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Author	Gyeongjin Kim1#, Miyoung Won3#, Tabita Dameria Marbun1, Yoogyung Lee4, Jaeyong Song5, Seongho Choi6, Eun Joong Kim1,2* # These authors contributed equally to this work.
Affiliation	1 Department of Animal Science and Biotechnology, Kyungpook National University, Sangju 37224, Korea 2 Research Institute for Innovative Animal Science, Kyungpook National University, Sangju, 37224, Korea 3 Subtropical Livestock Research Center, National Institute of Animal Science, Rural Development Administration, Jeju, 63242, Korea 4 Smart Livestock Environment Division, National Institute of Animal Science, RDA, Wanju, 55365, Korea 5 Livestock Research Institute, Nonghyup Agribusiness Group Inc., Anseong, 17558, Korea 6 Department of Animal Science, Chungbuk National University, Cheongju, 28644, Korea
ORCID (for more information, please visit https://orcid.org)	Gyeongjin Kim (https://orcid.org/0000-0003-2202-126X) Miyoung Won (https://orcid.org/0000-0001-7127-5034) Tabita Dameria Marbun (https://orcid.org/0000-0002-3360-9715) Yookyung Lee (https://orcid.org/ 0000-0002-9896-4152) Jaeyong Song (https://orcid.org/0000-0002-8613-5605) Seongho Choi (https://orcid.org/0000-0001-8869-0218) Eun Joong Kim (https://orcid.org/0000-0002-5962-6994)
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CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Eun Joong Kim
Email address – this is where your proofs will be sent	ejkim2011@knu.ac.kr
Secondary Email address	
Address	2559 Gyeongsang-daero, Sangju-si, Gyeongsangbuk-do, 37224, South Korea
Cell phone number	+82-10-2550-3706
Office phone number	054-530-1228
Fax number	054-530-1229

Abstract

Methane emissions from enteric fermentation in ruminants contribute substantially to greenhouse gas emissions from the agricultural sector. Therefore, effective strategies to mitigate enteric methane production are required. This study aimed to investigate the effects of kaolinite-based clay mineral (KCM) supplementation on methane emissions, rumen fermentation, and the rumen microbiome in goats. Eight Boer goats (39.7 ± 2.81 kg) were used in a 2 × 2 crossover design. The goats were fed a total mixed ration at 2% of body weight (dry matter basis), either without CM (CON) or with 1% KCM supplementation (TRT). Goats were adapted to the diets for 10 days. Each goat was housed in an open-circuit respiration chamber to measure methane emissions, dry matter intake (DMI), total-tract apparent digestibility, and rumen microbial composition. After methane measurements, rumen fluid samples were collected via stomach tube for fermentation analysis. Methane emissions (g/day) did not differ between CON (20.37 g/day) and TRT (21.29 g/day). The methane yield per unit of DMI in CON and TRT was 27.20 and 29.70 g/kg DMI, respectively, and did not differ between KCM and control. Except for a significant reduction in ruminal ammonia-N concentration with KCM supplementation (CON 19.58 mg/100 ml, TRT 16.21 mg/100 ml), rumen pH, volatile fatty acid profiles, and total-tract apparent digestibility were not altered. KCM supplementation induced minor shifts in rumen microbial community composition but did not significantly alter microbial diversity or rumen fermentation, owing to functional redundancy within the rumen microbiome. In conclusion, KCM supplementation at 1% of the diet did not reduce enteric methane emissions in goats, despite minor alterations in ammonia-N concentration and microbial composition. These results suggest that additionalinclusion levels, longer adaptation periods, or combined feed additive strategies may be required to achieve methane-mitigating effects in goats.

Keywords: Kaolinite-based clay mineral; Methane mitigation; Goat; Rumen fermentation; Microbiome

INTRODUCTION

Methane emissions from agriculture contribute substantially to global greenhouse gas emissions, accounting for approximately 2,800 Mt CO_2 equivalent, with enteric fermentation by ruminants accounting for 47–48% of these emissions [8]. Enteric methane is produced from feed digested by ruminants, particularly carbohydrates such as fiber, resulting in a loss of

approximately 2–12% of feed energy during animal production [21]. Because the global warming potential of methane is 28 times that of CO_2 over a 100-year horizon, mitigating enteric methane emissions from ruminants is critical to improve animal productivity. Various nutritional strategies have been proposed to reduce enteric methane emissions, including manipulating the forage-to-concentrate ratio, lipid supplementation, improving forage quality, and using feed additives such as 3-nitrooxypropanol (3-NOP), algae, saponins, and electron sinks (e.g., nitrate and fumarate) [4]. In particular, increasing attention has been given to eco-friendly and safe feed additives to mitigate enteric methane emissions [4].

Clay minerals (CMs), including montmorillonite, illite, and kaolinite, originate from naturally occurring rock or soil materials [30] and are aluminosilicates characterized by a phyllosilicate structure composed of tetrahedral layers of silicon and oxygen and octahedral layers of aluminum, oxygen, and hydroxyl groups [9]. CMs are intrinsically negatively charged, thereby attracting cations, resulting in high cation-exchange capacity (CEC), pH-buffering capacity [18], and adsorption capacity [13]. CMs can alleviate symptoms such as diarrhea and gastroenteritis [13] caused by mycotoxins—secondary metabolites produced by fungi in feed—thereby enhancing animal productivity [30]. The safety of CMs as feed additives in livestock has been proven [11]. Recently, Hosen et al. [16] reported that the affinity of CMs for methane is due to the aforementioned physicochemical properties, suggesting their potential as feed additives for enteric methane mitigation.

Despite this potential, studies evaluating the effects of CMs on methane emissions are scarce and limited to cattle [5], while studies involving goats have focused on toxin-binding effects [13] rather than methane production. Biswas et al. [5] observed a reduction in methane production when 1% CM was added in an *in vitro* ruminal experiment, which was attributed to alterations in rumen fermentation; however, no reduction in enteric methane emissions was observed *in vivo* in Hanwoo cattle when the same amount of CM was used. In a study by El-Nile et al. [12], supplementation with zeolite and nano-zeolite—materials similar to CMs—at 2% and 0.4%, respectively, resulted in methane mitigation *in vitro*. However, because only *in vivo* rumen fermentation characteristics have been evaluated in goats, the effect of CM supplementation on enteric methane emissions remains unclear. Overall, evidence regarding the methane mitigation potential of CMs in goats remains insufficient.

Thus, this study investigated the effects of kaolinite-based CM (KCM) supplementation on enteric methane emissions, rumen fermentation, and the rumen microbiome in male goats. This

study hypothesized that dietary inclusion of KCM could reduce methane emissions by altering rumen microbial composition and fermentation parameters associated with methane production.

MATERIALS AND METHODS

The experimental procedures of this study were approved by and conducted under the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University (Approved No. 2020-0061).

Experimental animals and diets

Eight castrated 1-year-old Boer male goats with an initial body weight (BW) of 39.7 ± 2.81 kg were used in a 2 × 2 crossover design. The goats were assigned to one of two dietary treatments—control (CON) or treatment (TRT)—based on BW. The CON group was fed a total mixed ration (TMR) without kaolinite-based CM supplementation, whereas the TRT group was fed a TMR supplemented with 1% kaolinite-based CM on a fed basis. Kaolinite-based CM consists of SIO_2 (73.0-74.0%), Al_2O_3 (13.8-14.9%), Fe_2O_3 (3.0-4.05%), CaO (2.34-3.14%), MgO (0.94-1.05%), K_2O (1.64-1.80%), Na_2O (4.04-5.00%), which have over 30 meq/100 g of CEC. The KCM was mixed with flour and sugar to enhance palatability. The ingredient and chemical composition of the TMR are presented in Table 1.

Goats were housed in individual pens and fed at 2% of BW on a dry matter (DM) basis. Animals were fed twice daily at 0800 and 1600 h in equal portions. Goats were adapted to their respective diets (CON or TRT) outside the respiratory chambers. Because 4 chambers were used simultaneously, this experiment consisted of 2 periods and 4 batches (2 batches per period). A total of 8 goats were used, with each goat assigned to a single batch per period. Four goats (two CON and two TRT) were moved to four individual simplified respiration chambers and adapted for an additional 3 days per 1 batch, resulting in a total adaptation period of 10 days. After feed and chamber adaptation, background methane concentration (ppm) was recorded for 1 day, after which methane emissions were measured for each goat over 24 h. Feces were collected during the last 3 days. Rumen fluid and blood samples were collected on the last day after methane measurement, 3 h post-feeding in the morning. TMR residual was recorded daily to determine dry matter intake (DMI). Each experimental period consisted of 10 days of adaptation and 6 days

of measurement, resulting in a total of 16 days per period. After the first period, goats were switched between the CON and TRT diets.

Methane measurement

Enteric methane emissions were measured using four simplified respiration chambers constructed from transparent acrylic panels, allowing visual contact among the goats. The dimensions of each chamber were 100 × 150 × 150 cm (width, depth, and height, respectively), with an internal volume of 2.25 m^3. The goats were housed in the chambers with free access to feed and drinking water. Before gas exchange measurements, a known concentration of methane (3000 μmol/mol in N_2; Air Korea Inc., Korea) was injected to evaluate methane recovery. The average methane recovery rate across the four chambers was 99.1 ± 6.89% (Fig. 1). Goats were housed individually in simplified respiration chambers for methane emission measurements over a 24-h period (1 day per goat). Methane concentration in the outlet gas was analyzed at 1-min intervals using a tunable diode absorption spectroscopy gas analyzer (Airwell+7, KINSCO Technology, Korea). Chamber temperature and humidity were recorded daily, and airflow rate was measured twice daily using a flow meter (VT210, KIMO Instruments, Korea). Daily methane emission (g/day) was calculated as described by Pinares and Waghorn [32]. Methane data were eliminated when chambers were opened for cleaning before morning feed, feeding of diets, or fecal collection.

Apparent nutrient digestibility

Fifty grams of feces among the total were collected from each goat twice daily before feeding during the last 3 consecutive days of methane measurement. Fecal samples were not pooled by day and were dried at 105℃ for 24 h for lignin analysis as an internal marker [41]. Hair was removed manually prior to grinding. Fecal samples were ground using a Cyclotec™ grinder (CT 293, FOSS, Denmark) equipped with a 2-mm screen. Dry matter digestibility (DMD) and apparent nutrient digestibility, including organic matter digestibility (OMD), crude protein digestibility (CPD), ether extract digestibility (EED), neutral detergent digestibility (NDFD), and acid detergent digestibility (ADFD), were calculated as follows:

$$\text{DMD (\%)} = 100 - [(\text{lignin concentrations in diets} / \text{lignin concentrations in feces})] \times 100$$

Apparent nutrient digestibility (%) = 100 − [(lignin concentrations of diets × nutrient concentration of feces) / (lignin concentrations of feces × nutrient concentration of diets)] × 100

Collection of blood and rumen fluid

Blood and rumen fluid samples were collected on the final day after methane measurement, 3 h post-feeding in the morning. Prior to rumen fluid collection, blood was collected first from the jugular vein into EDTA vacutainers (Becton Dickinson, USA). Blood urea nitrogen (BUN), alanine transaminase (ALT), aspartate aminotransferase (AST), glucose (GLU), and total protein (TP) concentrations were immediately analyzed using the VS2 analyzer (Zoetis, USA) with a preventive care profile plus rotor. Rumen fluid was collected via stomach tube 3 h after morning feeding, and the first aliquot was discarded to avoid contamination with saliva. The pH of the rumen fluid was measured using a portable pH meter (Starter 300, Ohaus, USA). Subsamples (10 ml) were immediately separated into 15-ml conical tubes and frozen in liquid nitrogen for analysis of the rumen microbiome. For volatile fatty acid (VFA) analysis, 1 ml of rumen fluid was mixed with 0.2 ml of 25% metaphosphoric acid. For ammonia-N, 0.5 ml of rumen fluid was mixed with 0.5 ml of 0.2 N hydrochloric acid to prevent volatilization. All samples were immediately frozen in liquid nitrogen and stored at −20℃ until analysis.

Chemical analysis

Feed samples were collected weekly and dried at 60℃ for 72 h. Feed residuals and fecal samples were dried at 105℃ for 24 h. Dried samples were ground through a 2-mm sieve using a Cyclotec^{TM} grinder (CT 293, FOSS, Denmark). Samples were analyzed for dry matter (method 934.01), organic matter (method 942.05), crude protein (method 2001.11), ether extract (method 920.39A), neutral detergent fiber (method 2002.04; using alpha-amylase and sodium sulfite, including residual ash), and acid detergent fiber (method 973.18) following AOAC [2]. Lignin was analyzed according to Van Soest et al. [41]. DMD was calculated using lignin as an internal marker as described above.

VFAs were analyzed as described by Li et al. [24]. Rumen fluid samples containing 25% metaphosphoric acid were centrifuged at 9,425 ×g for 10 min. Pivalic acid solution (2 g/100 ml), 0.1 ml was added as an internal standard. VFA samples were analyzed by gas chromatography (Hewlett Packard 5890 series II GC) equipped with a silica capillary column (Nukol, Supelco, USA). The oven, injector, and detector temperatures were set to 120℃, 170℃, and 200℃, respectively.

Ammonia-N concentration was determined using the method of Chaney and Marbach [7]. Following centrifugation at 9,425 ×g for 10 min, 20 µL of supernatant was mixed with 1 ml each of phenol color and alkali hypochlorite reagent. After incubation at room temperature for 30 min, absorbance was measured using a spectrophotometer (Optizen pop, Mecasys, Korea) at 630 nm wavelength.

DNA extraction and 16S rRNA library generation

From the rumen fluid samples, total genomic DNA was extracted using the DNeasy PowerMax Soil Kit (Qiagen, USA) following the manufacturer's instructions. The V3–V4 region of the 16S rRNA gene was amplified using primers Bakt_341F/805R, and sequencing libraries were prepared using Herculase II Fusion DNA Polymerase and the Nextera XT Index V2 Kit at Macrogen (Seoul, South Korea). The prepared libraries were analyzed on an Illumina platform in paired-end mode. Raw sequence reads were processed using the DADA2 pipeline (v1.38) in R (v4.5.2) [6, 27]. The workflow included quality profiling, error-rate learning, denoising, paired-end read merging, chimera removal, and construction of an amplicon sequence variant (ASV) table. Taxonomic classification was performed using the SIVA v138.1 reference database [33]. Alpha diversity indices, including observed ASVs, Chao1, Shannon, and Simpson, were calculated using the phyloseq package. Beta diversity was assessed using principal coordinate analysis based on Euclidean distances, and group-level differences were tested using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations [1]. Differentially abundant genera were explored using the linear discriminant analysis (LDA)-based differential abundance approach after Kruskal-Wallis screening [35].

Statistical analysis

No significant period or carryover effects were observed when data were analyzed using a linear mixed-effects model with treatment, period, and sequence as fixed effects and animal as a random effect. Therefore, all data were analyzed using paired *t*-tests in SPSS (version 25, IBM, USA). Individual goats served as the experimental unit. Differences were considered significant at $p < 0.05$, and *p*-values between 0.05 and 0.10 were considered to represent a tendency.

RESULTS AND DISCUSSION

This study investigated the effects of kaolinite-based CM supplementation on enteric methane emissions, rumen fermentation, and the rumen microbiome in Boer goats.

Dry matter intake and methane mitigation effect

DMI was 751.8 g/d and 716.8 g/d in the CON and TRT groups, respectively, with no significant difference between groups. Supplementation with 1% KCM did not affect DMI, which is consistent with previous studies reporting no effect when KCM was added at 1% [5] or 1% and 2% of DM [12]. These results indicate that the KCM inclusion level in this study was insufficient to affect DMI.

Methane emissions were 20.37 g/d and 21.29 g/d in the CON and TRT groups, respectively, with no significant difference between treatments. In addition, methane emissions per DMI did not differ between CON and TRT (Table 2). Contrary to our hypothesis that KCM supplementation could reduce enteric methane emissions in goats, KCM did not affect methane emissions, consistent with the findings of Biswas et al. [5].

The mechanism by which CM reduces CH_4 production in the rumen is associated with properties, such as CEC and adsorption. CM adsorbs cations, such as H^+ or NH_4^+ of ammonia-N. Hosen et al. [16] reported that adsorption of CMs is associated with interactions with rumen microorganisms, potentially inhibiting protozoa and methanogenic archaea. Such changes in microbial composition shift the VFA production pathway toward propionate [12]. It reduces the hydrogen available to methanogens during propionate production, thereby reducing methane emissions [5, 12]. While this mechanism has been reported in previous studies conducted on bentonite, zeolite, etc., kaolinite-based CM requires further investigation.

Nevertheless, KCM did not reduce enteric methane in this study. This may be attributed to the level of KCM supplementation used in this study or its physicochemical properties. Kaolin-based CM differs structurally from bentonite or montmorillonite. Kaolin-based CM is a 1:1 phyllosilicate that forms strong bonds between the tetrahedral and octahedral layers, resulting in exposure sites that occur only on the external surface. In the 2:1 structure, an alumina octahedral layer is sandwiched between two silica tetrahedral layers, resulting in weaker interlayer bonding and exposing sites on both the external surface and the interlayer space [9]. Due to these structural differences, the 1:1 structure has lower CEC and adsorption capacity compared to the 2:1 structure [9]. According to prior research, these surface characteristics are reported to play a key role in CH_4 reduction through H^+ adsorption via negative surface charge and direct clay-

microbial interactions within the rumen [16]. The CEC of the kaolinite-based CM used in this study was 30 meq/100 g, which is significantly lower than that of montmorillonite (77.5 meq/100 g) [38]. It is plausible that the limitations of these complex surface characteristics are the primary reason for the insufficient methane reduction effect. The 1% addition level was determined based on preliminary *in vitro* results showing that methane reduction was greater with 1% KCM than with the control (unpublished). However, the lack of a significant methane-reduction effect in this *in vivo* study suggests that the effect observed under *in vitro* conditions may have been decreased by a combination of the structural limitations of kaolinite-based CM [9] and the complex rumen environment [15]. For example, *in vitro* rumen fermentation is a closed system that prevents ruminal outflow and maintains a feed additive concentration throughout the experiment. In contrast, *in vivo* conditions with live animals result in a continuous flow, leading to the feed additive being lost from the rumen via outflow [26]. In addition, because the volume of rumen contents is much larger than in the *in vitro* condition, the methane-reduction effect is relatively lower than in vitro, suggesting the methane-reduction effect in animals may tend to decrease with the same supplement level [17]. Meanwhile, goats have a faster passage rate than sheep and cattle due to their physiological properties, including chewing efficiency and gut morphology. Tsiplakou et al. [40] reported that when fed the same feed, the passage rate of goats (5.4-6.9%/h) was faster than that of sheep (2.9-3.3%/h), suggesting that the retention time of additives in the rumen of goats may be shorter than that of other ruminants. Therefore, even when the same additive concentration is applied, the effective CM concentration in goats is likely to decrease more rapidly than in other ruminants, which may explain the lower *in vivo* methane reduction effect compared to *in vitro*. Previous studies have reported that although CM supplementation significantly reduced methane production *in vitro* [3, 5, 12], no significant reduction was observed *in vivo* [5]. Further study is needed to determine the dose-response and long-term effects.

Apparent nutrient digestibility

No significant differences in DMD or OMD were observed with KCM supplementation. KCM supplementation also did not affect apparent nutrient digestibility (CPD, NDFD, and ADFD, Table 3). These findings are consistent with El-Nile et al. [12], who reported improved nutrient digestibility in an *in vitro* rumen fermentation experiment but no effect *in vivo*. El-Nile et al. [12] inferred that unaffected nutrient digestibility when supplemented with 0.4% nano zeolite or 2% natural zeolite resulted from more efficient zeolite activity in the rumen than in the post-ruminal

digestive tract. However, the effects of CMs on apparent nutrient digestibility have been inconsistent across studies. Some of the impacts of CM on nutrient digestibility have been explained. The large surface area of CMs enhances nutrient adsorption, thereby improving digestibility and slowing passage through the digestive tract [13]. CMs can bind dietary toxins, preventing their inhibitory effects on animal performance and thereby enhancing nutrient utilization [9]. Gouda et al. [13] observed enhanced nutrient digestibility when goats were fed diets containing 2% CM. In contrast, Ortiz et al. [30] observed a decrease in DMD as CM supplementation increased from 0% to 2%, which was attributed to increased silicon content, an indigestible component.

Blood parameters

BUN, ALT, AST, GLU, and TP did not differ between CON and TRT (Table 4). BUN concentrations were 17.25 mg/dL and 17.71 mg/dL in CON and TRT, respectively, with no significant difference. BUN is an indicator of protein intake in ruminants, and low BUN levels may indicate low protein intake or kidney disease [28]. ALT and AST are indicators of liver function, with elevated levels indicating liver damage [25]. No significant differences were observed in ALT (10.75 μ/L vs. 15.00 μ/L) and AST (74.75 μ/L vs. 70.67 μ/L) between CON and TRT, respectively. Furthermore, no differences were observed in GLU and TP. All measured blood parameters (BUN, ALT, AST, GLU, and TP) were within the ranges reported in previous studies involving goats [13, 22, 28]. These results indicate that KCM supplementation did not induce adverse effects on kidney or liver function. Blood glucose also remained unchanged, as neither organic matter digestibility nor propionate concentration, a precursor of glucose synthesis, was affected by KCM supplementation [13].

Rumen fermentation parameters

No difference in pH was observed between CON and TRT supplemented with KCMs, consistent with the findings of Biswas et al. [5], who evaluated the effect of CMs on methane mitigation and rumen fermentation in Hanwoo steers. In this study, ruminal pH values were 6.33 and 6.45 for CON and TRT, respectively (Table 5). CMs are known to act as buffering agents in the rumen by adsorbing hydrogen ions, which may contribute to pH stabilization [12]. Although ruminal pH sensitivity depends on the frequency and duration of ruminal pH variation, none of the measured values in this study were below 6.25 [10]. This result suggests that silicate supplementation does not adversely affect ruminal pH in goats.

Ammonia-N concentrations were 19.58 mg/100 ml and 16.21 mg/100 ml in CON and TRT, respectively, with TRT showing a significant reduction compared with CON ($p<0.05$; Table 5). KCMs act as cation exchangers and can adsorb ammonia-N in the rumen. Thus, the lower ammonia-N concentration observed in TRT likely reflects the CEC of the supplemented KCM. Gouda et al. [13] reported reduced ruminal ammonia-N concentrations in goats fed bentonite, accompanied by increased total ruminal N content. The author attributed these to the enhanced microbial protein synthesis resulting from enhanced CP digestibility. In this study, however, protein digestibility did not differ between CON and TRT, despite the reduction in ruminal ammonia-N concentration. Additional research on microbial protein synthesis will be required for accurate interpretation.

Total VFAs, acetate, and propionate did not differ between CON and TRT (Table 5). Except for total VFA, these results are consistent with those of Biswas et al. [5] and El-Nile et al. [12] in Hanwoo steers and goats, respectively. In contrast to the results of this study, several studies have reported increased total VFA concentrations following CM supplementation [5, 12, 13], which has been attributed to improved OMD. Because total VFA depends on nutrient digestibility, mainly on the digested organic matter [37], the absence of differences in OMD between CON and TRT in this study may explain the lack of differences in total VFA and, consequently, methane emission. Additionally, substantial inter-individual variation might have masked treatment effects. In this experiment, rumen fluid was collected via stomach tubing, which, although widely accepted for collecting rumen fluid, may result in saliva contamination, to some extent, and underestimation of total VFA concentration if samples are not fully representative of whole-rumen content [34].

Microbial species richness and diversity

The α-diversity index, including the observed ASVs Chao1, Shannon, and Simpson, did not show a significant difference between CON and TRT groups (Fig. 2). Phylogenetic diversity was not further interpreted in this analysis. After paired-read merging and chimera removal, a total of 1,809 ASVs remained in rumen samples. Among these, 1,793 ASVs were categorized into the phylum level and 1,508 ASVs at the genus level. The ASVs were classified into 25 phyla, including 23 bacterial and 2 archaeal phyla (Fig. 3). Although the ASVs identified in our study were comparable to those reported in the literature [31, 44], a sampling procedure for rumen contents may partially explain some of the variation among studies. Rumen fluid collection was performed using a stomach-tube method in this study, which could have led to contamination

with saliva and a risk of variation in sampling sites. Hagey et al. [14] demonstrated that stomach-tube samples were most representative of the "liquid phase". In addition, stomach tube samples had significantly lower abundances of certain bacteria than the cannulated grab sample, suggesting that ASVs could vary depending on the sampling method used for rumen fluids.

At the phylum level, the rumen microbial community consisted mainly of *Bacteroidota*, *Proteobacteria*, *Firmicutes*, and *Actinobacteriota*. *Bacteroidota* and *Proteobacteria* accounted for the largest proportion, with average relative abundances of 42.3% and 40.9%, respectively. *Firmicutes* and *Actinobacteriota* were detected as major phyla, while archaea, including *Crenarchaeota* and *Euryarchaeota*, were present with relatively low abundance. At the genus level, *Chryseobacterium*, *Pseudomonas*, *Acinetobacter*, and *Enterococcus* were the predominant (Fig. 4). Euclidean PCoA-based β-diversity analysis explained 60.6% and 12.7% of the variation along the first and second PCoA axes, respectively (Fig. 5). However, PERMANOVA result showed no significant difference in microbial community composition between CON and TRT (R^2 = 0.0757, p = 0.267). These results suggest that supplementation with 1% kaolinite-based CM did not significantly alter rumen microbial diversity.

Despite the lack of significant changes in α-diversity, we identified several genera that were differentially abundant between groups; specifically, *Acinetobacter* was more abundant in CON, while *Corynebacterium*, *Ochrobactrum*, *Sphingomonas*, and *Brevundimonas* showed higher abundance in TRT (Kruskal–Wallis test, p<0.05) (Fig. 6). However, none of these genera exceeded an LDA score of 2.0, indicating weak discriminatory effects. Therefore, these taxa should be regarded as suggestive compositional indicators rather than robust treatment-responsive biomarkers.

CMs, including montmorillonite, illite, and kaolinite, possess negatively charged surfaces and cation exchange capabilities, which can contribute to the adsorption of ammonium ions in the rumen [9, 13]. In this study, while KCM supplementation significantly reduced ammonia-N concentrations, this reduction did not coincide with significant changes in microbial diversity, total VFA concentration, VFA ratio, or methane emissions. Consequently, the observed reduction in ammonia-N concentration appears to be primarily driven by the physicochemical adsorption properties of KCMs rather than by extensive shifts in the rumen microbial community [29, 42].

Hydrogen generated during rumen microbial fermentation can be used for VFA synthesis or converted into methane by methanogenic archaea [20, 36, 43]. However, the low archaeal abundance, coupled with the lack of significant shifts in VFA profiles and methane emissions,

suggests that 1% CM supplementation resulted in minimal effect on hydrogen flow and fermentation pathways. This functional redundancy potentially buffered the rumen fermentation process against minor taxonomic shifts, thereby maintaining stable yields [15, 23, 39].In this study, the addition of kaolinite-based CM at a 1% level to TMR did not significantly reduce enteric methane emissions from Boer goats. Due to its 1:1 layered structure, kaolinite has a lower CEC and specific surface area than CMs with 2:1 layered structure, such as bentonite and zeolite [9]. Thus, the KCM structure is considered the primary reason for the lack of methane reduction. Furthermore, the 1% addition level used in this study may not have been sufficient to alter hydrogen availability or rumen fermentation pathways. Although ammonia-N decreased with KCM addition, VFA profiles, apparent digestibility, and the rumen microbiome were not altered. These results suggest that the supplemental kaolinite-based CM level is insufficient to reduce methane [12]. To elicit more pronounced effects in vivo, higher dosages, extended adaptation periods, or synergistic use with other methane-reducing additives may be warranted.

Conclusion

In conclusion, CH_4 emission was not reduced by kaolinite-based CM supplementation at 1% in the TMR of Boer goats. There were no significant changes in VFA profiles, apparent digestibility, and microbiome. Although supplementation with KCM resulted in selective changes in certain microbial genera and a lower Ammonia-N concentration, methane did not differ, suggesting that the 1% of KCM may not have been sufficient to reduce methane by altering rumen hydrogen availability and fermentation pathways. Although there was no significant difference in this experiment, numerical reductions were observed with KCM, suggesting caution is required when adding more than 1% KCM, as 1% KCM may reduce dry matter intake due to palatability. Further study is needed to evaluate optimal kaolinite-based CM addition levels and the potential to reduce enteric methane using a larger number of animals and an experimental design with a long adaptation period.

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396

397

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538

539 **Table 1. Ingredient and chemical composition of the experimental diet**

Items	%
Ingredient (%)	
Meshed corn	20.0
Corn grain F	13.0
Bran A	21.0
TMR base	5.0
Byproduct A	5.0
Byproduct B	6.1
Soybean hull pellet	2.0
Molasses	3.0
Water	3.0
Limestone	2.8
Salt	0.8
Buffer	0.8
Vitamin/mineral mix	0.1
Probiotics	0.6
Tall fescue hay	7.0
Alfalfa hay	2.0
Annual ryegrass	3.8
Oat hay	4.0
Chemical composition, % of DM	
Dry matter (as-fed)	86.37
Organic matter	90.28
Crude protein	15.53
Ether extract	3.45
Crude fiber	10.76
Neutral detergent fiber	30.44
Acid detergent fiber	14.98
Calcium	1.31
Phosphorus	0.50
Gross energy (kcal/g)	4.063

540 Byproducts A and B are unknown owing to company confidentiality
541

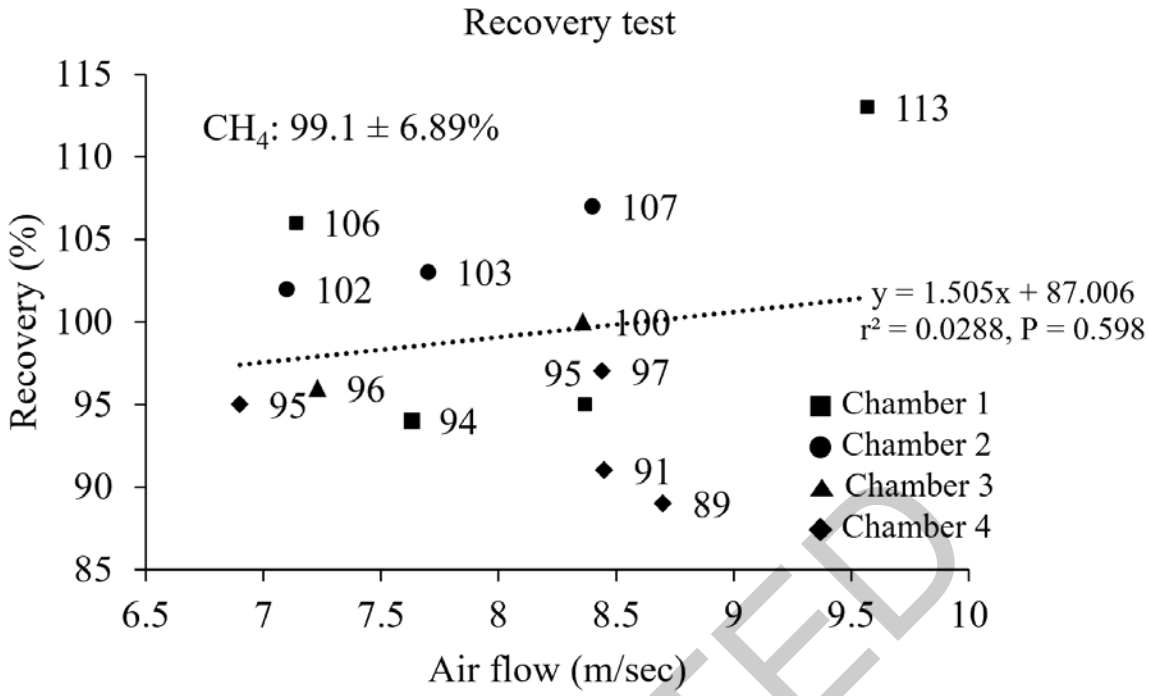

Fig. 1. Methane recovery ratio (%) of the four respiratory chambers

Table 2. Effects of kaolinite-based clay mineral supplementation on dry matter intake and methane emissions in Boer goats

Items	CON	TRT	SEM	*p*-value
DMI (g/d)	751.8	716.8	20.37	0.129
CH_4 (g/d)	20.37	21.29	1.543	0.568
CH_4/DMI (g/kg)	27.20	29.70	1.782	0.204

DMI: Dry matter intake

CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals

SEM: Standard error of the mean

Table 3. Effects of kaolinite-based clay mineral supplementation on nutrient digestibility in Boer goats

Items (% of DM)	CON	TRT	SEM	*p*-value
DMD	69.01	67.68	1.496	0.548
CPD	67.45	66.84	1.293	0.820
OMD	73.34	72.17	1.819	0.540
NDFD	45.76	41.68	2.452	0.277
ADFD	40.49	34.89	2.802	0.200

DMD: Dry matter digestibility, CPD: Crude protein digestibility, OMD: Organic matter digestibility, NDFD: Neutral detergent fiber digestibility, ADFD: Acid detergent fiber digestibility

CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals

SEM: Standard error of the mean

Table 4. Effects of kaolinite-based clay mineral supplementation on blood parameters in Boer goats

Items	CON	TRT	SEM	*p*-value
BUN (mg/dL)	17.25	17.71	0.522	0.604
ALT (μ/L)	10.75	15.00	3.137	0.281
AST (μ/L)	74.75	70.67	4.293	0.210
GLU (mg/dL)	74.13	72.43	3.262	0.802
TP (g/dL)	7.15	7.28	0.140	0.910

BUN: Blood urea nitrogen, ALT: Alanine transaminase, AST: Aspartate aminotransferase, GLU: Glucose, TP: Total protein

CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals

SEM: Standard error of the mean

Table 5. Effects of kaolinite-based clay mineral supplementation on rumen fermentation in goats

Items	CON	TRT	SEM	*p*-value
pH	6.33	6.45	0.103	0.307
Ammonia-N (mg/100 ml)	19.58	16.21	0.921	0.008
Total VFA (mg/ml)	51.96	50.44	7.337	0.842
Acetate (% of total VFA)	66.81	64.70	1.566	0.219
Propionate (% of total VFA)	19.11	18.76	1.580	0.829
Butyrate (% of total VFA)	10.94	12.74	1.043	0.127
Acetate/Propionate ratio	3.59	3.51	0.352	0.842

VFA: volatile fatty acid

CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals

SEM: Standard error of the mean,

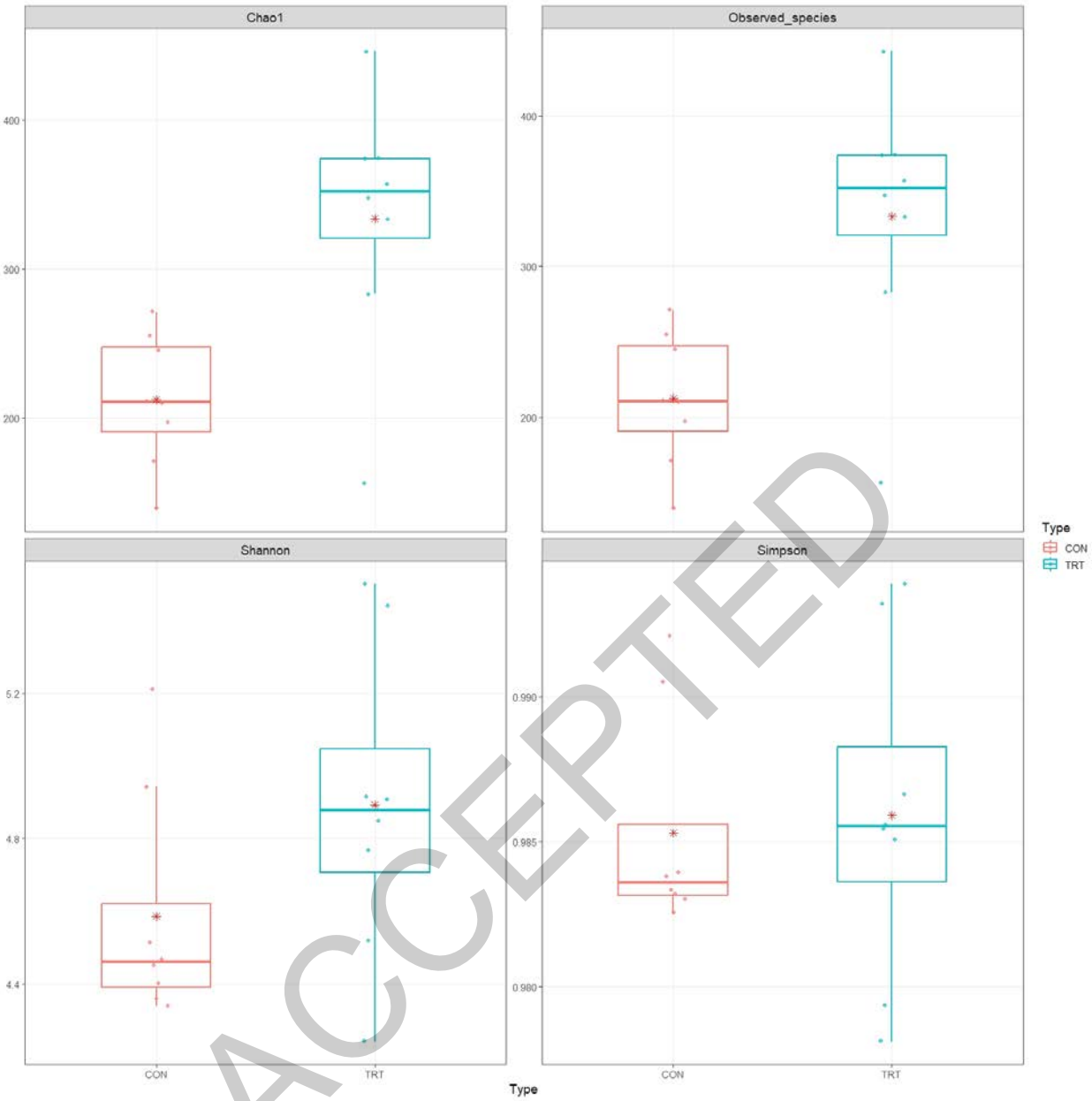

Fig. 2. Alpha diversity plots of CON and TRT. Rumen fluid samples were collected 3 h post-feeding. Panel shows (A) Chao1, (B) observed species, (C) Shannon, (D) Simpson (CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals)

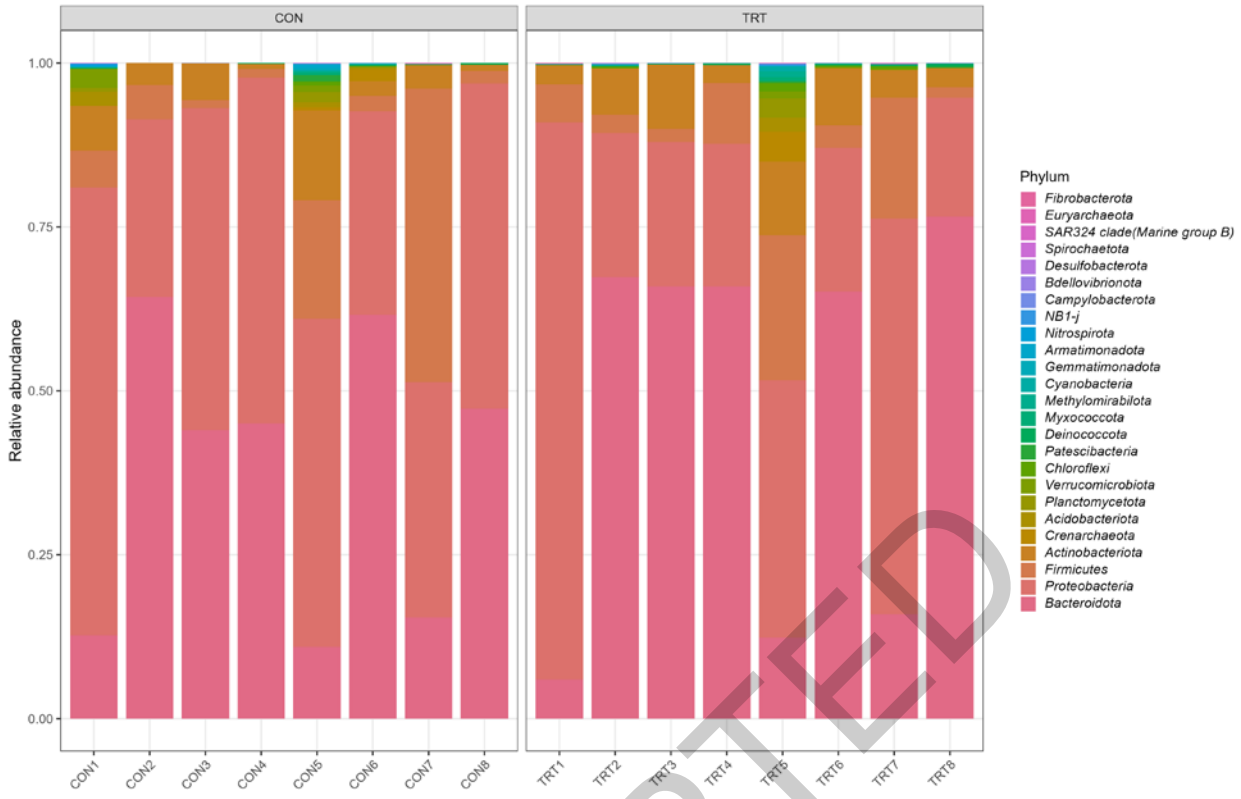

Fig. 3. Species diversity of rumen fluid at the phylum level. *Bacteroidota* and *Proteobacteria* were the predominant phyla in both CON and TRT. Twenty-five phyla were detected, including 2 archaeal and 23 bacterial phyla (CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals).

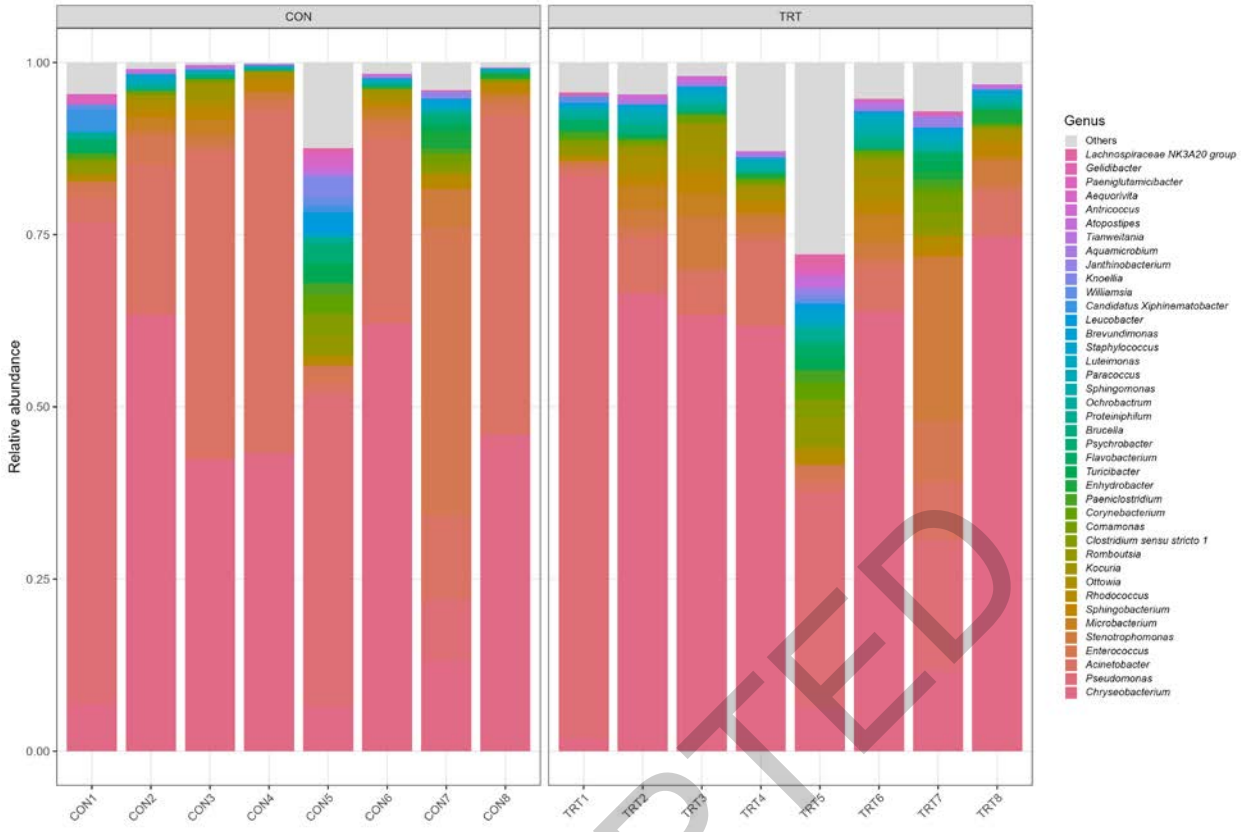

Fig. 4. Taxonomic composition of rumen microbial genera in CON and TRT.

Chryseobacterium, *Pseudomonas*, *Acinetobacter*, *Enterococcus*, and other low-abundance genera detected in both CON and TRT (CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals).

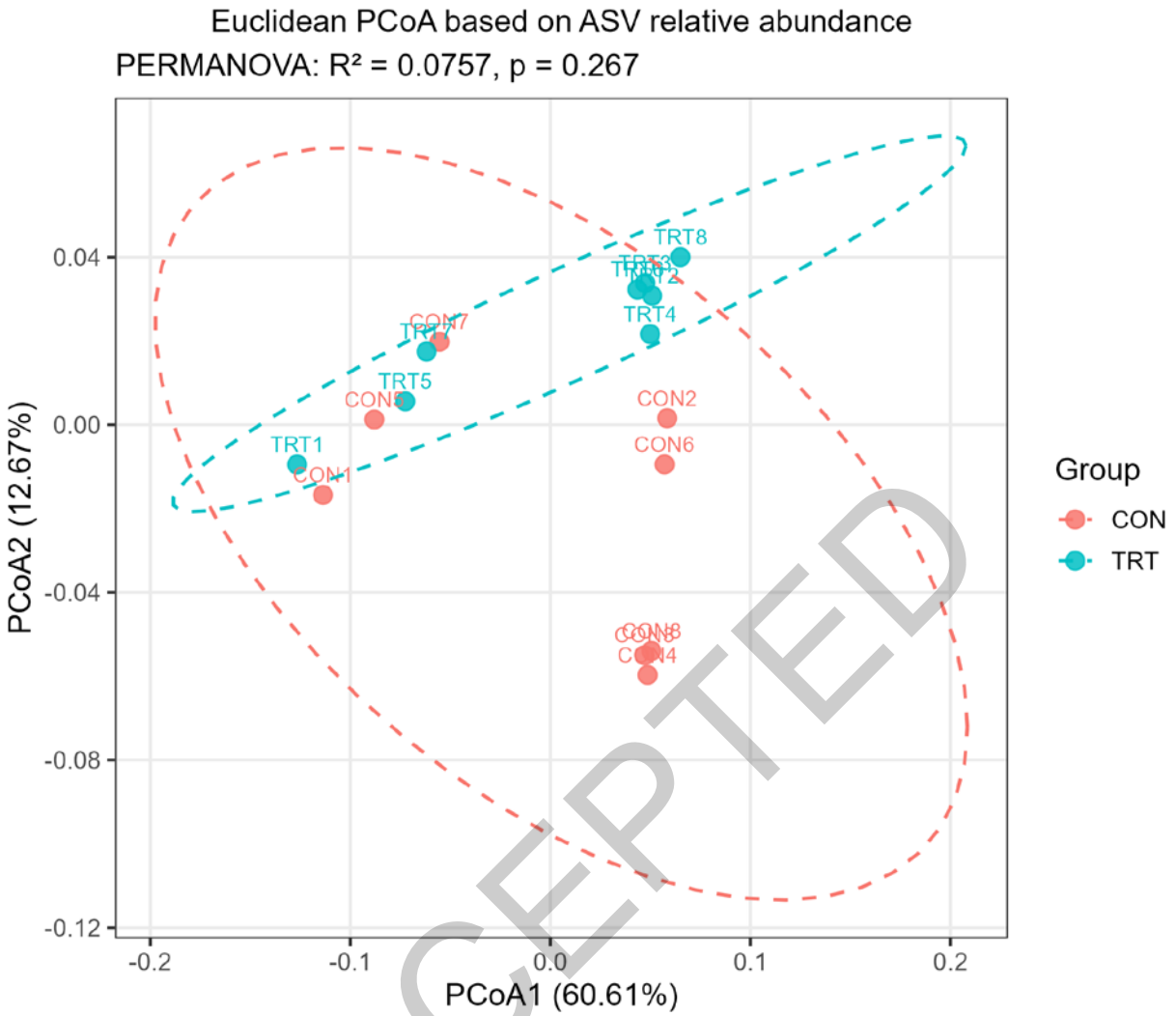

Fig. 5. Principal coordinate analysis (PCoA) plot of rumen microbial composition in CON and TRT. PCoA was performed based on Euclidean distances of ASVs relative abundance. The first and second axes explained 60.6% and 12.7% of the total variation, respectively. PERMANOVA showed no significant difference in microbial composition between CON and TRT (R^2 = 0.0757, p = 0.267, CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals).

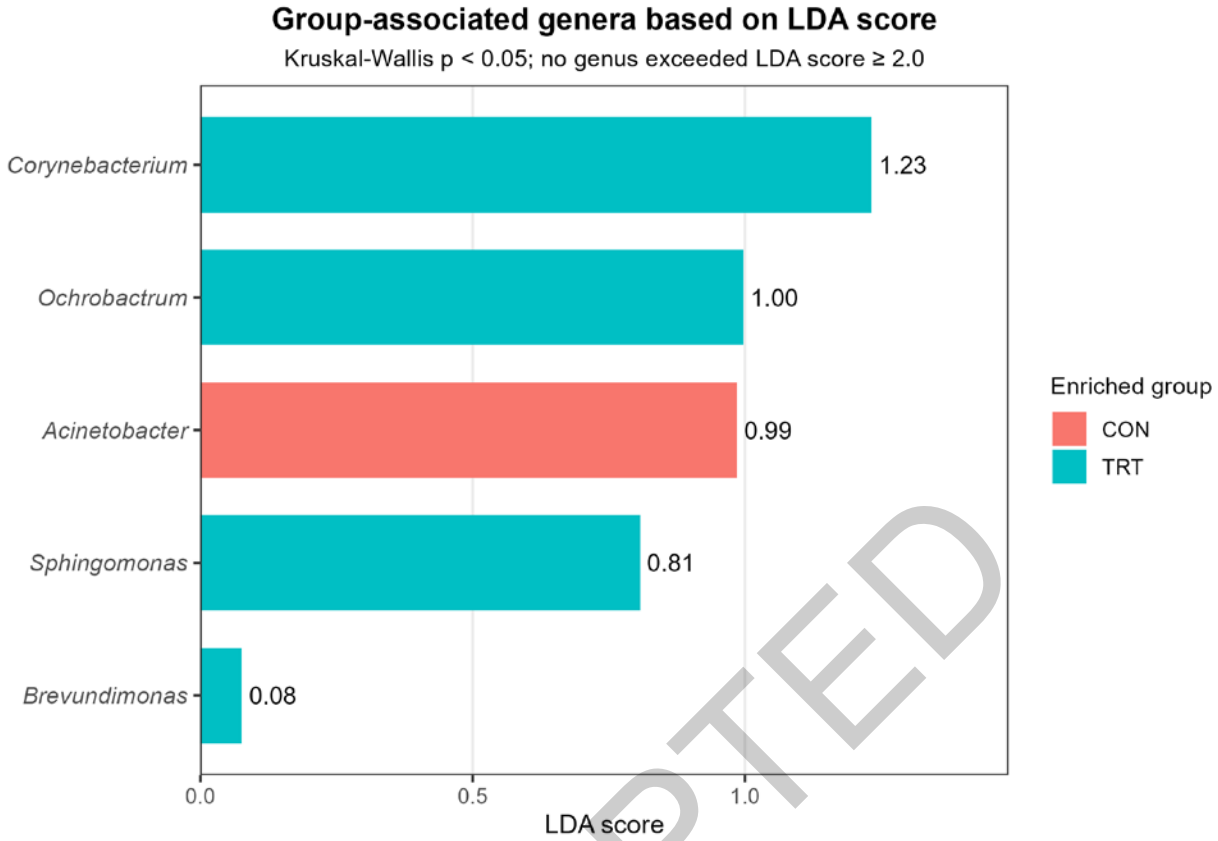

Fig. 6. Linear discriminant analysis (LDA) score plot of group-associated genera between CON and TRT. Genera were selected using Kruskal-Wallis screening ($p < 0.05$, CON: without kaolinite-based clay minerals, TRT: with kaolinite-based clay minerals).