Research Article

In vitro Gas Production and Rumen Microbiome Structural Shifts in Response to Graded Inclusion of *Indigofera zollingeriana* Pellet Concentrate

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Abstract | This study aimed to evaluate the dose-dependent effects of graded inclusion levels of *Indigofera zollingeriana* pellet concentrate on *in vitro* gas production, fermentation kinetics, and microbial community structure. A completely randomized design was applied with three treatments: T0 (0% Indigofera), T1 (13.57% Indigofera), and T2 (27.13% Indigofera), each with four replicates. *In vitro* gas production was measured over a 48 h incubation period, digestibility was determined post-incubation, and rumen microbiome composition was characterized using 16S rRNA gene sequencing targeting the V3–V4 region. Cumulative gas production decreased significantly with increasing Indigofera inclusion ($P < 0.05$), with the highest value observed in T0 (184.6 mL), followed by T1 (171.3 mL) and T2 (158.4 mL), indicating reduced fermentability at higher inclusion levels. At the genus level, the relative abundance of key fibrolytic taxa such as *Ruminococcus* and *Xylanibacter* decreased with increasing Indigofera inclusion, consistent with reduced gas production and altered fermentation kinetics. In addition, *Succiniclasticum*, involved in succinate conversion, declined at higher inclusion levels, suggesting shifts in downstream fermentation pathways. The increased representation of fiber-adaptive groups such as the *Christensenellaceae R-7* group indicates microbial restructuring under elevated fiber and tannin pressure rather than enhanced fermentative efficiency. Beta diversity analyses (PCoA and UPGMA) demonstrated clear separation of microbial communities in response to graded Indigofera inclusion. Overall, increasing levels of *Indigofera zollingeriana* in pellet concentrates were associated with reduced *in vitro* gas production and altered gas fermentation kinetics, accompanied by dose-dependent restructuring of the rumen microbiome, whereas low-level inclusion (T1) maintained relatively balanced fermentation characteristics and microbial stability.

Keywords | *Indigofera zollingeriana*, *in vitro* gas production, Fermentation kinetics, Rumen microbiome, 16S rRNA

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INTRODUCTION

Efficient nutrient utilization in ruminant production systems is strongly governed by rumen fermentation dynamics, particularly the capacity of microbial consortia to synchronize fiber degradation with the utilization of nitrogen. Under conventional feeding conditions, a substantial proportion of dietary protein is rapidly degraded in the rumen, resulting in excessive ammonia (NH_3) accumulation, of which only a fraction is incorporated into the microbial protein. The imbalance between ruminal nitrogen availability and fermentable energy leads to nitrogen losses via urea excretion, thereby reducing feed efficiency and increasing environmental

nitrogen emissions (Bueno *et al*., 2020; Hristov *et al*., 2022; Patra and Yu, 2015).

Plant secondary compounds, particularly tannins, have been widely investigated as nutritional modulators for mitigating excessive ruminal protein degradation. Tannins can reversibly bind to dietary proteins, forming tannin–protein complexes that are relatively stable at rumen pH but dissociate under the acidic conditions of the abomasum, thereby enhancing the flow of undegraded dietary proteins to the small intestine (Min *et al*., 2020; Mueller-Harvey, 2006; Patra and Saxena, 2011). This mechanism underpins the strategic use of tannin-containing forages and concentrates to improve the nitrogen utilization efficiency in ruminants. However, in the present study, the formation of tannin–protein complexes is presented as a conceptual background rather than a direct measured outcome (Sun *et al*., 2025). Bioactive plant-derived compounds have been widely explored as rumen modifiers, as *in vitro* studies have indicated their capacity to alter fermentation patterns and induce shifts in rumen microbial community structure in response to phytochemical exposure (Daning *et al*., 2020, 2022).

However, excessive tannin inclusion has been associated with adverse effects on rumen fermentation, particularly through the suppression of cellulolytic activity and the inhibition of fibrolytic microbial populations. High tannin concentrations may precipitate microbial enzymes, alter cell membrane integrity, and disrupt microbial metabolism, ultimately leading to reduced fiber digestibility and compromised animal performance (Costa *et al*., 2018; Jayanegara *et al*., 2020; Niderkorn and Jayanegara, 2021). Therefore, identifying the optimal inclusion levels that balance beneficial protein protection with minimal negative effects on fermentation remains a critical challenge.

Indigofera zollingeriana is a tropical legume forage characterized by its high crude protein content and the presence of condensed tannins, making it a promising alternative protein source for ruminant diets. Previous studies have demonstrated the potential of Indigofera to partially replace conventional protein sources while supporting acceptable animal performance (Ghzayel *et al*., 2025; Nuswantara *et al*., 2025; Somanjaya *et al*., 2025). Tannins modulate the rumen microbiome by altering the fibrolytic, amylolytic, and ureolytic bacterial communities, which can impact nutrient digestion and fermentation processes (Carrasco *et al*., 2017). Most available studies have focused on productive performance or chemical composition, with limited integration of fermentability parameters and rumen microbial ecology. Nevertheless, the dose-dependent effects of *Indigofera zollingeriana* on rumen fermentability and microbial ecological structure remain insufficiently characterized.

Advances in high-throughput sequencing of the *16S rRNA* gene have enabled the detailed characterization of rumen microbial communities and their responses to dietary interventions. These approaches provide critical insights into how dietary tannins and protein-rich forages reshape microbial structure and function beyond conventional fermentation indicators (Ahmad *et al*., 2020; Yanza *et al*., 2021). Despite this progress, comprehensive evaluations of graded Indigofera inclusion levels on *in vitro* fermentability and concurrent shifts in rumen microbiome structure are scarce.

Therefore, this study aimed to evaluate the dose-dependent effects of graded inclusion levels of *Indigofera zollingeriana* pellet concentrate on *in vitro* gas production, digestibility, and rumen microbial community structure of goats. We hypothesized that increasing levels of *Indigofera zollingeriana* would progressively alter fermentability and induce structural shifts in the rumen microbiome.

MATERIALS AND METHODS

Experimental design

This study was conducted using a completely randomized design with three treatments: T0 (0% Indigofera), T1 (13.57% Indigofera), and T2 (27.13% Indigofera), each with four replicates. The experimental setup and treatment structure were designed to evaluate the dose-dependent effects of graded Indigofera inclusion on *in vitro* gas production and rumen microbiome structure. This study was approved by the Research Ethics Commission of Universitas Brawijaya (No. 035-KEP-UB-2023).

Chemical composition analysis

Proximate analysis and Van Soest fiber fraction tests (NDF and ADF) were conducted at the Livestock Assembly and Modernization Agency (BRMP) for Large Ruminants, Pasuruan. Gas production was carried out at the Nutrition Biochemistry Laboratory, Faculty of Animal Science, UGM. Proximate analysis was performed on all feed ingredients and concentrates, including dry matter (DM), organic matter (OM), crude protein (CP), crude fiber (CF), crude fat (EE), ash, and nitrogen-free extract (NFE), according to the AOAC (2006) method and results were presented in Table 1. NDF and ADF fiber fraction analyses were conducted using the Van Soest method (Van Soest *et al*., 1991).

Rumen fluid collection

Rumen fluid was obtained from fistulated Balinese cattle fed king grass and concentrate according to NASEM (2016). Samples were collected before morning feeding, placed in a 39 °C thermos, filtered through a four-layer gauze, and maintained under anaerobic conditions with CO_2 flow.

Table 1: Ingredient composition and chemical characteristics of experimental pellet concentrates across treatments (T0–T2).

Feed Ingredient	T0 (%)	T1 (%)	T2 (%)
Sorghum Silage	50.00	50.00	50.00
Indigofera zollingeriana	0.00	13.57	27.13
DDGS	10.85	5.40	1.80
Copra meal	9.04	4.53	1.81
Coffee hull	7.69	4.07	1.35
Soybean meal	7.23	6.33	3.62
Rice bran	4.53	4.53	4.53
Corn Gluten Feed (CGF)	3.62	3.62	3.62
Fat powder	2.72	2.72	2.72
Cassava meal	1.81	2.72	1.81
Molasses	1.81	1.81	0.91
Premix	0.45	0.45	0.45
Arginine–Tannin	0.25	0.25	0.25
Chemical content			
Dry Matter (%)	89.99	89.95	90.15
Organic Matter (%)	89.62	89.10	89.71
Crude Protein (%)	14.49	13.31	13.27
Crude Fiber (%)	22.10	27.74	28.96
Ether Extract (%)	4.82	3.80	4.40
Ash (%)	10.38	10.90	10.29
Nitrogen-Free Extract (%)	48.23	44.26	43.09
NDF (%)	44.88	38.47	38.01
ADF (%)	30.96	29.5	29.23

Note: DDGS: Dried Distillers Grains with Soluble; NDF: neutral detergent fiber; ADF: acid detergent fiber.

In vitro gas production technique

In vitro fermentation was performed according to the method Menke *et al.* (1979). A total of 300 mg of substrate was placed into a glass syringe, and 30 mL of fermentation medium (rumen fluid: McDougall buffer 1:2) was added. The incubation was carried out at 39 °C, and the gas volume was recorded at 0, 2, 4, 6, 12, 24, and 48 h. Gas production kinetics were described using the non-linear model proposed by (Ørskov and McDonald, 1979), expressed as: $G(t) = a + b\ (1 - e^{-ct})$, where $G(t)$ represents cumulative gas production (mL) at incubation time t (h), a is the gas production from the immediately soluble fraction, b is the gas production from the insoluble but fermentable fraction, and c is the fractional rate constant of gas production. Model parameters were estimated by non-linear regression using measured gas production data across incubation times.

Microbiome analysis (16S rRNA NGS)

Bioinformatics analyses were performed using several R packages. Three fermentation media samples were centrifuged at 7,000 rpm for 15 min. The resulting pellet was used for DNA extraction using the Purelink Genomic DNA Mini Kit (Invitrogen Cat No K 8200). The obtained pellet was resuspended in 80 µl lysozyme digestion buffer containing 20 mg/ml lysozyme and incubated at 37 °C for 30 min. Then, 20 µl of Proteinase K was added and homogenized using a vortex. Next, 20 µl of PureLink Genomic Lysis/binding buffer was added and homogenized again using a vortex. The mixture was then incubated at 55 °C for 30 min. After incubation, 200 µl of 96-100% ethanol was added, followed by DNA purification. The DNA isolation product was placed in a spin column and centrifuged at 7,000 rpm for 1 min at room temperature (RT). The collection tube was removed, and the spin column was placed in a clean PureLink Collection Tube. A total of 500 µl of wash buffer 1 was added to the spin column and centrifuged at 7,000 rpm for 1 min, at room temperature. The collection tube was removed, and the spin column was placed in a clean PureLink Collection Tube. Then, 500 µl of wash buffer 2 was added and centrifuged at maximum speed for 3 min at room temperature, after which the collection tube was discarded. The spin column was transferred to a sterile 1.5 ml microcentrifuge tube,25-200 µl PureLink Genomic Elution Buffer was added, and the mixture was incubated at room temperature for 1 min. The column was then centrifuged at maximum speed for 1.5 min at RT. The DNA isolation and purification results were checked using 2% agarose electrophoresis and stored at -20 ºC for further analysis in diversity testing using metagenomic analysis methods based on 16S rRNA sequences at the universal bacterial hypervariable (V4) region (Rifa'i *et al.*, 2025).

Metagenomic analysis was performed using next-generation sequencing (NGS). Metagenomic analysis of methanogenic archaea was based on the 16S rRNA sequence in the hypervariable V4 region (approximately 300 bp). The isolated DNA samples were sent to 1st BASE (Axil Scientific Pte. Ltd. Malaysia) for DNA sequencing. The 16S library preparation workflow included a first round of PCR with the Illumina 16S metagenomics library prep kit, followed by PCR clean-up with AMPure XP Beads (Beckman Coulter, cat. no. A63880) and 80% ethanol according to the product protocol to remove the adapter dimers. Library sequencing was performed using the Illumina MiSeq platform with the 2×301PE format and Illumina MiSeq reagent kit v2. The resulting sequence data were used for bioinformatics analysis. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). QIIME was used to filter raw tags to obtain higher quality data (http://qiime.org/scripts/split_libraries_fastq.html) (Bokulich *et al.*, 2015). The generated tags were compared to a database (http://drive5.com/

uchime/uchime_download.html) and (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimeric sequences (Edgar *et al.*, 2011).The final step in obtaining effective tags was to remove chimeras using the link below: http://www.drive5.com/usearch/manual/chimera_formation.html (Haas *et al.*, 2011).

Species cluster annotation and operational taxonomic units (OTU). All effective tags were used to analyze the sequences using Uparse software (Uparse v7.0.1001 http://drive5.com/uparse) (Edgar and Flyvbjerg, 2015). Identical OTUs were obtained with a similarity of > 97%. Mothur software was used for distinct OTU sequences, and the sorting results were compared with the SSUrRNA database through the SILVA Database (http://www.arb-silva.de/) . The phylogenetic relationships of all OTUs were analyzed using MUSCLE Version 3.8.31 (http://www. drive5. com/muscle) (Edgar, 2004). OTU abundance data were obtained through alpha and beta diversity analysis. Alpha Diversity (Intra-Sample Analysis). Alpha diversity consisted of observed species, Chao1, Shannon, Simpson, ACE, and Good coverage. All indices were analyzed using QIIME (Version 1.7.0), and data interpretation was performed using R software (Version 2.15.3). Beta Diversity Analysis, differences between groups based on species complexity were valuated using beta diversity analysis using QIIME (Version 1.7.0). Microbiome analysis in this study was intended to describe the microbial community structure rather than to infer specific metabolic fluxes or functional protein utilization pathways.

Statistical analysis

Gas production was analyzed using one-way ANOVA. Significant differences ($P < 0.05$) were further tested using Duncan's Multiple Range Test (DMRT). The analysis was performed using SPSS v26. Microbiome community composition data were analyzed descriptively based on relative abundance and beta diversity metrics (PCoA and UPGMA). No ANOVA was applied to sequencing data, as relative abundance data derived from high-throughput sequencing do not fulfill parametric assumptions and are conventionally interpreted using multivariate approaches.

RESULTS AND DISCUSSION

Gas production and gas kinetics

The results showed that the difference in the proportion *of Indigofera zollingeriana* in pellet concentrate had a significant effect on *in vitro* gas production, as presented in Figure 1. The decrease in gas production as the level of Indigofera increased reflected changes in substrate characteristics and microbial responses to the nutrient components.

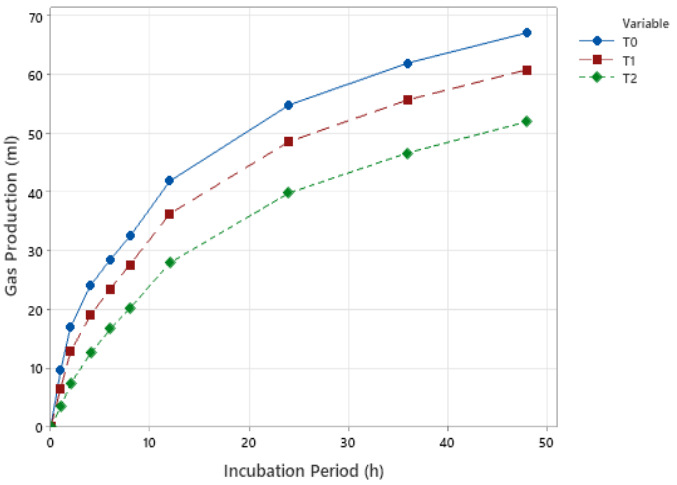

Figure 1: Gas production and incubation period.

The gas production curve (Figure 1) shows an increase in gas volume throughout the incubation period, but the rate and total gas production decreased consistently with increasing Indigofera levels. The T0 treatment produced the highest gas volume at all incubation times, followed by T1 and T2. This pattern indicates that the higher the fraction of Indigofera in the concentrate, the lower the fermentability of the substrate by the rumen microbes. The reduction in gas production at higher Indigofera levels reflects decreased substrate accessibility under increased fiber and tannin content rather than a complete failure of ruminal fermentation. These findings are in line with previous reports that increased structural fiber fractions and phenolic compound content, particularly condensed tannins, can limit the degradation of fermentable substrates and decrease *in vitro* gas production (Ramdani *et al.*, 2022).

The intermediate response shown by the T1 treatment indicates that at low levels of inclusion, Indigofera is still able to provide a relatively volatile fraction of nutrients, especially in the early stages of incubation. This is in line with the concept that the balance between available proteins, non-structural carbohydrates, and bioactive compounds determines the kinetic pattern of gases during *in vitro* fermentation (Getachew *et al.*, 2005). However, in the advanced incubation phase, the degradation ability of fibers tends to decrease because of the increased inhibitory effect of tannins on cellulolytic microorganisms.

Decreased fermentability at high Indigofera levels also has implications for changes in VFA patterns, where the reduced activity of acetate- and butyrate-producing microbes contributes to low total gas production. This phenomenon has been reported in various *in vitro* fermentation systems involving medium to high tannined feed materials, which consistently show gas degradation as an indicator of limited substrate degradation.

Composition of the rumen microbiome

The structure of the rumen microbiome at the phyla and genus levels is presented in Table 2.

Table 2: Distribution of microbiota taxonomy.

Taxonomy	Treatment		
	T0	T1	T2
Phyla	16	16	16
Class	21	22	21
Order	36	37	39
Family	66	68	72
Genus	108	110	121
Species	16	16	18

Description= P0 (control), P1 (low Indigofera), P2 (moderate Indigofera).

It shows the number of rumen microbiota taxa detected at different taxonomic levels (phyla to species) in each treatment group (T0, T1, and T2). In general, the number of phyla identified was relatively uniform between treatments, with 16 phyla in all groups. This suggests that the inclusion of *Indigofera zollingeriana* at the highest levels does not eliminate the main microbial groups in the rumen but rather influences the community structure at a lower taxonomic level. At the class and order levels, there was a gradual increase from T0 to T2, with the highest number of orders detected at T2. This pattern indicates the differentiation of microbial communities in response to changes in ration composition, particularly an increase in structural fiber fractions and bioactive compounds, such as tannins. This increase in microbial richness at higher inclusion levels reflects the adaptive restructuring of the rumen microbiome under phytochemical stress rather than improved fermentative efficiency. Previous studies have shown that nutrient and phytochemical pressures tend to drive the reorganization of microbial communities at the intermediate taxonomic level (class and order) without altering the dominance of the main phyla of the rumen (Susanto *et al*., 2024, 2025).

The most pronounced differences were observed at the family and genus levels, where T2 showed the highest number of families (72) and genera (121) compared to T0 and T1. The increase in taxonomic richness at this level reflects the adaptive response of the microbiome to a more complex and challenging fermentation environment owing to the increased fiber and tannin content. These conditions allow the emergence of tannin-tolerant microbial groups and microbes with high metabolic flexibility, although this is followed by a decrease in the dominance of major fibrolytic bacteria, as reflected in the results of fermentability and gas production. At the species level, the number of detected species was relatively stable at T0 and T1 (16 species) but increased at T2 (18 species). These improvements do not necessarily reflect improved fermentation function but indicate community fragmentation and the emergence of minor species capable of surviving suboptimal fermentation conditions (Hristov *et al*., 2012). Overall, the taxonomic distribution in Table 2 shows that increasing levels of *Indigofera zollingeriana* does not alter the basic framework of the rumen microbiome but promotes community restructuring at the family, genus, and species levels. These findings support the results of beta diversity and microbiome composition analysis, which suggest that T2 treatment undergoes the most pronounced shift in microbial communities in response to increased fiber and tannin fractions in the diet (Huws *et al*., 2021). The observed reduction in gas production and fibrolytic taxa at higher Indigofera inclusion levels may be attributed to increased fiber complexity and the presence of bioactive compounds, such as tannins, which can suppress cellulolytic activity and alter microbial metabolic pathways. These conditions likely promote microbial restructuring rather than enhanced fermentative efficiency.

Alpha and beta diversity

Alpha diversity in T2 tended to be lower than that in T0 and T1. Beta diversity analysis showed a clear separation of microbial communities between treatments, with T1 being in an intermediate position between T0 and T2 (Figure 2).

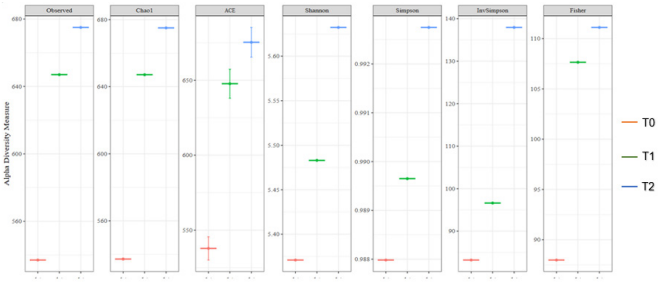

Figure 2: Alpha diversity.

Figure 2 shows the alpha diversity index of the rumen microbiome in the three treatments based on various metrics, namely observed species, Chao1, ACE, Shannon, Simpson, Inverse Simpson, and Fisher. Overall, there was a consistent pattern in which increased levels of *Indigofera zollingeriana* in the pellet concentrate resulted in gradual changes in rumen microbial diversity.

The T0 treatment exhibited the highest alpha diversity value across all indices. The high values of observed species and Chao1 indicate a greater number of species and a higher estimate of richness compared to T1 and T2. This reflects a stable rumen condition with optimal microbial activity in degrading fiber fractions and complex carbohydrates, in accordance with high gas production. The high Shannon, Simpson, and Inverse Simpson values indicate that

microbial communities at T0 are not only richer but also more evenly distributed, without the dominance of certain species being too strong. Increased levels of *Indigofera zollingeriana* in concentrated pellets decreased rumen fermentability, as reflected by decreased gas production. This decrease is consistent with the increased lignin and tannin content, which can limit microbial access to substrates and inhibit the activity of fibrolytic enzymes (Abid *et al*., 2025). This was reinforced by the reduced abundance of major fibrolytic taxa in the T2 treatment group. T1 treatment showed a relatively moderate decrease in gas production and maintained a stable microbial community structure (Barrett *et al*., 2022). The presence of a group of fermentative microbes at this level indicates that low doses of Indigofera can still be utilized without disturbing the balance of the rumen microbiome.

Rumen microbiome composition and diversity (NGS)

Relative abundance of Rumen Bacterial phyla

Figure 3 shows that Bacillota and Bacteroidota were the dominant groups at the phyla level in all treatments. Bacillota accounted for 51.67% at T0, dropping to 37.97% at T1, and then increasing again to 51.23% at T2. In contrast, Bacteroidota increased from 25.63% (T0) to 37.51% (T1) before decreasing slightly to 31.76% (T2). Other phyla with striking proportions are Planctomycetota, which gradually decreased from 7.94% (T0) to 5.67% (T1) and 2.94% (T2). An increase in Indigofera in the diet resulted in measurable changes in the community structure of the rumenic bacterial phyla. The decrease in Bacillota abundance at T1, followed by a return increase at T2, suggests that the microbial response to Indigofera levels is non-linear, possibly related to variations in the availability of fermentation substrates and phytochemical pressures. Higher abundance of Bacteroidota at T1 indicates that the fraction of hemicellulose and non-structural components at the low–medium level of Indigofera is more appropriate for this group, while the decrease in T2 reflects reduced fiber degradation efficiency due to increased tannins and lignification (Abo-Sherif *et al*., 2025).

Relative abundance of rumen bacterial class

Figure 4 shows that the microbial communities at the grade level were dominated by Clostridia and Bacteroidia in all treatments. The relative abundance of Clostridia was 40.77% at T0, which decreased to 31.84% at T1 and then increased to 43.17% at T2. In contrast, Bacteroidia increased from 25.63% (T0) to 37.51% (T1) before declining to 31.76% (T2). The Negativicutes group decreased sharply from 10.33% (T0) to 5.46% (T1) and slightly increased to 6.87% (T2). The class Planctomycetes also gradually declined from 7.94% (T0) to 5.67% (T1) and 2.94% (T2). Minor classes, such as Gammaproteobacteria, showed an increase from 6.07% (T0) to 10.11% (T1) and then decreased slightly to 8.26% (T2), whereas Anaerolineae, Synergistia, Lentisphaeria, and Verrucomicrobiia remained at low levels (<2%). Patterns of microbial composition changes at the grade level indicate that Indigofera levels affect the balance between fibrolytic and nonfibrolytic bacteria. A decrease in Clostridia at T1, followed by an increase at T2, indicates that this group is sensitive to variations in the fiber and tannin components. The decrease in Planctomycetes and Negativicutes in T2 indicates the sensitivity of this group to nutrient and chemical stress at high levels of *Indigofera* spp. (Norris *et al*., 2020). These patterns suggest that T1 represents a transitional inclusion level that maintains microbial balance, whereas T2 reflects a threshold-driven restructuring of the rumen microbial ecosystem.

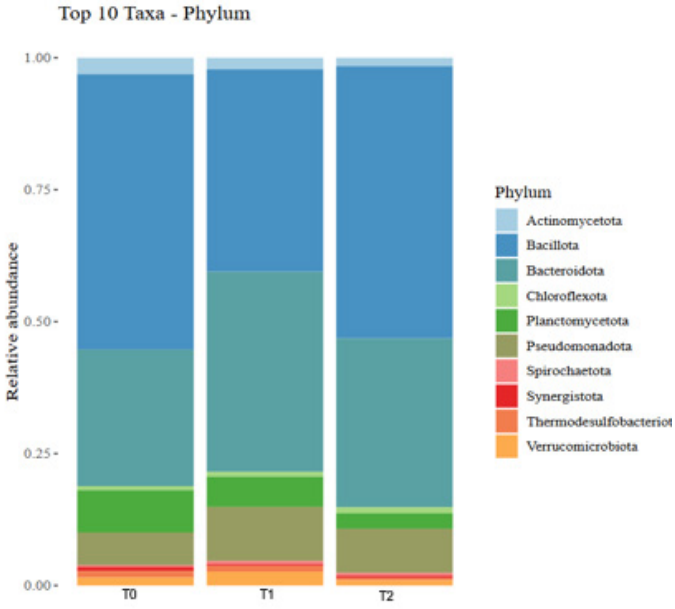

Figure 3: Relative abundance of rumen bacterial phyla.

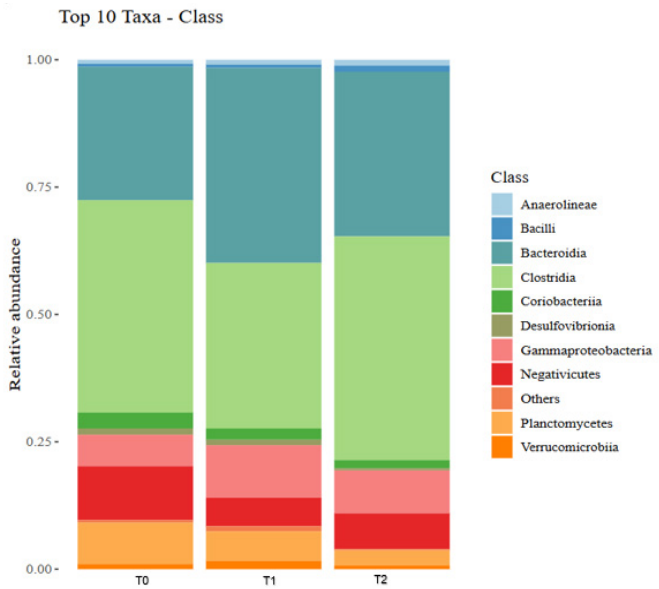

Figure 4: Relative abundance of rumen bacterial classes.

Relative abundance of rumen bacterial orders

The rumen microbiota community at the order level was dominated by Bacteroidales, Lachnospirales, and Oscillospirales across treatments (Figure 5). Bacteroidales increased from 25.63% (T0) to 37.51% (T1), and then decreased to 31.75% at T2. Lachnospirales decreased from 15.19% (T0) to 10.39% (T1) and then increased sharply to 19.66% (T2). Oscillospirales showed a gradual decreasing pattern from 12.26% (T0) to 9.84% (T1) and 9.17% (T2). The Christensenellales group remained high in all treatments (10.31%–12.09%), while Pirellulales experienced significant decreases from 7.94% (T0) to 5.67% (T1) and 2.94% (T2). Several minor orders underwent significant changes. For example, Burkholderiales increased from 3.41% (T0) to 6.59% (T1), and then decreased to 4.34% (T2).

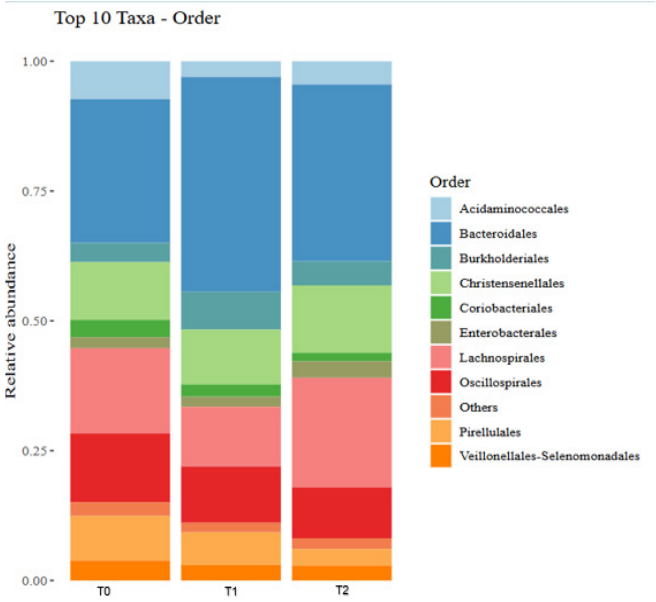

Figure 5: Relative abundance of the orders of ruminal bacteria.

Changes in microbial community patterns at the order level indicate that the proportion of Indigofera in the diet has a direct effect on the main fermentative groups in the rumen. An increase in T1 indicates that low–medium levels of Indigofera provide a substrate that supports the activities of this group of microorganisms. The decrease in T2 is consistent with the assumption that higher tannin fractions inhibit the degradation of carbohydrates. Lachnospirales (producers of butyrate and complex fiber degraders). A sharp increase in T2 (19.66%) indicated an adaptive response to phytochemical stress, supporting the findings of the significantly increased abundance of the *Lachnospiraceae* AC2044 group. The community structure at the order level reinforces the PCoA and genus analysis conclusions, namely that T2 treatment results in the most diverse microbial communities and is better adapted to phenolic and high-fiber stresses (Ammar *et al.*, 2023).

Relative abundance of rumen bacterial families

As shown in Figure 6, the composition of the rumen microbiota at the family level was dominated by *Lachnospiraceae*, *Rikenellaceae*, *Christensenellaceae*, and *Prevotellaceae* in all treatments. *Lachnospiraceae* decreased from 15.19% at T0 to 10.39% at T1 and then increased sharply to 19.66% at T2, indicating an adaptive response to the increased fiber fractions and complex components of Indigofera. *Rikenellaceae* increased from 14.18% (T0) to 19.48% at T1 before decreasing to 16.70% at T2, suggesting that this group is highly responsive to changes in non-structural carbohydrate availability. *Christensenellaceae* remained relatively stable across treatments, at 10.31% at T0, 9.57% at T1, and increased to 12.09% at T2, indicating a consistent role in fiber fermentation. Meanwhile, the abundance of *Prevotellaceae* increased slightly from 6.27% at T0 to 7.94% at T1 and remained stable at T2 (7.67%). Several other families showed noticeable changes. F082 nearly tripled from 2.25% at T0 to 6.04% at T1 before declining again to 3.95% at T2. Comamonadaceae also experienced an initial increase from 2.78% at T0 to 5.32% at T1, then decreased to 3.57% at T2. In contrast, Pirellulaceae showed a consistent decrease from 7.94% at T0 to 5.67% at T1 and 2.94% at T2, indicating sensitivity to the phenolic components of Indigofera. Selenomonadaceae also experienced a slight decrease from 3.55% at T0 to 2.74% at T1 and 2.71% at T2.

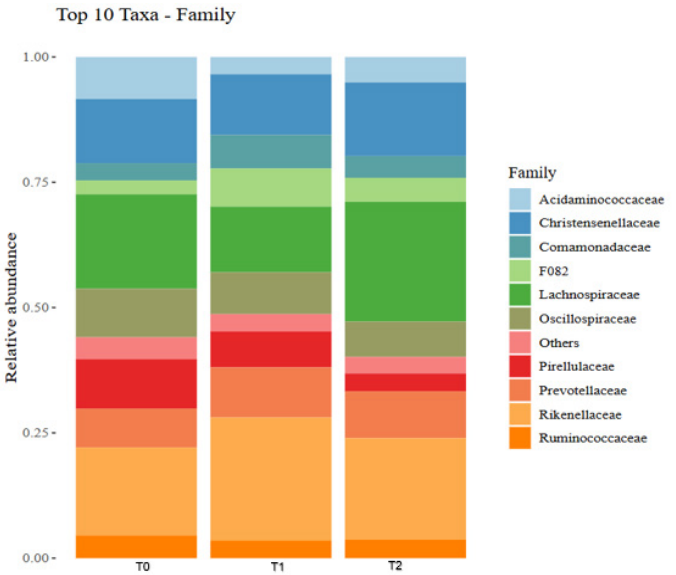

Figure 6: Relative abundance of rumen bacterial families.

Changes in the abundance patterns of the bacterial family suggest that increased levels of Indigofera in the diet exert different nutritional and chemical pressures on the rumen microbiome community. A sharp increase in *Lachnospiraceae* abundance at T2 suggests that this group, known as complex fiber degraders and butyrate producers, is well adapted to higher lignocellulose and tannin-producing contents. This is in line with the increase in several genera

under *Lachnospiraceae* in subsequent analyses. *Rikenellaceae* and *Prevotellaceae*, which play a role in the fermentation of non-structural carbohydrates, showed an increase in T1, indicating the availability of substrate fractions that support fermentative activity at low to moderate levels of Indigofera. However, the decrease in both at T2 suggests that the high tannin and fiber content begins to inhibit metabolism that relies on easily fermentable carbohydrates.

The progressive decline of Pirellulaceae from T0 to T2 suggests that this group is sensitive to increased concentrations of phenolic compounds, whereas the patterns of Comamonadaceae and F082 increased at T1 but decreased again at T2, indicating a short-term adaptation to changes in ration composition. The stability of *Christensenellaceae* in all treatments confirmed the important structural role of this family in maintaining the fermentation balance of the rumen, especially in the degradation of fibrous substrates (Bian *et al*., 2019). Overall, the pattern of change at the family level suggests that moderate levels of Indigofera provide more balanced fermentation conditions, whereas high levels (T2) result in a larger restructuring of microbial communities.

Relative abundance of rumen bacterial genera

Analysis of the composition of the microbiome at the genus level showed that the rumen bacterial community is dominated by the *Christensenellaceae R-7* group, the *Rikenellaceae RC9 gut group*, *Xylanibacter*, and several genera of the *Lachnospiraceae* family (Figure 7). The abundance of the *Christensenellaceae R-7* group remained relatively stable across the treatments, at 11.45% at T0, 11.16% at T1, and 13.54% at T2, indicating a consistent role in the fermentation of basic fibers. The *Rikenellaceae RC9 gut group* increased sharply at T1 (18.74%) compared to T0 (12.51%), before decreasing again at T2 (15.26%), indicating a response to changes in the availability of non-structural substrates in Indigofera-based diets.

The abundance of *Xylanibacter* also increased at T1 (from 5.91% to 7.55%) and remained at a similar level at T2 (7.54%), supporting its role in the degradation of the hemicellulose fraction. A different pattern was observed for *Comamonas*, which doubled from T0 (3.08%) to T1 (6.19%) before declining again to 3.97% at T2, suggesting a short-term adaptation to changes in nutrient components. The most prominent changes were observed in several genera that were sensitive to tannins and lignocellulose fractions. The *Lachnospiraceae* AC2044 group increased significantly at T2 (from 1.00% and 1.21% at T0 and T1 to 3.59%), reflecting the ability of the group to adapt to the increased components of the Indigofera complex. A similar trend was observed for *Ruminobacter*, which increased from 0.61% (T0) to 2.54% at T2. In contrast, *Succiniclasticum* experienced a sharp decrease in abundance in T1 (3.18%) compared to T0 (7.53%), and then slightly increased to 4.67% in T2, in line with the changes in the VFA profile, especially the shift in the propionate fermentation pathway.

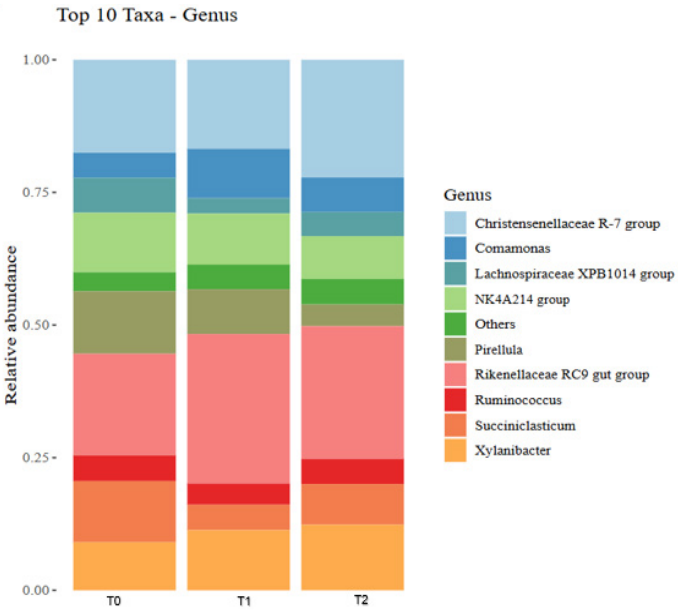

Figure 7: Relative abundance of the genera of rumen bacteria.

Changes in the composition of the microbiome at the genus level suggest that the addition of Indigofera at different levels results in a pronounced ecological response in the rumen microbial community. The stable abundance of the *Christensenellaceae R-7* group in all treatments with a slight increase in T2 illustrates that this group acts as a core genus that remains active in fiber fermentation, regardless of variation in ration composition. This is in line with the characteristics of the genus, which is known to play a role in the metabolism of structural fibers and maintain the stability of rumen communities (Cheng *et al*., 2025).

An increase in the *Rikenellaceae RC9 gut group* at T1 suggests that low–moderate levels of Indigofera support the activity of the complex carbohydrate breakdown group and metabolic intermediates in the rumen. However, the decrease in T2 reflects the presence of nutrients or chemical pressures (tannins and lignins) that begin to limit the fermentative activity of this group. A similar response was also observed in *Xylanibacter*, which increased at T1 and remained high at T2, reinforcing the interpretation that the hemicellulose fraction of Indigofera could still be utilized by certain groups despite an increase in the overall lignocellulose fraction (Anderson *et al*., 2021).

The genus that showed the sharpest changes was some members of *Lachnospiraceae*, notably the *Lachnospiraceae AC2044 group*, which increased significantly at T2. This shows that this group has the ability to adapt to more

challenging fermentation conditions due to the increased fiber and bioactive compounds of Indigofera. This increase is in line with the rise of *Ruminobacter* at T2, which tends to take advantage of simple sugars from the degradation of structural components, suggesting that fermentation is progressing towards a more intensive pattern of fiber degradation, even though total digestion is decreasing. In contrast, the decrease in *Succiniclasticum* abundance at T1 and partial recovery at T2 indicate that the propionate fermentation pathway is not optimally functioning in the early stages of Indigofera supplementation. Considering that *Succiniclasticum* is a key genus in the conversion of succinate to propionate, this decrease is in line with the changes in the VFA composition observed during the *in vitro* fermentation stage.

Overall, changes in genus composition in T2 showed that Indigofera accounted for 27.13% of the community, triggering a larger microbial community restructuring than that in T0 and T1. This shift consists of a decrease in the sensitive fermentative group (e.g., *Succiniclasticum*) and an increase in the adaptive group to high-fiber and tannin-rich conditions (e.g., *Lachnospiraceae AC2044* group). This pattern is in line with the decrease in digestibility in T2 and the differences in fermentation profiles observed in *in vitro* assays (Andersen *et al*., 2025; Chen *et al*., 2024). The relatively stable abundance of *Prevotellaceae* suggests that proteolytic activity was maintained at low-to-moderate Indigofera inclusion levels (Ramos *et al*., 2025). The observed microbial and fermentative responses to graded *Indigofera zollingeriana* inclusion are consistent with the known adaptive capacity of tropical forage legumes to varying nutrient availability and environmental stressors, which influence biomass production, structural composition, and subsequent rumen fermentability (Latief *et al*., 2018, 2020).

CONCLUSION

Graded inclusion of *Indigofera zollingeriana* in pellet concentrate exerted a dose-dependent influence on *in vitro* rumen fermentation and microbial community structure, as reflected by a progressive reduction in cumulative gas production and fermentability at higher inclusion levels of the feed. These changes were accompanied by distinct shifts in the rumen microbiome, characterized by a decreased abundance of key fibrolytic taxa (*Ruminococcus*, *Fibrobacter*, and *Succiniclasticum*) and an increased representation of tannin-tolerant bacterial groups under high Indigofera inclusion. Low-level inclusion (T1) maintained relatively favorable fermentation characteristics and microbial stability, whereas high-level inclusion (T2) induced pronounced microbial restructuring with reduced fermentative efficiency. Overall, these results demonstrate that *Indigofera zollingeriana* can be utilized at controlled inclusion levels, while higher levels require careful consideration due to their effects on fermentability and microbial ecological structure.

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NOVELTY STATEMENT

This study provides integrated evidence linking *in vitro* gas production kinetics with graded inclusion levels of *Indigofera zollingeriana* pellet concentrate and concurrent structural shifts in the ruminal microbiome. Unlike previous studies, this study combined fermentability parameters and 16S rRNA-based microbial community analysis to elucidate dose-dependent microbial responses to a tannin-containing forage.

AUTHOR'S CONTRIBUTION

MFL conceived and designed the study, conducted the experiments, performed data analysis, and drafted the manuscript.
SS, OS, and JAS, supervised the research, provided critical scientific input, and reviewed the manuscript.
All authors read, revised, and approved the final manuscript.

Generative AI and AI-assisted technology statement

The authors declare that no generative artificial intelligence or AI-assisted technologies were used to generate data, analyze results, or write the scientific content of this manuscript. AI tools were not used to produce figures, tables, or interpretation

Conflict of interest

The authors have declared no conflict of interest.

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