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Screening the CLA Producing Lactic Acid Bacteria Isolated from Rumen Goats and Their Effects on Ruminal *In Vitro* Fermentation

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ABSTRACT

Ruminant-Derived products are a major dietary source of conjugated linoleic acid (CLA), a bioactive fatty acid associated with numerous health benefits. However, the natural biosynthesis of CLA in the rumen is often limited by extensive biohydrogenation of unsaturated fatty acids, necessitating strategies to enhance its synthesis through microbial manipulation. This study aimed to identify CLA-producing lactic acid bacteria (LAB) from goat rumen fluid and evaluate their effects on *in vitro* rumen fermentation characteristics. A total of 140 LAB isolates were obtained from rumen fluid of lactating goats, with 23 isolates demonstrating CLA production ranging from 9.0 to 12.3 µg/mL after 24 h of incubation in MRS broth supplemented with linoleic acid. Molecular identification revealed five unique LAB species: *Lactobacillus fermentum* (n=15), *Lactobacillus plantarum* (n=4), *Pediococcus acidilactici* (n=2), *Lactobacillus argentoratensis* (n=1), and *Lactobacillus pentosus* (n=1). *L. plantarum*-R11 was identified as the highest CLA producer (>12.0 µg/mL) and adopted for *in vitro* ruminal fermentation assessment. Four treatments: control (TMR), TMR supplemented with *L. plantarum*-R11 (LP; 10^8 CFU/mL), TMR supplemented with 2% sunflower oil (SFO), and TMR supplemented with both SFO and LP (LPSFO). The LPSFO group significantly ($p<0.05$) increased cumulative gas production at 72 h (95.51mL/0.2 g DM) and 96 h (103.47 mL/0.2 g DM) compared to the control. Similarly, the LPSFO group had the highest ammonia-nitrogen content (13.15 mM), while pH remained stable across all treatments. The volatile fatty acid (VFA) profiles, fatty acid profiles, and total ruminal CLA content were similar across treatments. *Butyrivibrio fibrisolvens* appears more prominent in the LPSFO group on heatmap visualization, a bacterium associated with ruminal biohydrogenation and CLA metabolism. Our findings suggest that *L. plantarum*-R11 primarily functions as a rumen modulator, influencing rumen microbial ecology and promoting beneficial shifts in microbial community composition that may improve fermentation capacity.

***Keywords:** conjugated linoleic acid; Lactobacillus plantarum; probiotic; sunflower oil; microbial ecology*

INTRODUCTION

Conjugated linoleic acid (CLA) is a complex structure that comprises a group of positional and geometric isomers of linoleic acid (LA) that have gained attention due to the numerous therapeutic benefits, including anticarcinogenic (Viladomiu *et al*., 2016), antiatherogenic (den Hartigh, 2019), body mass (Fuke *et al*., 2017), and muscle-damage (Lee *et al*., 2017). Ruminants are the major source of CLA from their meat and milk through the process of microbial biohydrogenation of dietary polyunsaturated fatty acids in the rumen, followed by endogenous synthesis in animal tissues (Salsinha *et al*., 2023). However, the required therapeutic dose of CLA is low in meat or milk from ruminant animals, which has driven the development of nutritional strategies to modulate rumen microbial activity and enhance the nutritional quality of ruminant-derived products.

Biohydrogenation in the rumen is key to CLA synthesis, and microbes are responsible for the process. Among rumen microorganisms, lactic acid bacteria (LAB) have been investigated previously for their potential roles in CLA synthesis and rumen fermentation (Astuti *et al*., 2018; Tyagi *et al*., 2020). Those LAB species that possess linoleic acid isomerase activity can convert LA to CLA in numerous *in vitro* studies (Kuhl & De Dea Lindner, 2016; Tyagi *et al*., 2020). However, LAB are generally minor and transient members of the rumen ecology, and their functional relevance is highly dependent on diet composition. Balancing the LAB population is crucial for ruminant health and feed digestion by enhancing nutrient degradation and fermenting high-starch substrates into lactic acid, which is subsequently converted into propionic and butyric acids (Astuti *et al*., 2018). LAB could influence microbial distribution, altering fermentation parameters, such as increasing volatile fatty acid (Astuti *et al*., 2018) and fiber digestion (Fitriyah *et al*., 2024; Izuddin *et al*., 2018). Similarly, Astuti *et al*. (2018) reported improved dry matter and neutral detergent fiber digestibility on supplementation of rumen fluid with *L. plantarum* at 10^9 CFU mL^{-1}. In addition, a reduction in methane production associated with LAB supplementation promotes the shifts in hydrogen utilization, stimulation of alternative hydrogen sinks, or changes in microbial community structure (Doyle *et al*., 2019).

However, previous studies have focused on CLA-producing lactic acid bacteria (LAB) under pure culture systems, with limited studies under ruminant conditions. Some LAB species, including *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Bifidobacterium spp*., have been identified as CLA producers (Yang *et al*., 2017); however, CLA synthesis is highly strain-dependent (Chen *et al*., 2024), underscoring the need for targeted screening to identify high-performing candidates. Moreover, laboratory screening media are widely used for screening but differ substantially from the rumen environment in terms of substrate availability, microbial competition, and physicochemical conditions. Consequently, CLA production under rumen-simulated or *in vivo* conditions may not accurately reflect the functional outcomes observed *in vitro*. This discrepancy marks the importance of integrating microbial screening with fermentation-based evaluations to assess the rumen ecology of candidate strains. Therefore, the present study aimed to screen lactic acid bacteria isolated from the rumen microbiome of lactating goats for their CLA-producing potential under laboratory conditions and to evaluate the effects of a selected strain on *in vitro* ruminal fermentation characteristics and microbial community composition.

MATERIALS AND METHODS

Isolation and Screening of LAB for CLA Production

Isolation and screening of LAB for CLA production from goat rumen fluid. The Chiang Mai University Institutional Biosafety Committee approved this protocol (CMUIBC0666001, Approval No. A0666001) before the commencement of this experiment. Rumen fluid was collected from three randomly selected lactating goats (Anglo-Nubian × Saanen; average body weight 30 kg; age 3 years) at the Mae-Hia Agricultural Training and Research Center, Mueang, Chiang Mai (AUP). A suction pump was used to collect 100 mL of rumen fluid. Rumen fluid was stored in sterile containers that had been CO_2-flushed to remove any oxygen and homogenized before further processing. For 24 h at 37 °C, each sample was incubated in deMan-Rogosa-Sharpe (MRS) broth with 0.5 mg/mL of LA (GK5161-25G; Glentham Life Sciences, >90% purity) (Dahiya & Puniya, 2017). After being serially diluted in 0.1% peptone water, the samples were plated on MRS agar and incubated at 37 °C for 72 h. Randomly picked colonies from the MRS agar plates were transferred to MRS broth, and the selected isolates were streaked on MRS agar plates for further purification. The cultures were microscopically examined for purity after Gram staining and then stored at -20 °C in 10% glycerol stocks.

Biochemical and morphological identification. All isolated colonies were subjected to Gram staining and biochemical tests (Bell *et al*., 2005). The selected bacterial isolates were smeared onto glass slides, Gram-stained following standard procedures, and examined microscopically to determine cell morphology and Gram reaction. We used a catalase test with 3% hydrogen peroxide (H_2O_2) added to day-old bacterial cultures on glass slides.

UV-based spectrophotometric screening of LAB for CLA production. Based on colony morphology and Gram staining, a total of 140 presumptive LAB isolates were selected. After the selection process, these isolates were subcultured and screened for CLA-producing potential in MRS broth supplemented with LA. The isolates were rapidly screened using a UV-based spectrophotometric method to evaluate their CLA production (Tyagi *et al*., 2020). However, the approach was used to identify candidate strains with relative CLA-producing ability; it does not quantify individual CLA isomers with the precision of gas chromatography. A 0.2 µm syringe filter was used to filter a solution of LA (5 mg/mL) in sterile distilled water supplemented with 1% (w/v) Tween-80 (Hi-media, Mumbai, India). To conduct the screening process, 10 mL of MRS broth was inoculated with bacterial cultures at 1% (v/v) and supplemented with 0.05% Lcys-HCl as a reducing agent and 0.5 mg/mL LA as the substrate. The cultures were then incubated at 37 °C for 24 h. The capacity of lactic acid isolates to synthesize CLA was evaluated following incubation using the methodology of Barrett *et al*. (2007). The enriched samples were centrifuged at 13,000 × g for 5 min at 4 °C. Subsequently, 1 mL of the supernatant from the sample was vigorously mixed with 2 mL of isopropanol and allowed to settle for 3 min. Subsequently, 1.5 mL of hexane was introduced to facilitate the extraction of FAs, which was subsequently allowed to settle for an additional 3 min. A UV-Vis spectrophotometer (Specord-200, Schönwalde-Glien, Germany) was used to analyze a 1 mL aliquot at 233nm. The trans-10, cis-12 CLA isomer was used to establish a standard curve for quantifying total CLA, with a range of 0.5 to 10 µg/mL. The blank was represented by hexane layers that contained only LA during the assay.

Based on screening results, isolates with CLA-producing activity below 9.0 μg/mL were discarded, and R-11 was selected for further evaluation due to its comparatively higher CLA-producing activity in MRS medium. Furthermore, the DNA sequencing results revealed that the isolated R-11 strain is *Lactobacillus plantarum* and was selected for the *in vitro* study.

Genomic DNA isolation. DNA was extracted from 3 mL of bacterial cultures confirmed to produce CLA after 24 h of incubation using the NucleoSpin® Tissue Kit from MACHEREY-NAGEL GmbH & Co. KG, Germany. The efficiency and quality of the extracted DNA were tested using a 1% (W/V) agarose gel electrophoresis at 100 V for 15 min in 1 × TBE buffer. The DNA concentration and purity were assessed using the NanoDrop™ 2000/2000c Spectrophotometer. Isolated DNA was stored at -20 °C until further use.

16S rRNA gene sequencing and identification. The DNA samples from isolates confirmed to produce CLA were amplified using universal 16S rRNA gene primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). These primers specifically targeted portions of the 16S rRNA gene during PCR amplification. The PCR reaction mixture consisted of PCR buffer, 10 μM of each primer, 10 mM of each dNTPs mix, 1 unit of Taq DNA polymerase, and 100 ng of template DNA. The reaction was conducted in a total volume of 50 μL, following PCR cycling conditions for 35 cycles. Following amplification, the PCR products were purified from the gel using the QIAquick Gel Extraction Kit (Cat#28704) according to the manufacturer's instructions (QIAGEN, Hilden, Germany). We outsourced purified products for bidirectional sequencing to Gibthai Co., Ltd. (Bangkok, Thailand). The bidirectional sequencing output was examined and confirmed in BioEdit, and alignment of both strands was performed using CLUSTAL W v2.0. Finally, these sequences were BLAST against the NCBI GenBank database to identify similarities with other *Lactobacillus* sequences. Phylogenetic analysis was performed to confirm the taxonomic identification of CLA-producing LAB isolates. The 16S rRNA gene sequences of the isolates and reference strains retrieved from the NCBI GenBank database were aligned using ClustalW implemented in the RStudio v2023.06.0 Build 421 (Posit Software, PBC, Boston, MA, USA). A genetic distance matrix was calculated using the Kimura 2-parameter (K80) model. A Maximum Likelihood tree was constructed. Bootstrap analysis with 1,000 replicates was performed to assess branch support, and bootstrap values ≥70% were considered reliable. The phylogenetic tree was visualized using the ggtree package in RStudio (version 2023.06.0).

In Vitro Degradation of TMR Supplemented with *Lactobacillus plantarum*-R11

Production of *L. plantarum*-R11. To rejuvenate the bacteria, *L. plantarum*-R11 in a 10% glycerol stock was streaked onto de Man, Rogosa, and Sharpe (MRS) agar and incubated at 37 °C for 48 h. A pure single colony was picked, then placed in 10 mL MRS broth and incubated for 24 h at 37 °C. The enriched culture (1% v/v) was transferred to MRS broth in a sterile Erlenmeyer flask at 37°C with agitation at 100 rpm, then incubated for 15 h. The bacteria-media solution was separated using an Allegra X-22R Benchtop Centrifuge (Beckman Coulter, USA) at 10000 × g for 5 min, then the pellet was kept at 4 °C before supplementation.

***In vitro* gas production method.** The *in vitro* degradation was performed using the gas production technique (Menke *et al*., 1979). Goat rumen fluid was collected from randomly selected three goats in an abattoir, Hamza Farm, located in 117/314 Soi 22 Pa Daet Sub-district, Mueang Chiang Mai District, Chiang Mai, under ethics license No. AG01005/2566. Rumen fluid was pooled in equal portions, then filtered through two layers of cheesecloth before the *in vitro* degradation experiment. The basal feed used for *in vitro* is a total

Table 1. Chemical composition of total mixed ration used in the *in vitro* experiment (DM basis, %)

Item	TMR
Feed ingredients (%)	
Corn silage	18.84
Pangola hay	12.81
Water	30.14
Corn husks	18.84
Rice bran	4.52
Corn meal	6.03
Soybean meal	8.67
Premix	0.15
Chemical composition	
Dry matter (%)	41.78
Ash (%)	7.98
Crude protein (%DM)	16.85
Ether extract (%DM)	4.08
NDF (%DM)	66.04
ADF (%DM)	37.80
ADL (%DM)	10.72
Hemicellulose	28.24
Cellulose	27.08

Note: Chemical composition values are represented on a dry matter basis (%DM); TMR: total mixed ration; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin.

Table 2. Fatty acid composition of TMR (g/100 g total fat)

Fatty acid	Concentration
C12:0	0.51
C14:0	0.23
C16:0	13.48
C18:0	5.07
C18:1n9c	27.81
C18:2n6	34.71
Total SFA	19.28
Total MUFA	27.81
Total PUFA	34.71

Note: TMR: total mixed ration; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

mixed ration (TMR) with chemical composition listed in Table 1. The fatty acid profile of TMR is shown in Table 2. Treatments in this experiment were: CON: TMR; LP: TMR + 10^8 CFU / mL *L. plantarum*-R11; SFO: TMR + 2% sunflower oil; LPSFO: TMR + 2% sunflower oil + 10^8 CFU/mL *L. plantarum*-R11.

Each treatment has three replications for measuring gas production. The TMR was weighed at 230 ± 5 mg, then placed in a 100 mL plunger-equipped syringe (FORTUNA®; Poulten and Graf GmbH, Wertheim, Germany). Blanks, standard hay, and standard concentrate (Hohenheimer Futterwerttest (HFT); University of Hohenheim, Germany) syringes were prepared according to the method of Menke *et al.* (1979). The syringes were prewarmed at 39 °C before being filled with 30 mL of rumen fluid and buffered mineral mixture. Rumen fluid and buffered mineral solution were mixed in a ratio of 1:2. Buffered mineral solution was made as described by Menke *et al*. (1979). During the mixing process, the buffered mineral solution was kept in a flask flushed with constant CO_2, stirred with a magnetic stirrer at 39 °C. Rumen fluid was added after the buffered mineral solution turned clear. For the LP and LPSFO groups, 3 mL of *L. plantarum*-R11 in MRS broth was added to the rumen-buffered mineral solution. The sunflower oil (Naturel brand, Lam Soon Ltd.; Bangkok, Thailand) (2%, w/w) was incorporated into the TMR before use in the experiment. The syringes were incubated in a water bath at 39 °C, equipped with a rotor at 30 RPM. Gas production was recorded at 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 h during the incubation period.

The accumulated gas production data were adopted to calculate gas kinetic parameters using an exponential model as described in (Ørskov & McDonald, 1979):

$$GP = a + b\,(1 - e^{-ct})$$

where GP was the volume of gas produced at time "t" (mL/0.2 g DM), a was the production of gas from the immediately soluble fraction (mL), b was the production of gas from the insoluble fraction (mL/0.2 g DM), c was the rate of gas production from insoluble fraction (mL/h), and t = incubation time (h).

***In vitro* ruminal pH, total ammonia (NH_3), and volatile fatty acids (VFA)**. Rumen fluid pH was measured after 24 h of incubation with a portable pH meter (Model 205, Testo, Lenzkirch, Germany). Ammonia nitrogen (NH_3-N) concentration in rumen fluid was determined using the phenol–hypochlorite colorimetric method. Following 24 h of incubation, rumen fluid samples were centrifuged at 10,000 × g for 5 min, and the supernatant was collected for analysis. Briefly, the phenol and hypochlorite reagents were thoroughly mixed with the 50 µL supernatant, which was then diluted with distilled water. The mixture was incubated for 10–15 min to allow color development. Ammonia-N concentration was calculated from a standard curve prepared using ammonium chloride, and absorbance was measured at 625 nm using a UV-Vis spectrophotometer (Chaney & Marbach, 1962). Furthermore, rumen fluid was prepared according to Tanakorn *et al*. (2026) for the quantification of volatile fatty acids (VFA) using gas chromatography (GC). One microliter of the sample was injected in split mode into a Shimadzu Nexis GC-2030 gas chromatograph equipped with an automatic injector (Shimadzu AOC-20i Plus) and a Zebron ZB-FAME capillary column (30 m × 0.25 mm i.d. × 0.20 µm film thickness; Phenomenex, USA). Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}, with the injector temperature maintained at 160 °C. The oven temperature was programmed to increase from 60 °C to 115 °C at 5 °C/min, then to 130 °C at 3 °C/min, and finally to 230 °C at 15 °C/min for 3 min. The FID was maintained at 250 °C. The samples were classified by comparing their peak retention time to those of the VFA standard (Volatile Free Acid Mix; Supelco, Laramie, Wyoming, USA).

***In vitro* dry matter degradability (IVDMD) and organic matter degradability (IVOMD).** The syringes were systematically arranged into separate sets for individual sample incubations to assess *in vitro* substrate degradation. The analysis concentrated on assessing *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD). Approximately 230 ± 5 mg of each sample was measured and subjected to the previously described *in vitro* gas production techniques. After a 24 h incubation period, the contents of the glass syringes for each treatment and control were filtered using a Buchner funnel lined with the Whatman filter papers No. 41. The residue in the filter paper was dried at 105 °C for 24 h, and the organic matter (OM) content of substrates was determined by ashing samples at 550 °C (Hnokaew & Yammuen-Art, 2021). The *in vitro* substrate degradability was determined using the following formula:

IVDMD (%) = [(dry matter initial weight - dry matter final weight) / (dry matter initial weight)] × 100

IVOMD (%) = [(organic matter initial weight - organic matter final weight) / (organic matter initial weight)] × 100

Fatty acid analysis. The rumen fluid was collected after 24 h incubation for fatty acids analysis. Approximately 1.0 mL of rumen was converted to fatty acid methyl esters (FAMEs) by direct transesterification as described by O'Fallon *et al*. (2007). Before gas chromatography analysis, the collected hexane extract was concentrated under nitrogen and preserved at -20 °C. The composition of fatty acids was determined using a GC-7820A (Agilent Technologies Inc., USA) following the protocol by (Teng *et al*., 2017) and a CP-Sil 88 fused-silica capillary column (100 m length × 0.25 mm diameter (i.d) and film thickness 0.20 **µm**; Agilent Technologies Inc., USA). The samples were classified by comparing their peak retention times with those of the FAME mixture standard (Food Industry FAME Mix, 30 mg/mL; RESTEK, Pennsylvania, USA), which provided isomeric profiles in the chromatogram. Total CLA is accumulated from identified isomers, which are *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA.

Rumen microbial community analysis. Rumen fluid samples (n = 3 per treatment) were collected after 24 h of incubation for microbial community analysis. Amplicon metagenome sequencing targeting the V3-V4 hypervariable regions of the 16S rRNA gene was performed on an Illumina NovaSeq 6000 platform by Gibthai Co., Ltd. (Bangkok, Thailand) following their standard protocols (Sahatsanon *et al*., 2025). Briefly, genomic DNA was extracted, and the V3-V4 region was amplified using universal bacterial primers (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). Raw sequences were quality-filtered, and operational taxonomic units (OTUs) were clustered at 97% sequence similarity (Barfod *et al*., 2013). Taxonomic classification was performed using the SILVA reference database. Relative abundances of bacterial taxa at the phylum, family, genus, and species levels were visualized using heatmaps and bar plots (Barfod *et al*., 2013).

Statistical Analysis

The *in vitro* data, such as gas production, *in vitro* degradation, kinetics of gas production, total NH_3, volatile fatty acids (VFA), fatty acids composition, were analyzed with RStudio 2023.06.0 Build 421 (Posit Software, PBC, Boston, MA, USA), using analysis of variance (ANOVA) based on the completely randomized design (CRD) (Akhmad, 2019). Normality of data distribution was assessed before analysis. The mathematical model used is a linear model for CRD: $Y_{ij} = \mu + \alpha_i + \boldsymbol{\varepsilon_{ij}}$, where Yij is the observation value, μ is the overall mean, αi is the treatment effect, and **εij** is the experimental error. The Duncan multiple-range test was used to determine the significance of differences among the groups. The outcomes were presented as the mean ± standard error of the mean (SEM). Statistical differences were considered significant at $p<0.05$.

RESULTS

Screening and Isolation of CLA-producing LAB

A total of 140 isolates were obtained from selective media (MRS Agar) enriched with LA derived from goat rumen fluid. The pure isolates exhibited rod-shaped and spherical colonies, and microscopic examination confirmed that all isolates were Gram-positive. Additionally, they tested negative for catalase and oxidase, which are characteristic of lactic acid bacteria (Table 3). A phylogenetic tree was generated to visualize the genetic similarities among sequencing results (Figure 1). All 140 isolates were evaluated for CLA synthesis in MRS broth enriched with LA using an active culture technique based on UV spectrophotometry. Following 24 h of incubation, 23 isolates were identified as producing CLA concentrations ranging from 9.0 to 12.3 µg/mL from LA (Figure 2). The highest CLA production was observed in isolate RI-11, whereas the lowest production was recorded for RI-8. Only two isolates (RI-11 and RI-41) produced CLA concentrations exceeding 12.0 µg/mL, while the majority of isolates exhibited CLA production of approximately 9.0 µg/mL in MRS-supplemented broth. Therefore, RI-11 was selected as the potential CLA-producing bacterium to be incorporated into

Table 3. Biochemical characteristics of rumen isolates

Isolates	Biochemical characteristics		16s rRNA Sequencing
	Catalase/oxidase	Shape	Identified based on sequencing
RI-3	-	R	*Lactobacillus fermentum*
RI-6	-	R	*Lactobacillus fermentum*
RI-8	-	R	*Lactobacillus fermentum*
RI-11	-	R	*Lactobacillus plantarum*
RI-21	-	R	*Lactobacillus fermentum*
RI-22	-	R	*Lactobacillus fermentum*
RI-24	-	R	*Lactobacillus fermentum*
RI-26	-	R	*Lactobacillus fermentum*
RI-35	-	R	*Lactobacillus fermentum*
RI-41	-	C	*Pedicoccus acidilactici*
RI-45	-	R	*Lactobacillus fermentum*
RI-48	-	R	*Lactobacillus fermentum*
RI-55	-	R	*Lactobacillus plantarum*
RI-59	-	C	*Pedicoccus acidilactici*
RI-67	-	R	*Lactobacillus fermentum*
RI-76	-	R	*Lactobacillus fermentum*
RI-81	-	R	*Lactobacillus fermentum*
RI-83	-	R	*Lactobacillus pentosus*
RI-90	-	R	*Lactobacillus fermentum*
RI-93	-	R	*Lactobacillus fermentum*
RI-97	-	R	*Lactobacillus argentoratensis*
RI-99	-	R	*Lactobacillus plantarum*
RI-140	-	R	*Lactobacillus plantarum*

Note: Rumen Isolates: RI, Rod: R, Cocci: C

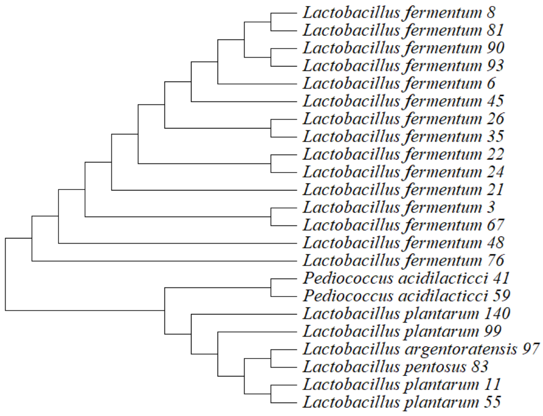

Figure 1. Phylogenetic analysis of LAB strains isolated from rumen fluid based on 16S rRNA gene sequencing

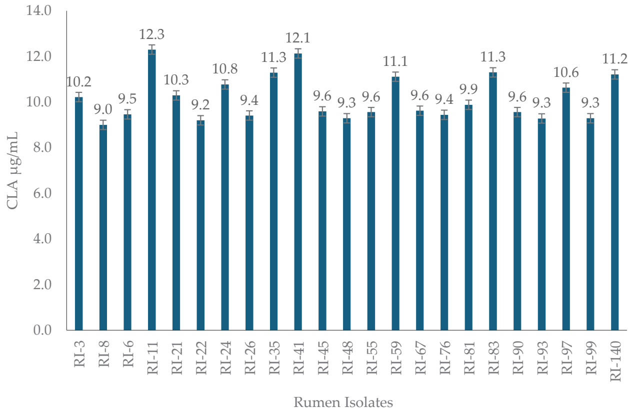

Figure 2. Comparative conjugated linoleic acid (µg/mL) assay of the goat isolates after 24 h incubation

the *in vitro* gas production technique for evaluating its fermentation characteristics. The positive isolates underwent molecular confirmation, yielding five strains of lactic acid bacteria, as detailed in Table 3: *Lactobacillus fermentum* (15), *Lactobacillus plantarum* (4), *Pediococcus acidilactici* (2), *Lactobacillus argentoratensis* (1), and *Lactobacillus pentosus* (1).

In Vitro Gas Production (IVGP) and *In Vitro* Rumen Fermentation

Supplementation with sunflower oil (SFO) and *L. plantarum*-R11 (LP) affected *in vitro* degradability and gas production (Table 4). The inclusion of 2% SFO significantly suppressed early gas production at 2 h and 6 h of incubation; the LP and LPSFO groups produced significantly more gas than the control group. Additionally, the LPSFO combination significantly yielded the highest gas production at 72 and 96 h. Simultaneously, the asymptotic gas potential (b) of the LPSFO group was higher compared to other groups. However, the LPSFO group exhibited the lowest IVDMD and IVOMD at 24 h and maintained lower dry matter degradability at 48 h, whereas the SFO group restored degradability to control levels at this time point.

Fermentation parameters are affected by the combination of *L. plantarum*-R11 and sunflower oil (Table 5). The LPSFO produced the highest ammonia (NH_3) levels, and the pH remained stable. The VFA and ruminal fatty acids profile (Table 6) were not significantly affected by treatments.

Heatmap analysis illustrated differences in relative abundance patterns of selected bacterial taxa at the species level among treatments (Figure 3). The microbial profiles were similar across the group, with the baseline community structure characterized by a higher relative abundance of *Ruminococcus*. *Acinetobacter indicus* and butyrate-producing bacteria show higher relative abundance in the LP group. In contrast, a distinct abundance pattern, with *Blautia* spp. and *Selenomonas ruminantium* appearing more, was

Table 4. *In vitro* gas production and kinetics of gas production of total mixed ration supplemented with *Lactobacillus plantarum*-R11 and sunflower oil

Variables	Treatments					
	CON	LP	SFO	LPSFO	SEM	p-value
IVGP (mL/0.2 gDM)						
2 h	15.92^{a}	15.87^{a}	10.96^{b}	11.93^{ab}	1.299	0.040
4 h	19.89	18.85	16.96	19.88	0.693	0.417
6 h	18.90^{b}	21.83^{a}	18.94^{b}	20.90^{a}	0.729	0.007
8 h	20.88	23.81	20.95	22.88	0.727	0.168
10 h	23.87	26.78	24.93	26.86	0.732	0.162
12 h	58.15	60.51	53.84	58.70	1.412	0.391
24 h	75.07	72.41	71.80	77.60	1.331	0.621
48 h	77.05	77.37	73.78	84.57	2.274	0.135
72 h	81.02^{b}	80.34^{b}	76.76^{b}	95.51^{a}	4.141	0.021
96 h	83.03^{b}	86.30^{b}	78.73^{b}	103.47^{a}	5.421	0.008
Kinetics of Gas Production						
a (mL)	-2.16	-1.11	-5.83	-2.28	1.029	0.539
b (mL/0.2mgDM)	85.85^{b}	85.37^{b}	84.99^{b}	102.48^{a}	4.274	0.050
c (mL/h)	0.06	0.06	0.07	0.05	0.004	0.111

Note: IVGP: *In vitro* gas production, CON (control): Total Mixed Ration (TMR), LP: TMR + 10^8 CFU / mL of *L. plantarum* supplementation; SFO: TMR + 2% of sunflower oil; LPSFO: TMR + 2% of sunflower oil + 10^8 CFU / mL of *L. plantarum* supplementation; SEM: standard error mean (n= 3 replicates per treatment) n; superscript a-b connote values among treatment groups are significantly different at p<0.05 or p<0.01; a: production of gas from the soluble fraction; b: production of from the insoluble fraction; c: rate of gas production from insoluble fraction.

Table 5. *In vitro* rumen degradability and rumen fermentation of total mixed ration supplemented with *Lactobacillus plantarum*-R11 and sunflower oil

Variables	Treatments					
	Control	LP	SFO	LPSFO	SEM	p-value
Fermentability						
Total NH_3 (mM)	10.76^{d}	12.51^{b}	12.28^{c}	13.15^{a}	0.506	<0.001
pH 24 h	7.09	6.84	6.95	7.02	0.053	0.083
pH 48 h	6.70	6.71	6.67	6.74	0.014	0.702
Acetate (mM)	26.24	30.04	36.10	32.88	2.095	0.425
Propionate (mM)	8.53	8.26	11.61	8.69	0.785	0.697
Butyrate (mM)	9.78	9.46	9.33	7.97	0.399	0.574
Total VFA (mM)	44.55	47.76	57.04	49.54	2.647	0.410
A:P	3.35	3.61	3.94	4.46	0.241	0.898
Degradability (%)						
IVDMD 24 h	62.31^{a}	56.86^{b}	57.86^{ab}	47.51^{c}	3.110	0.001
IVOMD 24 h	67.71^{a}	61.80^{b}	62.88^{ab}	51.63^{c}	3.380	0.001
IVDMD 48 h	64.39^{a}	61.80^{b}	63.48^{a}	60.53^{b}	1.856	0.001
IVOMD 48 h	68.35	67.16	68.99	65.78	2.017	0.149

Note: CON (control): Total Mixed Ration (TMR), LP: TMR + 10^8 CFU / mL of *L. plantarum* supplementation; SFO: TMR + 2% of sunflower oil; LPSFO: TMR + 2% of sunflower oil + 10^8 CFU / mL of *L. plantarum* supplementation; VFA: Volatile fatty acids; A:P: Acetate:Propionate; SEM: standard error mean (n = 4 replicates per treatments); superscript a-d connote values among treatment groups are significantly different at p<0.05 or p<0.01; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability.

Table 6. Ruminal fatty acids profile *in vitro* (% Total FA)

Fatty acids	Treatments					
	Control	LP	SFO	LPSFO	SEM	p-value
cis-9, *trans*-11 C18:1, CLA	0.45	0.61	0.45	1.25	0.190	0.217
trans-10, *cis*-12 C18:1, CLA	0.41	0.61	0.40	1.00	0.140	0.128
C12:0, lauric	5.40	1.06	2.94	0.75	1.069	0.443
C14:0, myristic	6.89	3.44	4.08	2.70	0.914	0.506
C16:0, palmitic	28.53	27.15	20.36	26.20	1.799	0.203
C16:1, cis-9, palmitoleic	0.58	0.89	2.72	0.79	0.495	0.360
C18:0, stearic	32.79	35.30	29.79	35.70	1.363	0.692
C18:1n9t, octadecenoic	8.63	13.94	13.20	16.97	1.722	0.119
C18:1n9c, oleic	10.19	12.29	9.93	11.27	0.540	0.706
C18:2n6c, linoleic	3.66	4.71	7.59	4.44	0.858	0.459

Note: CLA: conjugated linoleic acid; CON (control): Total Mixed Ration (TMR), LP: TMR + 10^8 CFU / mL of *L. plantarum* supplementation; SFO: TMR + 2% of sunflower oil; LPSFO: TMR + 2% of sunflower oil + 10^8 CFU / mL of *L. plantarum* supplementation; SEM: Standard Error Mean (n = 4 replications per treatment).

observed in the LPSFO group compared with the other treatment groups. Additionally, a prominent display in *Butyrivibrio fibrisolvens* in the SFO and LPSFO groups, followed closely by the CON group. *B. fibrisolvens* is a key microbe involved in ruminal biohydrogenation and CLA synthesis. All observations are descriptive and based on relative abundance patterns derived from heatmap visualization.

A clear difference in community structure between the sample groups in the taxonomic analysis at the species level (Figure 4). Variation in the relative abundances of key taxa, including *Streptococcus lutetiensis*, *Streptococcus macedonicus*, *Ruminococcus flavefaciens*, and unclassified members of *Ruminococcus* and *Prevotella*. A substantial unassigned fraction and other categories indicated a considerable degree of microbial diversity that requires further investigation. The compositional changes suggest that experimental treatments exerted significant pressure on the microbial community.

DISCUSSION

Our present study screened rumen-derived lactic acid bacteria (LAB) from goats for their potential to produce CLA under laboratory conditions. *L. plantarum*-R11 was evaluated for its effects on *in vitro* ruminal fermentation and microbial community structure. A total of 140 rumen-derived isolates, 23 LAB strains, were identified to produce CLA concentrations ranging from 9.0 to 12.3 µg/mL after 24 h of incubation. According to Sosa-Castañeda *et al.* (2015), a higher CLA value was observed when LAB were cultured in skimmed milk supplemented with 2% LA at 36 °C for 48 h. Numerous factors may have contributed to the difference in CLA content produced, including incubation time, LA availability, and culture medium. Skimmed milk provides a more favorable substrate and reduces microbial competition compared with MRS-based systems, while longer incubation allows greater enzymatic conversion of LA to CLA.

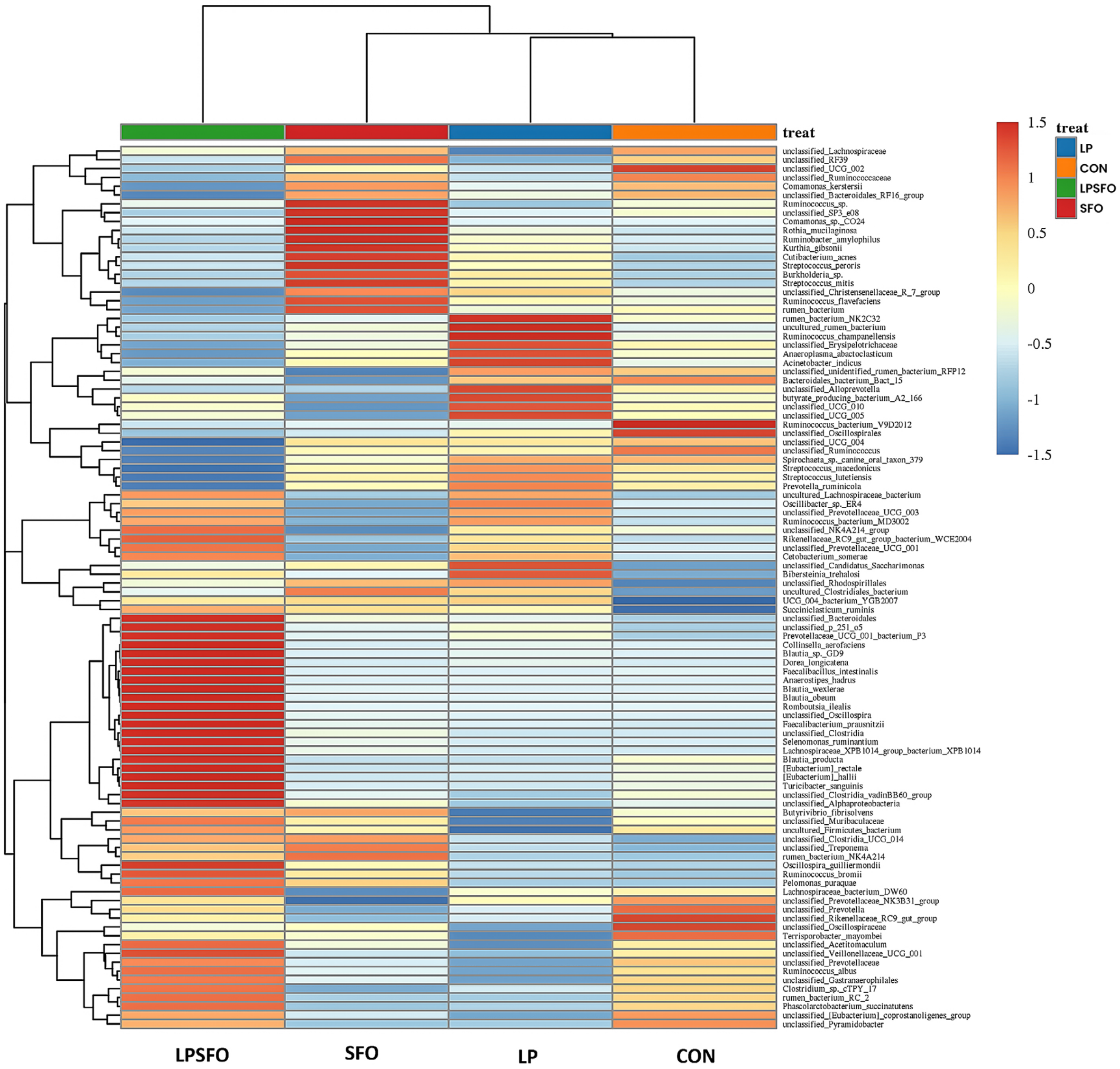

Figure 3. Heatmap illustrating species-specific abundance patterns among treatments. CON (control): total mixed ration (TMR), LP: TMR + 10^8 CFU / mL of *L. plantarum* supplementation; SFO: TMR + 2% of sunflower oil; LPSFO: TMR + 2% of sunflower oil + 10^8 CFU / mL of *L. plantarum* supplementation.

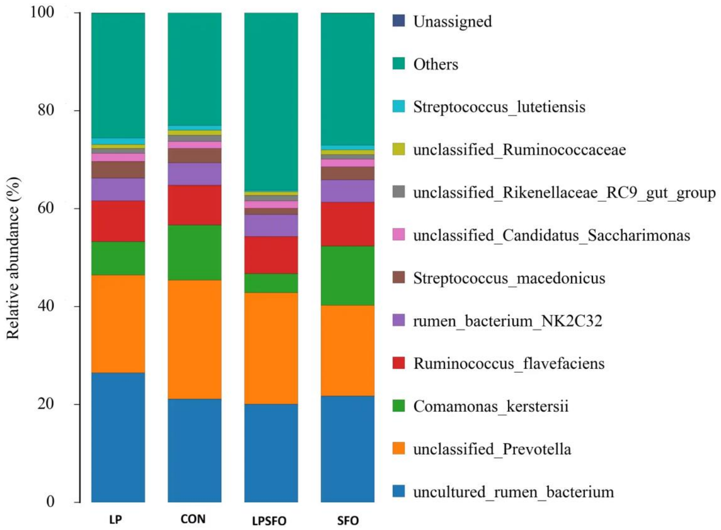

Figure 4. Species abundance bar plot among treatments. CON (control): total mixed ration (TMR), LP: TMR + 10^8 CFU / mL of *L. plantarum* supplementation; SFO: TMR + 2% of sunflower oil; LPSFO: TMR + 2% of sunflower oil + 10^8 CFU / mL of *L. plantarum* supplementation.

We identified five distinct LAB species: *Lactobacillus fermentum* (15 isolates), *Lactobacillus plantarum* (4 isolates), *Pediococcus acidilactici* (2 isolates), *Lactobacillus argentoratensis* (1 isolate), and *Lactobacillus pentosus* (1 isolate), demonstrating the diversity of CLA-producing bacteria within the LAB of the rumen microbiome. The predominance of *L. fermentum* among CLA producers aligns with the findings of Tyagi *et al*. (2020), who reported that this species possesses robust linoleic acid isomerase activity. LAB strains isolated from the rumen exhibit variation in CLA production efficiency, even within the same species, underscoring the specific metabolic capabilities of each strain (Chen *et al*., 2024).

These findings align with the need for systematic screening to identify efficient CLA-producing strains rather than relying solely on species-level identification. Based on our preliminary CLA-producing ability in MRS medium, *L. plantarum*-R11(12.3 μg/mL) was selected using a UV-based spectrophotometric method. However, the introduction of *L. plantarum*-R11 into the *in vitro* system did not enhance ruminal CLA accumulation during *in vitro* fermentation. Still, it did exert measurable effects on gas kinetics and microbial community composition. The UV-based method used during initial isolate screening is a rapid, cost-effective preliminary screening tool. However, it has some limitations in resolving individual CLA isomers compared with gas chromatography. The disparity observed between the MRS medium and the rumen conditions for CLA production might be attributed to the media used in both experiments. The MRS condition provides abundant substrates and minimal microbial competition, thereby favoring LA conversion. In contrast, the rumen environment is surrounded by intense microbial competition and extensive biohydrogenation, which likely explains the lack of increased CLA under rumen-simulated conditions. Previous studies observed discrepancies between pure-culture screening and rumen systems, restating that CLA production observed in laboratory media does not necessarily translate to ruminal conditions (Özer & Kılıç, 2021).

Inoculation with LP and SFO, individually and in combination, influenced *in vitro* gas production parameters. We observed suppressed gas production at 2 h in the SFO and LPSFO in the earlier phase. Due to the antimicrobial effects of the medium-chain fatty acids present in SFO, which temporarily slow microbial activity during the early fermentation phase, as suggested by Jenkin (1993). However, by 6 h, the LPSFO and LP groups marked enhanced gas production compared to the control, suggesting the *L. plantarum*-R11 promotes microbial adaptation and fermentation despite the presence of dietary oil. Elevated asymptotic gas potential (b) and the highest cumulative gas production at 72 and 96 h were observed in the LPSFO group, indicating improved and sustained fermentation of the insoluble fiber fraction during prolonged incubation. An increase in the asymptotic gas parameter (b) reflects substrate degradation, particularly slowly fermentable components such as cell wall polysaccharides, and its increase indicates improved utilization of residual fibrous substrates. Previous studies reported that gas production increases in the late phase when dietary lipids are combined with microbial additives, reducing lipid biohydrogenation and inhibiting microbial activity in the early phase, thereby promoting fermentation more efficiently at later incubation times (Fitriyah *et al*., 2024; Kim *et al*., 2020). The study suggests a synergistic interaction between *L. plantarum*-R11 and SFO that enhances long-term fermentative capacity, possibly through selective modulation of the microbial community.

Despite improved gas production, the LPSFO group exhibited lower IVDMD and IVOMD at 24 h than the control. This contradiction between gas production and digestibility reflected a shift in fermentation rather than a reduction in microbial activity. The PUFA's inhibitory effects on fiber-degrading rumen bacteria might contribute to the reduced degradability in the LPSFO group (Gao *et al*., 2019; Jenkins, 1993). Sunflower oil is rich in LA; thus, its presence could disrupt microbial cell membranes and interfere with cellulolytic activity, particularly among gram-positive fiber-degrading species. Interestingly, by 48 h, the SFO group had restored degradability, suggesting that the microbial community had adapted to the oil supplement over time. Moreover, the LPSFO group recorded a high ammonia-nitrogen concentration (13.15 nM), suggesting enhanced protein degradation. The NH_3 level does not exceed the optimal range for microbial growth (5-20 mM), which may support microbial protein synthesis (McDonald *et al*., 2010). Supplementation with *L. plantarum*-R11 did not disrupt rumen buffering capacity, which is critical for maintaining optimal microbial function; thus, this is reflected in the stable pH across all treatments (6.67-7.09). The VFA and fatty acid profiles remained stable across the groups.

LAB is not the most dominant member of the rumen microbiota, but its presence might influence microbial interactions under *in vitro* conditions. Rumen microbial community analysis provides insight into the potential of *L. plantarum*-R11 to shift microbial communities. The relative abundances of the treatments suggest selective microbial modulation of the rumen ecosystem; however, these differences are descriptive and based on relative abundance patterns rather than formal differential abundance or beta-diversity analyses. *Ruminococcus spp.* and *Prevotella spp* are the dominant in the control group, representing fibrolytic and amylolytic bacteria essential for plant polysaccharide degradation (Flint *et al*., 2008). The LP treatment showed dominance of *Ruminococcus*, with *Acinetobacter indicus* and butyrate-producing bacteria also present. The LPSFO group showed a distinct abundance pattern, with *S. ruminantium* and *Blautia* spp. appeared more prominent (Figure 4). We observed a stable pH across the treatment, even though *L. plantarum*-R11 is associated with lactic acid production (Liu *et al*., 2021; Lu *et al*., 2023). Both taxa are associated with lactate utilization, which may partly explain the stable ruminal pH. However, as lactate concentration was not measured, the interpretation is based on a potential mechanism rather than a confirmed effect.

The established relationship between lactate-producing and lactate-utilizing bacteria likely contributed to the extended gas production and improved fermentative stability in the LPSFO group. *Butyrivibrio fibrisolvens*, a key bacterium responsible for CLA production through LA isomerization in the rumen (Hussain *et al*., 2016), was enriched in both the SFO and LPSFO. The degree of *B. fibrisolvens* on the relative abundance in oil-supplemented groups confirms that sunflower oil provided sufficient LA substrate to stimulate CLA-producing bacterial populations. However, stable CLA content in the ruminal fatty acid profile at 24 h suggests that either the sampling time did not capture peak CLA levels or that rapid conversion to stearic acid via complete biohydrogenation occurred. The combined supplementation of *L. plantarum*-R11 and sunflower oil appears to produce a synergistic effect on long-term gas production and microbial community structure.

Overall, the potential mechanism is that *L. plantarum*-R11 produces antimicrobial compounds, such as bacteriocins, which may selectively inhibit competing bacteria and modulate rumen microbial activities (Urban *et al*., 2024). This modulation could create a more favorable fermentative ecology characterized by stable pH, reduced microbial competition, and enhanced cross-feeding. It could promote the activities of lactate-utilizing bacteria such as *S. ruminantium* to limit lactate accumulation and support the activities of fibrolytic and biohydrogenating bacteria, including *Butyrivibrio* spp. (Guo *et al*., 2020). However, these mechanisms remain hypothetical because bacteriocin production and interspecies interactions were not confirmed in the study. Alternatively, the presence of *L. plantarum*-R11 in rumen ecology could enhance the bioavailability of LA from sunflower oil through lipase activity, providing a substrate for biohydrogenation bacteria (Tyagi *et al*., 2020). The observed changes in microbial community composition validate the hypothesis that *L. plantarum*-R11 acts as a modulator of rumen ecology rather than only a CLA producer in the complex rumen environment.

CONCLUSION

Our study demonstrates that rumen-derived lactic acid bacteria can be effectively screened in the laboratory for their potential to produce CLA. Moreover, such potential does not necessarily translate into increased CLA accumulation under a rumen-simulated environment. *L. plantarum*-R11, selected based on its CLA-producing ability in MRS medium, but the properties did not reflect on ruminal CLA concentration. Still, it influenced gas production kinetics and rumen microbial community composition, playing a key role in microbial modulation rather than in direct CLA enhancement. Our findings underscore the difference between pure-culture screening outcomes and functional responses in rumen-like environments. Future research should focus on *in vivo* studies with robust fatty acid tracing alongside comprehensive microbial community analyses to confirm the ecological role of LAB supplementation.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used Claude.ai, QuillBot, and Grammarly to paraphrase, edit language, and proofread. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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