
Mortierella	1.97 ± 1.03	1.50 ± 0.71	0.35
unclassified_Didymosphaeriaceae	1.71 ± 0.53	1.71 ± 1.30	0.92

[1]CON = Control group.

[2]CORD= the group supplemented with Cordycepin.

[3]Means are declared significantly different when $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

Table 6 KEGG pathway enrichment analysis identified the significantly different metabolites between control group (CON) and the group with addition of 0.64 g L^{-1} cordycepin (CORD)

ID	Description	Metabolite Ratio[1]	Bg Ratio[2]	Enrich Factor[3]	*P*-value[4]
ko01040	Biosynthesis of unsaturated fatty acids	3.66%	1.21%	3.02	0.001
ko00230	Purine metabolism	9.15%	5.05%	1.81	0.004
ko00591	Linoleic acid metabolism	3.66%	1.41%	2.59	0.006

[1]Metabolite Ratio = the number of differential metabolites associated with this pathway / the number of differential metabolites annotated by KEGG.

[2]Bg Ratio = the ratio of the number of all metabolites associated with this pathway to the number of all metabolites annotated by KEGG.

[3]Enrich factor = Metabolite Ratio/Bg Ratio.

[4]Means are declared significantly different when $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

Table 7 Identification of significantly different metabolites in rumen fluid *in vitro* between control group (CON) and the group with addition of 0.64 g L^{-1} cordycepin (CORD)

KEGG pathway ID	metabolite ID	metabolite name	FC	*P*-value[1]	VIP	CON vs. CORD[2]
ko01040	neg_3629	(13Z,16Z)-Docosadienoic acid	1.3168	< 0.001	1.5650	up
	pos_2794	Dihomo-gamma-linolenate	1.5118	0.004	1.4317	up
	pos_2837	Arachidonate	1.5824	0.044	1.1551	up
	pos_3034	(6Z,9Z,12Z)-Octadecatrienoic acid	1.4179	0.008	1.3455	up
	pos_3126	Arachidonic acid	1.2149	0.042	1.0796	up
	pos_3668	(5Z,8Z,11Z,14Z,17Z)-Icosapentaenoic acid	0.7736	< 0.001	1.5934	down
ko00230	neg_1570	Allantoic acid	1.7192	0.018	1.2843	up
	neg_251	Adenine	1.7223	0.003	1.5434	up
	neg_2530	Xanthine	0.7731	0.048	1.0628	down
	neg_264	Uric acid	2.0718	< 0.001	1.7395	up
	neg_295	Adenosine 3′-monophosphate	3.0318	< 0.001	1.7960	up
	neg_383	Phosphoribosylformylglycineamidine	0.1355	< 0.001	1.8221	down
	neg_4055	GDP[5]	0.1687	0.004	1.5303	down
	neg_4110	Guanosine diphosphate	0.0611	0.049	1.1722	down
	neg_486	2′-Deoxyadenosine	0.1439	< 0.001	1.7038	down
	pos_2751	Xanthosine	1.1116	0.030	1.1719	up
	pos_338	dGMP[6]	7.2924	0.002	1.6376	up
	pos_339	2′-Deoxyguanosine-5′-monophosphate	2.5262	< 0.001	1.6941	up
	pos_417	2′-Deoxyguanosine	0.6094	< 0.001	1.7567	down
	pos_521	Deoxyinosine	409.3818	< 0.001	1.8228	up
	pos_530	Hypoxanthine	370.8304	< 0.001	1.8221	up
ko00591	neg_2645	Pinellic acid	1.1184	0.030	1.1476	up
	pos_2577	9-OxoODE[7]	1.6773	0.002	1.4914	up
	pos_2794	Dihomo-gamma-linolenate	1.5118	0.004	1.4317	up
	pos_2837	Arachidonate	1.5824	0.044	1.1551	up
	pos_3034	(6Z,9Z,12Z)-Octadecatrienoic acid	1.4179	0.008	1.3455	up
	pos_3126	Arachidonic acid	1.2149	0.042	1.0796	up

dGMP, deoxyguanosine monophosphate; FC, fold change; GDP, guanosine-5′-diphosphate; 9-OxoODE, (10E,12Z)-9-oxooctadeca-10,12-dienoic acid; VIP, variable importance projection.

[1]Means are declared significantly different when $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

[2]CON = Control group; CORD = the group supplemented with Cordycepin; up = up-regulated; down =down-regulated.