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ORIGINAL PAPER

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Supplementation with *Gymnopodium floribundum* alters rumen bacterial function and nitrogen utilisation in lambs: a potential role for condensed tannins

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Abstract. This study evaluated the effects of *Gymnopodium floribundum* Rolfe on feed intake, nitrogen metabolism, fermentation, and the rumen microbiome of lambs. After a 14-day adaptation period, eight male lambs were assigned to two groups and fed their respective diets for 22 days. The control diet (CD, n = 4) consisted of grass and concentrate (55:45), whereas the experimental diet (ED, n = 4) of grass, concentrate, and *G. floribundum* (25:45:30). Ruminal fluid was collected during two sampling periods. Relative to the CD group, the ED group had higher dietary crude protein intake ($P = 0.030$), 1.68-fold lower nitrogen utilisation ($P = 0.030$), and 2.26-fold greater faecal nitrogen excretion ($P = 0.030$). Redundancy analysis ($P = 0.031$) identified propionate and butyrate as the volatile fatty acids most strongly associated with the dietary treatment, whereas nitrogen retention and dry matter intake were the variables contributing most to the observed variation. Diet explained 11% of the total variation ($R^2 = 0.11$) and was associated with changes in bacterial taxa

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involved in structural carbohydrate degradation and volatile fatty acid metabolism. The relative abundance of *Saccharofermentans*, *Lachnospiraceae_ND3007_group*, and *UCG-004* decreased in ED lambs, together with reduced activity of the L-arginine degradation II (P = 0.010) and L-methionine salvage cycle III (P = 0.007) pathways. These changes may be related to the presence of condensed tannins and other bioactive compounds. Overall, *G. floribundum* altered the rumen microbiota, nitrogen utilisation, volatile fatty acid metabolism, and predicted bacterial metabolic functions. These findings suggest that dietary inclusion of *G. floribundum* may shift bacterial metabolism towards alternative pathways involved in butyrate production.

Key words: bacteria, condensed tannins, fermentation, metagenome, nitrogen balance, volatile fatty acids

Introduction

Bacteria and other microorganisms in the rumen are essential for feed fermentation and contribute to the metabolism and homeostasis of ruminants (Russell and Hespell, 1981; Petri et al., 2013; Henderson et al., 2015). Therefore, studying the rumen microbiome may improve our understanding of microbial processes related to methane emissions, fibre digestibility, feed efficiency, and the use of natural feed resources (Russell and Hespell, 1981; Firkins and Yu, 2015).

Due to their high abundance and functional diversity, bacteria are among the most important members of the rumen microbiome, contributing substantially to the degradation of structural carbohydrates in the ruminant diet (Krause and Russell, 1996; Xie et al., 2025). The composition and activity of these bacterial communities are influenced by various factors, particularly diet, which can alter bacterial composition, diversity, and metabolic functions within the rumen ecosystem (Wang et al., 2024; Nueraihemaiti et al., 2025). Consequently, changes in the microbial population structure may affect animal production traits (Sanjorjo et al., 2023). In some cases, the rumen microbiome predicts animal

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performance as accurately as diet, and combining both improves prediction accuracy (Gleason and White, 2018).

In tropical regions, ruminant diets are mainly based on grasses and other native forages, which are often characterised by low digestibility and high concentrations of structural carbohydrates, including cellulose, haemicellulose, and lignin. As a result, gross energy losses range from 2 to 12% (Kurihara et al., 1999; Ku-Vera, et al., 2013; Piñeiro-Vázquez et al., 2017). The composition of the diet, particularly the inclusion of tropical forage plants, plays an important role in determining the composition of the rumen microbiome and determining energy efficiency (Mccann et al., 2014).

Gymnopodium floribundum Rolfe (*Polygonaceae*) has several practical uses. It is used as timber and as a nectar source for honey production (Ortiz Ocampo et al., 2019). More recently, it has also been evaluated as a forage resource for small ruminants. Under browsing conditions in low deciduous forest (LDF), sheep may consume up to 11% of *G. floribundum* foliage (Ventura-Cordero et al., 2017; Jaimez-Rodríguez et al., 2019). In housed lambs, *G. floribundum* has been included in diets at levels of up to 40% (Méndez-Ortiz et al., 2019).

Although *G. floribundum* leaves have a fibre content comparable to that of tropical grasses, it contains higher concentrations of crude protein (CP; 9–12%), making it a medium-protein forage with greater nutritional value (Méndez-Ortiz et al., 2022). In addition, its leaves contain high concentrations of secondary metabolites, including polyphenolic compounds and condensed tannins (CT), throughout the year (Ortíz-Ocampo et al., 2022).

Evidence from studies on other plant species suggest that these compounds can alter the rumen microbiome composition (Bodas et al., 2012; Omondi et al., 2024). Under *in vitro* conditions, tannins and the relatively high protein content of tropical foliage affect fibre and protein fermentation as well as the diversity of fibre-degrading bacteria (Rira et al., 2022).

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These compounds have also been associated with changes in rumen fermentation products (Rira et al., 2022; Alayón-Gamboa et al., 2023).

The use of alternative forage resources, such as *G. floribundum*, may contribute to the conservation of natural areas by promoting the sustainable use of local plant species. It may also diversify ruminant diets and reduce feeding costs, particularly for small- and medium-scale producers in regions with limited infrastructure or restricted access to conventional feedstuffs. This study tested two hypotheses: 1) the volatile fatty acid profile and nitrogen utilisation in lambs are influenced by the nutritional composition of *G. floribundum* and by the effects of its condensed tannins on rumen fermentation and protein metabolism; and 2) supplementation with *G. floribundum* alters the composition of the rumen bacterial community, with nutrient availability and condensed tannins influencing the abundance of specific bacterial taxa. Therefore, the objective of this exploratory study was to characterise the rumen bacterial community in growing lambs fed diets containing *G. floribundum* and to analyse its relationship with rumen metabolic functions.

Materials and methods

Experimental design

Leaves of *Gymnopodium floribundum* Rolfe were collected from an area belonging to the Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán (UADY) (located at 20.8666° N, 89.6223° W; 8 m a.s.l.). The area is characterised by low deciduous forest (LDF) vegetation and a tropical subhumid climate (García, 2004). Plant material was collected from February 27 to November 22, 2023, between 7:00. and 11:00. During the sampling period, the mean air temperature was 28.89 °C, relative humidity was 66.22%, evaporation was 234.87 mm, and cumulative rainfall was 911.3 mm (Servicio

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Meteorológico Nacional-CONANGUA, 2023). The leaves were shade-dried in a well-ventilated enclosure and inspected daily for signs of spoilage. Drying was completed after approximately 3–4 days, when the dehydrated leaves became brittle and olive green in coloration. The dried material was then stored in polyethylene bags.

Animals

The animal study protocol was approved by the Institutional Bioethics Committee of the CCBA, Universidad Autónoma de Yucatán (UADY), México (protocol code CB-CCBA-D-2023-002, 14 July 2023).

Eight male commercial-breed hair sheep (21.01 ± 1.43 kg body weight (BW); 8 months old) were used, with each animal serving as an experimental unit. Lambs were randomly assigned to either the control (CD; n = 4) or experimental (ED; n = 4) group. The study employed a repeated-measures design consisting of a stabilisation period followed by an experimental period. To distinguish samples collected during these phases, groups are referred to as CDs and EDs during the stabilisation period and as CDep and EDep during the experimental period.

The stabilisation phase (s) lasted 21 days and consisted of 14 days of acclimation to the metabolic cage followed by 7 days of adaptation to the basal diet, comprising chopped grass and concentrate (55:45). During the final 7 days, lambs were gradually adapted to the experimental diets. The experimental period (ep) lasted 22 days, during which the experimental group (EDep) received a ration containing chopped grass, concentrate and *G. floribundum* (25:45:30), while the control group (CDep) continued to receive the basal diet (55:45).

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Diets (Table 1) were adjusted every 14 days based on BW to maintain a constant feed allowance. Due to the exploratory nature of the study and the limited sample size, conservative filtering criteria and non-parametric effect size measures were applied to improve the robustness of the analyses.

Collection of rumen fluid

Rumen fluid samples (~30 ± 10 ml) were collected at two time points: at the end of the stabilisation period (9 January 2024; day 21) and at the end of the experimental period (19 February 2024; day 22). Samples were collected 4 h after feeding to standardise rumen fermentation conditions. Rumen fluid was obtained using an oro-ruminal tube adapted to the size of the lambs (Martín et al., 2005). All procedures were performed under veterinary supervision to ensure animal welfare. A separate sterile oro-ruminal tube (5 mm diameter) was used for each lamb at each sampling to prevent cross-contamination. The first 5–15 ml of rumen fluid were discarded to minimise saliva contamination. Samples showing visible saliva contamination were discarded, and the procedure was repeated. Eight samples were collected at each sampling time (4 lambs per treatment group).

Volatile fatty acids (VFAs) in rumen liquid

A sub-sample of 4 ml of rumen liquid, previously filtered using gauze paper, was obtained. Subsequently, 1 ml of 25% metaphosphoric acid was added for preservation. The samples were stored at −4 °C until analysis by gas chromatography (Barros-Rodríguez et al., 2015).

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Feed intake and diet digestibility

During the experimental period, dry matter (DM) intake was recorded daily for lambs in both groups (CD and ED). Representative sub-samples of the offered feed were collected daily and stored at −20 °C for subsequent chemical analysis. These analyses included crude protein (CP) (method 990.03) (AOAC, 2006), neutral detergent fibre (NDF) (ANKOM method 6), acid detergent fibre (ADF) (ANKOM method 5), total phenols (TP), total tannins (TT) (Makkar, 2000), and condensed tannins (CT) (Makkar and Becker, 1993).

Apparent diet digestibility was determined by total faecal collection during the final five days of the experimental period according to the methodology described by Méndez-Ortiz et al. (2022).

Nitrogen balance and purine derivative analysis

A 10% sub-sample of the total faecal output was collected from each lamb. Similarly, a 10% sub-sample of the total urine excreted by each lamb was collected during the last 5 days of the experimental period. The pH of the urine samples was adjusted to 3.0 by adding 10% sulphuric acid, following the method of Méndez-Ortiz et al. (2022). Nitrogen consumed (N_C) faecal nitrogen (F_N), and urinary nitrogen (U_N) were used to calculate nitrogen retention (N_R), following the formula proposed by Rodríguez and Llamas, (1990):

$$N_R \text{ (g/day)} = N_c - (F_N + U_N).$$

Nitrogen efficiency (N_E) was calculated according to Méndez-Ortiz et al. (2022) as the ratio of nitrogen retained (N_R) to nitrogen consumed:

(N_C) $$N_E = \left({}^{N_R}/_{N_C}\right).$$

Purine derivatives were quantified in urine samples using the colorimetric method described by Chen et al. (1996).

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The amount of purine bases (X) absorbed in the duodenum (α, mmol/day) was estimated using the following equation:

$$\alpha = 0.84X + 0.150\text{kg}^{0.75}\exp^{(-0.25X)},$$

where: X – total urinary excretion of purine derivatives (mmol/day).

Microbial N absorbed in the duodenum (β, g/day) was estimated using the following equation:

$$\beta = 0.768 + 1.01x,$$

where: x – total urinary excretion of purine derivatives (Chen et al., 1996, Samaniego et al., 1997).

DNA extraction and 16S rRNA gene sequencing

A 1 ml sub-sample of ruminal fluid per lamb was mixed with 500 µl of DNA/RNA Shield™ (Zymo Research Corp., Irvine, California, USA) to preserve DNA integrity. Metagenomic DNA was extracted using the ZymoBIOMICS™ DNA Miniprep® Kit (Zymo Research Corp., Irvine, California, USA) according to the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Samples containing at least 200 ng of DNA were used for library preparation and sequencing of the V4–V5 region of the 16S rRNA gene. Sequencing was performed on an Illumina NovaSeq 6000 System (2×150 bp; Illumina, Inc., San Diego, California, USA) at a target depth of approximately 200 000 reads per sample. Sequencing was carried out by Novogene Co., Ltd.

Statistical analysis

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A linear mixed model (LMM) was fitted using the lme4 package in R to analyse volatile fatty acid (VFA) concentrations and voluntary feed intake in the experimental periods (CDs, EDs, CDep, and EDep) (Bates et al., 2015). The individual lamb was included as a random effect. Feed intake was analysed using the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \epsilon_{ijk},$$

Where: Y_{ijk} – response variables (feed intake), μ – overall mean, α_i – effect of diet, β_j – fixed effect of period, γ_k – random effect of lamb, and ϵ_{ijk} – residual error. For VFA concentrations, the period effect was excluded because it was highly collinear with diet. Therefore, the following model was used:

$$Y_{ijk} = \mu + \alpha_i + \gamma_k + \epsilon_{ijk}.$$

Degrees of freedom and *P*-values were estimated using the Satterthwaite approximation to ensure robust inference for the small sample size (Luke, 2017).

The normality of the residuals was tested using the Shapiro-Wilk test, and homoscedasticity was assessed using the Breusch-Pagan test and visual inspection of the residual plots. Residual diagnostics were further performed using the DHARMa package (version 0.4.7) based on 1 000 simulated residuals (Hartig et al., 2024). Formal tests for overdispersion and outliers, as well as visual inspection of Q-Q and residual-versus-predicted plots, confirmed that the model assumptions were met. Pairwise comparisons of estimated marginal means were performed using Tukey's honestly significance difference (HSD) test. The level of statistical significance was set at $P < 0.05$ for all tests.

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Nitrogen balance testing was evaluated only during the final week of the experimental period (CDep and EDep); therefore, one observation was obtained from each lamb (n = 4 per group). Treatment groups were compared using a two-tailed Mann–Whitney U test (Conover, 1999). Cliff's delta (δ) was calculated to estimate the magnitude of the treatment effect, as it is appropriate for non-parametric comparisons based on small sample sizes (Macbeth et al., 2010). A negative δ indicates higher values in the EDep group, whereas a positive δ indicates higher values in the CDep group.

A partial redundancy analysis (pRDA) was performed to evaluate the contribution of rumen metabolism and nitrogen balance variables to the total variation. Because the experimental design included repeated measures, animal identity was used as a conditioning variable to account for individual variation (Capblancq and Forester, 2021). Homogeneity of multivariate variances was validated using a permutation test (PERMDISP). Additionally, Spearman's rank correlation was performed separately for each experimental group to determine associations between variables (Siegel and Castellan, 1998). Each lamb in all analyses in this section was considered the experimental unit.

Evaluation of microbial diversity and functional prediction

The results concerning VFAs, N balance variables, purine derivatives, feed intake, apparent digestibility, BW, and average daily gain (ADG) were integrated with the 16S rRNA gene sequencing data for each lamb and experimental group.

Sequence quality filtering and adapter trimming were performed using DADA2 software (Callahan et al., 2016). Taxonomic classification was performed using the RESCRIPt package (Robeson II et al., 2021) based on the SILVA 138.2 database (Chuvochina et al., 2026), with a Naive Bayes classifier trained using the q2-feature-

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classifier plugin in QIIME2 (Robeson II et al., 2021). Sequences assigned to mitochondria, chloroplasts, or eukaryotes were removed using the *filter-seqs* plugin. An optimal sampling depth was selected based on rarefaction curves to ensure a uniform number of sequences in all samples.

The taxonomic composition of the microbial communities in the CDs, EDs, CDep, and EDep groups was compared using the Bray-Curtis (Bray and Curtis, 1957), Manhattan (Sokal and Michener, 1967), Jaccard (Jaccard, 1908), Canberra (Lance and Williams, 1967), Kulczynski (Kulczyński, 1927), and weighted UniFrac (Lozupone et al., 2011). Differences among groups were evaluated by permutational multivariate analysis of variance (PERMANOVA) using the adonis2 function in the vegan package in R (Oksanen et al., 2001), with statistical significance declared at $P \leq 0.05$. To account for the repeated-measures design, permutations were restricted within lambs using the how function from the permute package (Simpson et al., 2026). Homogeneity of multivariate dispersion was assessed using the betadisper function.

To examine the relationships between taxonomic abundance, metabolic variables, and nitrogen balance, a partial redundancy analysis (pRDA) was carried out. Similar to diversity analysis, permutations were restricted within lambs to account for the repeated-measures design. To reduce technical noise, taxa were filtered using the phyloseq package. Only taxa with a prevalence of at least 50% and a minimum relative abundance of 1% were retained (Cao et al., 2021). This conservative threshold reduced the influence of low-abundance taxa and potential sequencing artifacts (McMurdie and Holmes, 2013).

Differential abundance analysis was performed using ANCOM-BC2 (Lin and Peddada, 2020). Comparisons were made between the CDep and EDep groups and between the EDs and EDep groups to evaluate changes associated with dietary supplementation.

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Statistical significance was determined using a false discovery rate (FDR) threshold of $q \leq 0.05$. Functional prediction between the CDep and EDep groups was performed using *PICRUSt2* (Douglas et al., 2020), with the *MetaCyc* metabolic pathways database (Caspi et al., 2020). Functional profile analysis and visualisation were conducted using the ggpicrust2 package in R (Yang et al., 2023), employing the LinDA method (Zhou et al., 2022). Pathways with an FDR-adjusted $P < 0.05$ were considered significantly different. Log2 fold-change values were calculated relative to the EDep group. In all analyses described in this section, each lamb was considered an experimental unit.

Results

Feed intake

VFA production

Total dry matter (DM) intake did not differ significantly between the CDep and EDep groups (80.84 ± 2.13 and 79.40 ± 2.13 g DM/kg $LW^{0.75}$, respectively; $P = 0.656$), and lambs in both groups consumed approximately 92% of the total ration offered on a DM basis (Table 2). In the EDep group, *G. floribundum* foliage was included at 30% of the total ration offered (DM basis). Lambs consumed 90.3 ± 0.8% of the foliage offered, equivalent to 23.80 ± 0.26 g DM/kg $LW^{0.75}$ (Table 3). Consequently, *G. floribundum* represented 29.4% of the total DM actually consumed by the EDep group. Foliage intake remained stable throughout the experimental period, with no significant differences among days ($P > 0.05$; Table 3).

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Feed intake was calculated daily as the difference between the amount offered and the refusals collected the following morning, both corrected for their respective DM content. In the ED group, *G. floribundum* foliage was offered separately from the grass–concentrate mixture, allowing foliage intake to be quantified independently. Intake is expressed both as g DM/kg $LW^{0.75}$ and as the proportion (%) of the corresponding amount offered.

Nitrogen balance

The EDep group consumed 20% more nitrogen than the CDep group ($P = 0.03$, $\delta = -1.00$). Faecal nitrogen excretion was 2.27-fold higher, whereas urinary nitrogen excretion was 0.67-fold lower in the EDep group than in the CDep group ($P = 0.03$, $\delta = -1.00$). N efficiency was lower in the EDep group (27.7%) than in the CDep group (46.6%) ($P = 0.03$, $\delta = -1.00$). No significant differences were observed in purine derivatives between the groups (Table 5).

CP intake was approximately 20.41% higher in the EDep group than in the CDep group ($P = 0.03$, $\delta = -1.00$), reflecting the inclusion of *G. floribundum* in the diet. In contrast, no significant differences were observed in the ADF or NDF intake. *G. floribundum* dry leaves contained total phenols (TP), total tannins (TT), and condensed tannins (CT), whereas these compounds were not detected in *Pennisetum purpureum*. CP digestibility was 25% lower in the EDep group than in the CDep group ($P = 0.03$, $\delta = -1.00$), whereas no significant differences were found in DM digestibility (Table 5).

Interaction between dietary treatments and ruminal fermentation

In the EDep group, Spearman's rank correlations showed that acetate, propionate and butyrate concentrations were positively associated with total VFA, valerate and nitrogen retention ($P < 0.001$). However, butyrate and propionate were negatively correlated with the

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acetate-to-propionate ratio ($P < 0.001$). Faecal CP was negatively correlated with DM and CP digestibility ($P < 0.001$) (Figure 1).

The partial redundancy analysis (pRDA), based on feed intake, rumen fermentation, and nitrogen balance variables, explained 76.05% of the total variation ($P = 0.015$). The first axis explained 66.1% of the variation, whereas the second axis explained an additional 9.95% ($P = 0.015$), accounting for 76.05% of the total variation (Table 6).

The variable contributing most to the ordination were N in ration, CP intake, DM digestibility, DM intake and NDF intake. Variance partitioning showed a significant pure effect of dietary treatment (adjusted $R^2 = 0.6358$, $P = 0.006$), while the VFA profile explained an additional adjusted $R^2 = 0.2363$ ($P = 0.001$). Because nitrogen balance and intake data were available only during the experimental period (CDep and EDep), their individual contributions could not be separated from the dietary effect.

Ruminal microbial diversity

Following the quality control of 3 247 465 sequences from 16 samples, 248 710 high-quality sequences with a mean read length of 328 bp were retained. The sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1241704 and BioSample accession numbers SAMN55283468–SAMN55283483. Rarefaction analysis indicated sufficient and uniform sequencing depth across samples, with a final value of 10 878 reads. A total of 3 201 amplicon sequence variants (ASVs) were identified.

The results indicated slight differences in the composition of the rumen bacterial communities. Significant differences were detected using the abundance-based Bray–Curtis, Kulczynski, and weighted UniFrac distance metrics (global pseudo-$R^2 = 0.23$, $P \leq 0.05$;

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Table 6). However, pairwise comparisons between experimental groups were not statistically significant (Table 7).

Furthermore, similar effect sizes were observed for the incidence-based Jaccard and Canberra metrics (pseudo-$R^2 = 0.23$), supporting the consistency of the beta-diversity analysis.

Across the four analysed groups, 18 phyla, 24 classes, 31 orders, 64 families, and 123 bacterial genera were identified. The ED group contained 111 genera during the stabilisation period and 94 genera during the experimental period, representing a 15% decrease in genus richness. Conversely, the CD group contained 91 genera during the stabilisation period, and 112 genera during the experimental period, corresponding to a 23% increase in genus richness. A total of 76 genera (61.8%) were shared among all four groups (Figure 2).

Composition of the rumen microbial community and predicted functional profiles in lambs fed *Gymnopodium floribundum*

Partial redundancy analysis (pRDA), which integrated bacterial taxonomic abundances with fermentation (VFA) and nitrogen balance variables, showed that RDA1 explained 76.63% of the restricted variance ($P = 0.031$), whereas RDA2 explained 23.37% and did not contribute significantly to the model (Table 8, Figure 3).

Variance partitioning identified a significant pure effect of dietary treatment ($R^2_{adj} = 0.117$; $P = 0.031$) whereas the metabolic variables did not contribute significantly ($P = 0.114$).

Compared to the EDs group, the EDep animals showed lower abundance of *Saccharofermentans* (LFC = −2.67), *Lachnospiraceae_ND3007_group* (LFC = −2.18), and

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probable_genus_10 (LFC = −1.84) (Figure 4, Table 9). Similarly, the comparison between the EDep and CDep groups showed lower numbers of *Lachnospiraceae_ND3007_group* (LFC = −2.70), and *UCG-004* (LFC = −2.55), a genus previously not found to differ in population size, and *Incertae_Sedis_33* (LFC = −1.66), comprising 809 markers that could not be confidently assigned to a genus (Figure 5, Table 10).

Functional prediction identified differences between the CDep and EDep groups in pathways related to precursor biosynthesis, bioactive compound production, and bacterial cell growth (Figure 6). Compared to the CDep group, the EDep group showed lower predicted enrichment of the following pathways: L-arginine degradation II (AST pathway) ($P = 0.001$), superpathway of dTDP-glucose-derived O-antigen building blocks biosynthesis ($P = 0.045$), polymyxin resistance ($P = 0.001$), succinate fermentation to butanoate ($P = 1.87 \times 10^{-8}$), S-methyl-5-thio-α-D-ribose 1-phosphate degradation ($P = 0.001$), L-methionine salvage cycle III ($P = 0.001$), formaldehyde assimilation (serine pathway) ($P = 0.002$), and glycolysis V (*Pyrococcus*) ($P = 0.007$).

Collectively, these results indicate lower predicted abundances of pathways related to nitrogen and amino acid metabolism in the EDep group than in the CDep group.

Discussion

According to our analysis, DM intake and digestibility (DM and CP) were the one of the most important variables and the main factors that structured the data matrix. Therefore, many of the effects observed in this study are likely attributable to the diet. A key finding was that intake of *G. floribundum* exceeded 90% of the supply, indicating high acceptability and moderate palatability in lambs despite the presence of secondary metabolites. A high level of consumption by lambs has also been reported previously, although the diet in that

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study was offered as ground leaves and acceptability reached only 40% (Méndez-Ortiz et al., 2019).

Evidence of higher faecal N excretion in the EDep group suggests lower nutrient utilisation by the lambs. However, N balance did not differ significantly between the groups, possibly due to the higher protein content provided by the *G. floribundum* diet. This plant material also supplied CT to the diet, which can bind dietary and microbial proteins in the rumen, forming protein-tannin complexes that are less susceptible to enzymatic degradation by ruminal bacteria (Besharati et al., 2022). Increased faecal N excretion associated with CT-containing diets has previously been reported in ruminants (Costa et al., 2021; da Silva Aguiar et al., 2023; Méndez-Ortiz et al., 2022). Similarly, Méndez-Ortiz et al. (2022) found that lambs fed *G. floribundum* leaves had lower CP digestibility and N utilisation than those fed a diet without *G. floribundum*. When polyethylene glycol (PEG), a tannin-binding agent, was added to the *G. floribundum* diet, both CP digestibility and N utilisation increased, supporting the role of CT in reducing protein utilisation. In this study, the presence of CT in the EDep diet may likewise explain the higher faecal N excretion, although limited fermentable energy may also have contributed to this response.

In the present study, intake of the fibre fractions (ADF and NDF) showed no significant differences between the groups. However, CP digestibility was lower in the EDep group consuming *G. floribundum* despite its higher protein intake. The presence of CT in the DM of the EDep diet is consistent with the reduced CP digestibility and higher faecal N excretion observed in this group. However, due to the small sample size (n = 4), these findings should be interpreted with caution. These results suggest that an appropriate combination of dietary fibre and CT content may play a key role in optimising nutrient utilisation efficiency (Mangan, 1988; Méndez-Ortiz et al., 2022).

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Redundancy analysis (RDA) and Spearman correlations suggest an association between the VFA profile and nitrogen balance variables, largely mediated by DMI and nitrogen retention efficiency. Specifically, the association between decreasing butyrate concentrations and lower nitrogen retention in the EDep group, suggests a potential limitation in the availability of fermentable energy required for microbial protein synthesis under high inclusion levels of *G. floribundum*. This pattern indicates that, although fermentation remains active, the metabolic synchronisation between energy availability and nitrogen utilisation (Henning et al., 1993), may be disrupted in the EDep group. This interpretation is consistent with ruminant digestive physiology, where substrates with higher carbohydrate solubility favour greater proportions of butyrate and propionate (Li, 2021; da Silva Macêdo et al., 2022; Durango et al., 2021), thereby improving dietary nitrogen utilisation. It is also consistent with previous findings that animals consuming *G. floribundum* lose more energy in faeces and retain less energy (Méndez-Ortiz et al., 2022).

Furthermore, the results suggest that diet may substantially influence the ruminal microbiome. Although DMI was an important explanatory variable in the multivariate analysis, NDF intake had a greater contribution to the RDA. This finding is biologically significant because tropical grass and *G. floribundum*, both rich in low-digestible structural carbohydrates (Ventura-Cordero et al., 2017;Lee, 2018;Ortíz-Ocampo et al., 2022), stimulate the development of bacterial taxa involved in fibre degradation and the production of butyrate, propionate and other VFAs (Wang et al., 2016; Kumar et al., 2022; Kaminsky et al., 2023; Zhang et al., 2024; Torres-Velázquez et al., 2025). Among the identified genera, only the population of *Quinella* increased in the EDep group and has previously been associated with reduced methane production (Kumar et al., 2022).

While condensed tannins (CT) in *G. floribundum* appear to influence DM and CP

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digestibility, as suggested by the response to PEG in previous studies, the fibrous nature of the plant material may have a greater influence on the bacterial community in this context. These results suggest a functional adaptation of the microbiome to a substrate where the complexity of the plant cell wall is the primary limiting factor (Chesson, 1988; Li, 2021; Zhang et al., 2022). However, interactions with dietary protein, including that provided by *G. floribundum* (Li, 2021), and phenolic compounds such as CT (Thompson et al., 2025), may also contribute to the composition of the bacterial community. Nevertheless, further studies involving larger experimental populations are needed to confirm these observations.

Despite the dietary intervention, the EDs, CDs, EDep and, CDep groups shared a core microbiome comprising 76 genera (61.8%) that was present in both the stabilisation and experimental periods. This finding indicates a high degree of structural stability of the ruminal microbiome in these lambs. In contrast, taxonomic richness decreased by 15% in the EDep group, suggesting that CT selectively affected the abundance of certain taxa (Figures 2–3) and associated functional pathways (Figure 4), rather than causing extensive restructuring or loss of the core bacterial community.

Given the limited sample size, the associations, metabolic profiles and microbiological changes reported here should be regarded as exploratory and require confirmation in larger studies. The bacterial communities differed only slightly between the study groups. However, lambs fed *G. floribundum* had lower levels of several bacterial genera. Among these, *Saccharofermentans*, which degraded starch in yaks (*Bos grunniens*) fed high-energy diets containing maize and barley (Hu et al., 2020), was less prevalent. In cattle, high population levels of this genus have also been associated with laminitis (Dai et al., 2016; Opdahl et al., 2018). The population of *probable_genus_1* also declined; this genus has been positively correlated with high concentrations of NH_3-N and increased total VFA

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production in the rumen of Hu sheep fed rice straw silage, cabbage, and diets enriched with *Lactobacillus plantarum* (Li et al., 2022)), as well as with increased VFA levels in Holstein dairy cows (Liu et al., 2022). Other genera that declined included *Lachnospiraceae_ND3007_group*, which has been associated with energy metabolism and VFA production in cattle (Shi et al., 2021; Xu et al., 2023). The closely related *Lachnospiraceae_K4A136_group* has been linked to propionate and butyrate production in yaks and young goats (Dai et al., 2021; Wang et al., 2023), while *UCG-004* has been positively associated with average daily gain in cattle fed highly fermentable diets containing maize, wheat bran, and barley (Lee et al., 2024), as well as with lysine degradation in Hu sheep (Li et al., 2022).

Consumption of *G. floribundum* dry leaves appears to alter the ruminal bacterial community in the rumen in a manner that may affect specific fermentation pathways. Molan et al. (2001) demonstrated that CT can inhibit the growth of amylolytic bacteria in the rumen, including *Saccharofermentans* (known for starch degradation), as well as proteolytic bacteria. A reduction in the population of *probable_genus_1*0, which has been positively correlated with NH_3-N concentration and increased VFA production in the rumen, may also be consistent with this effect. Reduced populations of these bacterial taxa could contribute to the VFA profile observed in the present study, particularly the increase in acetate and the decrease in iso-butyrate and iso-valerate levels.

The functional profiles presented here are bioinformatic predictions and, given the limited sample size (n = 4), they should be regarded as preliminary rather than a definitive characterisation of microbial metabolism.

The L-arginine degradation II (AST) pathway, originally described in *E. coli*, has been linked to the dissimilation of the L-arginine carbon skeleton (Stalon et al., 1987). This

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pathway enables certain microorganisms to use arginine as their sole source of nitrogen and carbon (Cunin et al., 1986). The process begins with the succinyl-coenzyme A-dependent succinylation of L-arginine and culminates in its conversion of arginine to L-glutamate and succinate, with the concomitant release of ammonia (NH_3) and carbon dioxide (CO_2) (Itoh, 1997; Wu et al., 2024). In the rumen, L-glutamine and L-glutamate are essential for bacterial metabolism and the maintenance of host homeostasis, serving as precursors for the synthesis of protein structural blocks, glutathione (tripeptide), alanine, ornithine, citrulline, arginine, and aspartate, as well as for purine and pyrimidine (Wu et al., 2024). As discussed above, the genus *probable_genus_10* was positively correlated with NH_3-N concentrations in the rumen, supporting the proposed involvement of the L-arginine degradation II pathway.

During succinate fermentation to butanoate, succinate is converted to crotonyl-CoA through a series of enzymatic reactions, primarily producing acetate, butyrate, and hexanoate. *Clostridium kluyveri* utilises propanol and succinate as substrates in reactions yielding propionate, valerate, butyrate, and hexanoate as the main products, whereas fermentation of ethanol and succinate produces acetate, butyrate, and hexanoate (Bartsch and Barker, 1961). This metabolic pathway is an alternative route also resulting in the synthesis of β-oxidation intermediates and VFAs such as acetate and butyrate (Kenealy and Waselefsky, 1985). Genera belonging to the family *Lachnospiraceae*, including *Lachnospiraceae_ND3007_group* and *probable_genus_10*, all associated with butyrate and propionate production, were significantly decreased in lambs fed *G. floribundum*. Nevertheless, the proportion of butyrate remained unchanged. In contrast, functional profile analysis predicted significant differences in the succinate fermentation to butanoate. Taken together, these findings suggest that changes in the bacterial community did not translate into altered butyrate proportions, indicating a degree of metabolic plasticity and potential

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functional redundancy within the ruminal microbiome (Duncan et al., 2014).

The S-methyl-5-thio-α-D-ribose 1-phosphate degradation I pathway is part of the L-methionine salvage cycle III superpathway and is involved in the biosynthesis of several important metabolites, including polyamines and bacterial autoinducers. It involves the conversion of L-methionine via S-adenosyl-L-methionine (SAM), resulting in S-methyl-5-thioadenosine (MTA) as a byproduct (Albers, 2009). In bacteria, recycling of MTA to methionine generates 2-oxoglutarate, a key intermediate in the citric acid cycle that is essential for amino acid biosynthesis, gene regulation, and cellular signalling (Ellens et al., 2015). The pathway requires the coordinated activity of multiple enzymes, which increases the likelihood of interactions with condensed tannins, potentially reducing its activity in microorganisms. This interpretation is consistent with the lower predicted activity of the pathway in lambs fed G. *floribundum*.

The formaldehyde assimilation I (serine pathway) in type II methanotrophic bacteria oxidises methane and methanol to formaldehyde (de Vries et al., 1990). Formaldehyde subsequently reacts with glycine to form L-serine, which participates in a series of enzymatic reactions, generating several metabolic intermediates (O'Connor and Hanson, 1975). These compounds take part in CO_2 fixation and the formation of compounds such as oxaloacetate and malate, which can subsequently be converted to acetyl-CoA and glyoxylate (Quayle, 1980). The activity of key enzymes involved in the conversion of formaldehyde to intermediates leading to acetyl-CoA may be inhibited by the formation of tannin–protein complexes in lambs consuming *G. floribundum*, potentially altering ruminal energy and metabolism. As acetyl-CoA is a central intermediate in energy metabolism and a key precursor for VFA synthesis, reduced activity of this pathway could influence VFA levels.

The biosynthesis of the superpathway of dTDP-glucose-derived O-antigen building

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blocks, which generates precursors required for O-antigen synthesis and lipopolysaccharide formation in the outer membrane of Gram-negative bacteria, was significantly reduced in lambs fed *G. floribundum*. A similar pattern was observed for the polymyxin resistance pathway. The reasons for the higher predicted activity of these pathways in the CDep group remain unclear and could not be explained by the variables examined in this study. Bacteria may activate resistance mechanisms in response to environmental factors such as ruminal pH, limited carbohydrate availability or temperature, all of which can affect cell integrity and metabolism (Poole, 2012). However, the lower predicted activity of these pathways in lambs fed *G. floribundum* may also reflect the effects of bioactive compounds, such as CT on proteins involved in these metabolic processes, although this interpretation remains speculative.

The absence of tannins in the CDep diet likely increased protein availability for ruminal bacteria. In an *in vitro* study, Sayd et al. (2022) investigated the effects of tannins on plant proteins during sheep digestion and reported that tannins reduced the formation of final fermentation products in the rumen, whereas protein degradation increased in the simulated abomasum despite the presence of tannins. In the present *in vivo* study, the higher faecal N excretion observed in lambs fed *G. floribundum* suggests the formation of tannin-protein complexes that may resist dissociation under abomasal conditions. Consequently, protein availability to ruminal bacteria is restricted, which may also limit protein utilisation by the host (Besharati et al., 2022).

In an *in vitro* study, Gram-positive bacteria isolated from the rumen of cattle, including *Butyrivibrio fibrisolvens* A38 and *Streptococcus bovis* 45S1, developed morphological changes in their cell walls following exposure to condensed tannins from *Onobrychis viciifolia* (Smith et al., 2005). In the current study, condensed tannins may have

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caused similar effects, potentially contributing to the reduced populations of members of the family *Lachnospiraceae*, which are also Gram-positive (Fitzgerald et al., 2025).

The reduced populations of *Saccharofermentans, Lachnospiraceae_ND3007_group*, and *probable_genus_10* genera associated with energy and amino acid metabolism, coincided with a lower predicted activity of metabolic pathways involved in arginine, methionine, and serine metabolism, as well as in succinate fermentation to butanoate, an important route for butyrate production. This metabolic pathway has been reported to be sensitive to dietary tannins, which can influence the ruminal microbiota and VFA production (Vasta et al., 2010; Hackmann, 2024). Despite the lower predicted activity of these metabolic functions and the reduced populations of bacteria associated with *G. floribundum* intake, the proportion of butyrate remained unchanged in the EDep group. These findings suggests functional plasticity within the rumen microbiome, allowing sustained butyrate production, despite changes in bacterial populations and predicted metabolic pathways (Zhuang et al., 2024).

Conclusions

This study provides preliminary evidence that a *Gymnopodium floribundum* diet influences ruminal fermentation, nitrogen balance, and key biosynthetic pathways in growing lambs. Condensed tannins appear to contribute to nitrogen partitioning and to changes in the ruminal microbiota. However, these responses are also influenced by the structural composition of the diet, particularly its neutral detergent fibre (NDF), acid detergent fibre (ADF) and crude protein (CP) contents.

Due to the exploratory nature of this research and the limited sample size (n = 4), these findings should be considered preliminary. Although *G. floribundum* demonstrates

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potential as a botanical resource for tropical sheep production, further studies involving larger cohorts and longitudinal designs are required to fully validate its impact on metabolic efficiency and long-term productive performance.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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Table 1. Percentage of dry matter (DM) and chemical composition of the feed used for diets, %

Indices	DM	CP	Ash	NDF	ADF	EE	TP	TT	CT
Gymnopodium floribundum	90.52	14.50	6.57	44.46	22.38	0.80	16.335	11.575	23.205
Grass	31.41	6.49	6.20	66.81	43.41	1.46	ND	ND	ND
Concentrate	90.41	19.66	4.37	13.06	7.24	2.17	ND	ND	ND

The inclusion levels of concentrate-forage were calculated for each lamb to achieve an average daily weight gain of 100 g/day; DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, ADF – acid detergent fibre, EE – extract ether, TP – total phenols, TT – total tannins, CT – condensed tannins, ND – not detected

Table 2. Diet consumption of the experimental (ED) and control (CD) diets during the experimental period (average of 22 days) and during sampling for purine derivative analysis (days 16 to 22)

Total DM intake, g DM/kg $LW^{0.75}$							
Factor	SEM	*P*-value					
CDep diet and Day 16 (baseline)	1.864	$1.82e^{-13}$					
EDep	2.266	0.7501					
Day 17	1.454	0.508					
Day 18	1.454	0.105					
Day 19	1.454	0.048					
Day 20	1.454	0.206					
Day 21	1.454	0.094					
Day 22	1.454	0.076					
Pairwise comparison							
Diets	EDep (7 days)				CDep (7 days)		
	80.2 ± 1.6[a]				79.5 ± 1.6[a]		
Days	16	17	18	19	20	21	22
	81.5 ± 1.48[a]	82.4 ± 1.48[a]	79.1 ± 1.48[a]	78.5 ± 1.48[a]	79.6 ± 1.48[a]	79.01 ± 1.48[a]	78.87 ± 1.48[a]
Whole experimental period intake							
Factor	SEM	*P*-value					
Diet CD (base line)	4.03	2e-16					
Diet ED	3.01	0.656					
Pairwise comparisons, g/kg $LW^{0.75}$							
Diets	ED				CD		
	80.84 ± 2.13[a]				79.40 ± 2.13[a]		

CD – control diet, ED – experimental diet, DM – dry matter, ep – experimental period; g/kg $LW^{0.75}$ – grams per kilogram of metabolic live weight. Data are presented as mean value ± SEM; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$ according to Tukey's mean comparison test. Two multiple comparisons are presented because the group x time interaction was significant

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Table 3. *Gymnopodium floribundum* intake with the experimental diet during the experimental period (average of 22 days) and during sampling for purine derivative analysis (days 16 to 22)

Gymnopodium floribundum intake, g/kg $LW^{0.75}$							
Day	SEM				*P*-value		
Day 16 (baseline)	0.420				$2e^{-16}$		
Day 17	0.582				0.796		
Day 18	0.582				0.523		
Day 19	0.582				0.077		
Day 20	0.582				0.902		
Day 21	0.582				0.738		
Day 22	0.582				0.738		
Pairwise comparison							
Days	16	17	18	19	20	21	22
ED diet , g/kg $LW^{0.75}$	24.8 ± 0.42[a]	24.7 ± 0.42[a]	24.4 ± 0.42[a]	23.7 ± 0.42[a]	23.2 ± 0.42[a]	23.5 ± 0.42[a]	23.3 ± 0.42[a]
	Mean ± SEM along the experimental period				*G. floribundum* intake as % of foliage offered (DM basis)		
Whole experimental period ED diet, g/kg $LW^{0.75}$	23.80 ± 0.26				90.34 ± 0.80		

ED – experimental diet, DM – dry matter, g/kg $LW^{0.75}$ – grams per kilogram of metabolic live weight. Data are presented as mean value ± SEM; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$ according to Tukey's mean comparison test.

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Table 4. Volatile fatty acids in the ruminal liquid of lambs from all groups and periods, mMol

VFA		EDs (base line)	CDs	CDep	EDep
acetic acid	SEM	4.713	6.665	6.665	6.185
	P-value	8.10e^{-05}	0.619	0.271	0.173
propionic acid	SEM	1.4	1.98	1.98	1.98
	P-value	0.000104	0.586	0.471	0.127
butiric acid	SEM	0.749	1.059	1.059	1.059
	P-value	6.25e^{-06}	0.7872	0.035	0.01
valeric acid	SEM	0.068	0.097	0.097	0.097
	P-value	4.75e^{-05}	0.599	0.097	0.149
isobutiric acid	SEM	0.036	0.05	0.025	0.08
	P-value	0.0002	0.739	0.635	0.59
isovaleric acid	SEM	0.142	0.174	0.174	0.2
	P-value	0.008	0.966	0.889	0.248
total VFA	SEM	6.238	8.822	8.22	8.505
	P-value	2.04e^{-05}	0.793	0.213	0.101
acetic/propionic	SEM	0.478	0.677	0.677	0.627
	P-value	8.96e^{-06}	0.374	0.548	0.401
Pairwise comparison					
VFA		EDs	CDs	CDep	EDep
acetic acid		18.1 ± 4.71^{a}	18.1 ± 4.71^{a}	18.1 ± 4.71^{a}	18.1 ± 4.71^{a}
propioinic acid		7.94 ± 1.4^{a}	9.05 ± 1.4^{a}	6.47 ± 1.4^{a}	4.70 ± 1.4^{a}
butiric acid		5.70 ± 0.749^{a}	5.41 ± 0.74^{a}	3.19 ± 0.74^{a}	2.47 ± 0.74^{a}
valeric acid		0.425±0.068^{a}	0.372 ± 0.068^{a}	0.250 ± 0.068^{a}	0.27 ± 0.068^{a}
isobutiric acid		0.205 ± 0.036a	0.222 ± 0.036a	0.230 ± 0.036a	0.16 ± 0.088a
isovaleric acid		0.49 ± 0.153a	0.49 ± 0.100a	0.46 ± 0.100a	0.24 ± 0.153a
Total VFA		42.2 ± 6.24a	39.9 ± 6.24a	30.6 ± 6.24a	25.7 ± 6.24a
acetic/propionic		3.55 ± 0.479a	2.93 ± 0.479a	3.14 ± 0.479a	4.12 ± 0.479a

CD – control diet, ED – experimental diet, DM – dry matter, s – stabilization period; ep – experimental period; VFA – volatile fatty acids. Data are presented as mean value ± SEM; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$ according to Tukey's mean comparison test.

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Table 5. Nitrogen balance, purine derivatives, intake, and diet digestibility in the experimental and control diets at the experimental period

Variables	CDep	EDep	*P*-value	Cliff's δ
nitrogen balance, g/kg $LW^{0.75}$				
N in ration intake	1.51 ± 0.01	1.82 ± 0.13	0.0303	-1.00
N in faeces	0.49 ± 0.01	1.11 ± 0.08	0.0303	-1.00
N in urine	0.31 ± 0.04	0.20 ± 0.01	0.0303	1.00
N retention	0.70 ± 0.04	0.49 ± 0.01	0.1123	0.62
Use of N (%)	46.63 ± 2.68	27.67 ± 4.36	0.0303	1.00
PD in urine, ppm				
Uric Acid	159.5 ± 45.33	253 ± 30.23	0.1939	-0.62
Allantoin	797.62 ± 288.38	1375 ± 161.6	0.4678	-0.50
Xanthine	56.5 ± 21.89	85.0 ± 13.64	0.4704	-0.375
Total PD	1013.63 ± 350.561732.75 ± 195.244		0.4704	-0.375
PD in urine, mmol/day				
Uric Acid	3.27 ± 0.45	2.41 ± 0.17	0.4704	-0.375
Allantoin	7.08 ± 0.50	7.77 ± 0.20	0.3123	0.50
Xanthine	2.10 ± 0.13	2.06 ± 0.05	0.8852	-0.375
Total PD	8.69 ± 0.80	9.65 ± 0.29	0.4704	-0.375
PD absorbed in the duodenum	8.69 ± 0.80	9.59 ± 0.29	0.4704	-0.375
Microbial N, g/day	2.74 ± 0.63	3.56 ± 0.35	0.4704	-0.375
intake of diet, g/kg $LW^{0.75}$				
CP intake	9.46 ± 0.23	11.39 ± 0.16	0.0303	-1.00
NDF intake	33.60 ± 2.28	30.54 ± 0.44	0.3123	0.50
ADF intake	21.34 ± 1.52	17.97 ± 0.29	0.1939	0.62
TP intake	ND	3.63 ± 0.01	--	--
TT intake	ND	2.57 ± 0.01	--	--
CT intake	ND	5.15 ± 0.02	--	--
digestibility of diet, %				
Digestibility of DM	57 ± 0.02	53 ± 0.003	0.1123	0.62
Digestibility of CP	66 ± 0.011	41 ± 0.006	0.0303	1.000

N – nitrogen, PD – purine derivates, CP – crude protein, NDF – neutral detergent fibre, ADF – acid detergent fibre; TP – total phenols; TT – total tannins; CT – condensed tannins; DM – dry matter; ND – not detected; g/kg $LW^{0.75}$ – grams per kilogram of metabolic live weight. When comparing CDep vs. EDep, a negative sign in Cliff's delta (δ) indicates that the values in the experimental group (EDep) are greater than those in the control group (CDep), and vice versa. Data are presented as mean value ± SEM. $P > 0.05$ (no statistically significant) according to Mann-Whitney U test.

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Table 6. Partial redundancy analysis of nitrogen balance, volatile fatty acids and intake (without bacterial abundance) of the four group (including: EDs, CDs, EDep and CDep)

Partial redundancy analysis			
ANOVA (model)		F	*P*-value
Data structure of lamb metabolic variables		9.1816	0.001
Axis of RDA			
RDA1		31.5892	0.0156
RDA2		5.1525	0.01562
Variance partitioning			
Factor	R^2_{adj}	*P*-value	
VFAs (propionate, butyrate, total VFA)	0.2363	0.001	
Diet (groups)	0.6358	0.006	
Variable with highest importance according to the RDA			
Metabolic variable	RDA1	RDA2	Importance
N ration, g/kg $LW^{0.75}$	−0.8859576	0.104671316	0.8859576
CP intake, g/kg $LW^{0.75}$	−0.8859576	0.104671316	0.8859576
Digestibility of DM, %	−0.8760577	0.190060873	0.8760577
CP faeces, g/kg $LW^{0.75}$	−0.8689977	−0.155504820	0.8689977
NDF intake, g/kg $LW^{0.75}$	−0.8689565	0.195235349	0.8689565
N faeces, g/kg $LW^{0.75}$	−0.8514252	−0.167030203	0.8514252
N retention, %	−0.8194281	0.332412557	0.8194281
Digestibility of CP, %	−0.7994056	0.388602610	0.7994056
Use of N, %	−0.7970056	0.381297527	0.7970056
N urine, g/kg $LW^{0.75}$	−0.7684829	0.357127496	0.7684829
CP urine, g/kg $LW^{0.75}$	−0.7684829	0.357127496	0.7684829
DM intake, g/kg $LW^{0.75}$	−0.7507302	0.009768637	0.7507302
Butyrate, mmol	0.6610317	0.037905735	0.6610317
CT intake, g/kg $LW^{0.75}$	−0.6270732	−0.556144616	0.6270732
TT intake, g/kg $LW^{0.75}$	−0.6270732	−0.556144616	0.6270732
TP intake, g/kg $LW^{0.75}$	−0.6269343	−0.556760494	0.6269343
Total VFA, mmol	0.4949333	0.182553415	0.4949333
Propionate, mmol	0.4872290	0.178293724	0.4872290
Isobutyrate, mmol	0.4501663	0.524129525	0.4501663
Acetate, mmol	0.3846129	0.174945298	0.3846129

RDA – redundancy analysis, ANOVA – analysis of variance, VFA – volatile fatty acids, N – nitrogen, CP – crude protein, NDF – neutral detergent fibre, DM – dry matter, TP – total phenols, TT – total tannins, CT – condensed tannins, g/kg $LW^{0.75}$ – grams per kilogram of metabolic live weight. R^2_{adj} **–** adjusted coefficient of determination represents the proportion of the total variance that is explained by the model while penalizing the inclusion of irrelevant variables. The significance level of the analysis is 0.05. RDA1 and RDA2: The sign (+ or −) on the axes indicates the direction of the association between the variables and the treatment groups in the ordination space. Importance: It quantifies the magnitude of each variable's impact on the multivariate structure. Higher absolute values indicate greater predictive power regarding variation in the data.

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Table 7. Beta diversity analysis of the from all groups and periods two periods and the two groups, and their pairwise comparisons

			Beta diversity metrics					
			Bray-Curtis	Jaccard	Manhattan	Canberra	Kulczynski	Weighted UniFrac
	All groups	R^2	0.23	0.23	0.23	0.23	0.23	0.22556
		P-value	0.015	0.015	0.015	0.015	0.015	0.03125
Pairwise comparison	EDs_vs_CDs	R^2	0.12838	0.12838	0.12838	0.12838	0.12838	0.06774
		q	1.000	1.000	1.000	1.000	1.000	1.000
	EDs_vs_EDep	R^2	0.15244	0.15244	0.15244	0.15244	0.15244	0.17374
		q	0.125	0.125	0.125	0.125	0.125	0.125
	EDs_vs_CDep	R^2	0.16812	0.16812	0.16812	0.16812	0.16812	0.12152
		q	1.000	1.000	1.000	1.000	1.000	1.000
	CDs_vs_EDep	R^2	0.22055	0.22055	0.22055	0.22055	0.22055	0.27167
		q	1.000	1.000	1.000	1.000	1.000	1.000
	CDs_vs_CDep	R^2	0.22218	0.22218	0.22218	0.22218	0.22218	0.19277
		q	0.125	0.125	0.125	0.125	0.125	0.25
	EDep_vs_CDep	R^2	0.13013	0.13013	0.13013	0.13013	0.13013	0.14701
		q	1.000	1.000	1.000	1.000	1.000	1.000

CD – control diet, ED – experimental diet, s – stabilization period, ep – experimental period, Pseudo-R^2 – variability attributed to the model; q – Benjamini-Hochberg adjusted *P*-value

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Table 8. Partial redundancy analysis of the ruminal bacterial community and metabolic parameters of EDs, CDs, EDep and CDep groups

Partial redundancy analysis			
ANOVA (model)		F	*P-value*
Data structure of bacteriome abundances		1.5953	0.03125
Data structure of lamb metabolic variables		1.5953	0.108
Axis of RDA			
RDA1	76.63%	2.4449	0.03125
RDA2	23.37%	0.7458	0.75781
Variance partitioning			
	R^2_{adj}		*P-value*
Diet group	0.117		0.031
Bacteria with the highest importance according to the RDA			
Name		RDA1	RDA2	Importance
g__*Xylanibacter*		0.27670017	0.0099288181	0.27687825
g__*Xylanibacter*		−0.23873617	0.0650072786	0.24742859
g__*Xylanibacter*		−0.20565522	0.0299668930	0.20782705
g__*Quinella*		0.16863658	0.1209616244	0.20753316
g__*Xylanibacter*		−0.11638522	−0.0773138547	0.13972456
g__*Ruminococcus*		0.04975763	−0.1055385131	0.11667990
g__*Xylanibacter*		0.09786559	0.0554470639	0.11248134
g__*Prevotellaceae_NK3B31_group*		0.09157549	0.0642945684	0.11189219
g__*Lachnospiraceae_NK3A20_group*		0.05437185	−0.0931220185	0.10783325
g__*Christensenellaceae_R-7_group*		0.10376332	0.0044119399	0.10385708

CD – control diet; ED – experimental diet; s – stabilization period; ep – experimental period; RDA – redundancy analysis; ANOVA – analysis of variance; R^2_{adj} – adjusted coefficient of determination represents the proportion of the total variance that is explained by the model while penalizing the inclusion of irrelevant variables. The significance level of the analysis is 0.05. RDA1 and RDA2: The sign (+ or −) on the axes indicates the direction of the association between the variables and the treatment groups in the ordination space. Importance: It quantifies the magnitude of each variable's impact on the multivariate structure. Higher absolute values indicate greater predictive power regarding variation in the data.

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Table 9. Differential abundance at the genus level between the experimental diet (*Gymnopodium floribundum*) at the stabilization and experimental periods (EDs vs EDep), stabilization period used as reference

Taxon	LFC IN	LFC G	*P*-value IN	*P*-value *G*	*q* IN	*q* G
Saccharofermentans	1.33 ± 0.45	–2.67 ± 0.64	0.003	3.09×10^{-05}	0.223	0.002
Lachnospiraceae_ND3007_group	1.09 ± 0.37	–2.18 ± 0.53	0.003	3.76×10^{-05}	0.223	0.002
f: Lachnosporace; g: probable_genus_10	0.92 ± 0.16	–1.84 ± 0.23	1.47×10^{-08}	1.13×10^{c15}	2.77×10^{-06}	2.13×10^{-13}

ED – experimental diet, s – stabilization period, ep – experimental period, LFC – log fold change, G – group; IN – intercept, *q* – *P*-value adjusted Benjamini-Hochberg ($P \leq 0.05$). Data are presented as mean value ± SEM

Table 10. Differential abundance at the genus level between the control and experimental diet at the experimental period (CDep vs EDep), control diet was used as a reference

Taxon	**LFC IN**	**LFC G**	***p* IN**	***p* G**	***q* IN**	***q* G**
Lachnospiraceae_ND3007_group	1.35 ± 0.54	–2.70 ± 0.77	0.013	4.89×10^{-04}	0.868	0.033
f: Erysipelatoclostridiaceae; g: UCG-004	1.27 ± 0.47	–2.55 ± 0.67	0.007	1.63×10^{-04}	0.785	0.6×10^{-08}
Incertae_Sedis_33	0.83 ± 0.18	–1.66 ± 0.26	1.03×10^{-05}	4.36×10^{-10}	0.002	8.89×10^{-08}

CD – control diet, ED – experimental diet, ep – experimental period, LFC – log fold change, G – group, IN – intercept, *q* – *P*-value adjusted Benjamini-Hochberg ($P \leq 0.05$). Data are presented as mean value ± SEM

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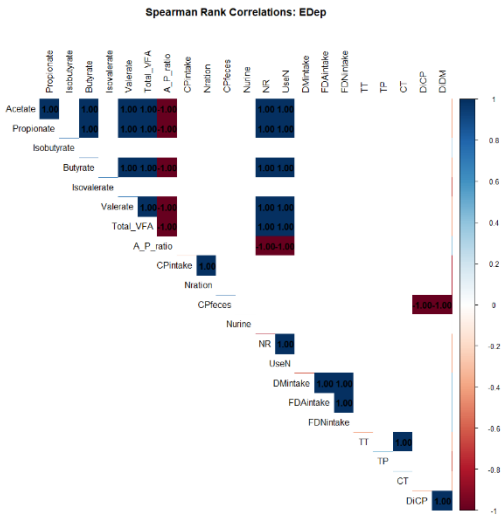

Figure 1. Spearman correlation analysis of the EDep group. Only significant correlations after FDR-BH correction are shown ($P < 0.001$)

total VFA – total volatile fatty acids, A/P ratio – acetate/propionate ratio, CP intake – crude protein intake, N ration – nitrogen ration, CP faeces – crude protein in faeces, N urine – nitrogen in urine, NR – nitrogen retention, Use N – use of nitrogen, DM intake – dry matter intake, FDA intake – acid detergent fibre intake, FDN intake – neutral detergent fibre intake, TP – total phenols, TT – total tannins, CT – condensed tannins, DiCP – digestibility of crude protein

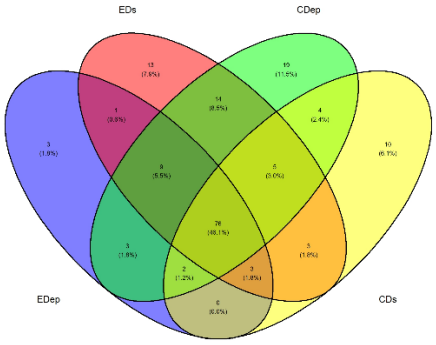

Figure 2. Venn Diagram of the shared and unique genera among the lambs from all groups and periods

CD – control diet, ED – experimental diet, s – stabilization period, ep – experimental period

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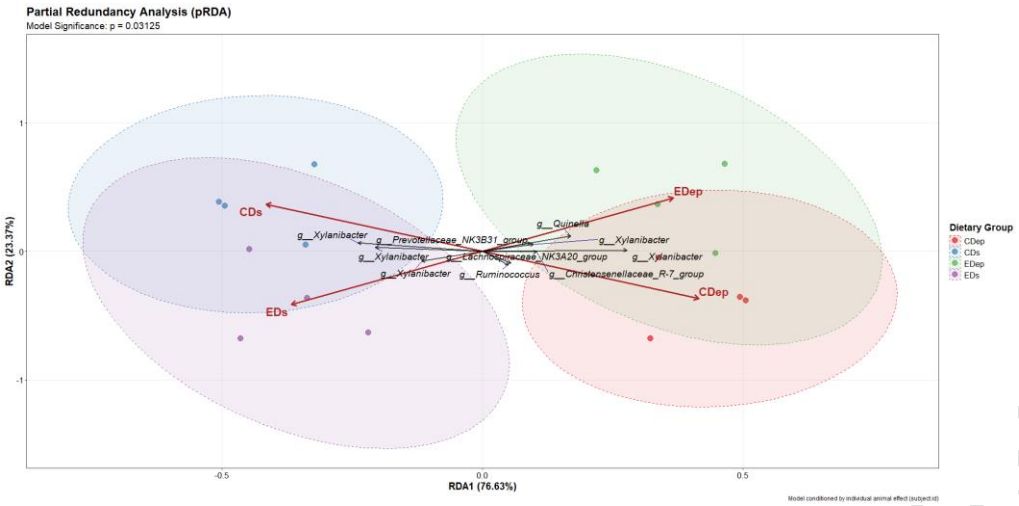

Figure 3. Partial redundancy analysis biplot of the top 10 most abundant bacterial taxa across all diets.

The first axis (RDA1) explains a significant proportion of the variance, while the second axis (RDA2) was non-significant.
CD – control diet, ED – experimental diet, s – stabilization period, ep – experimental period, RDA – redundancy analysis

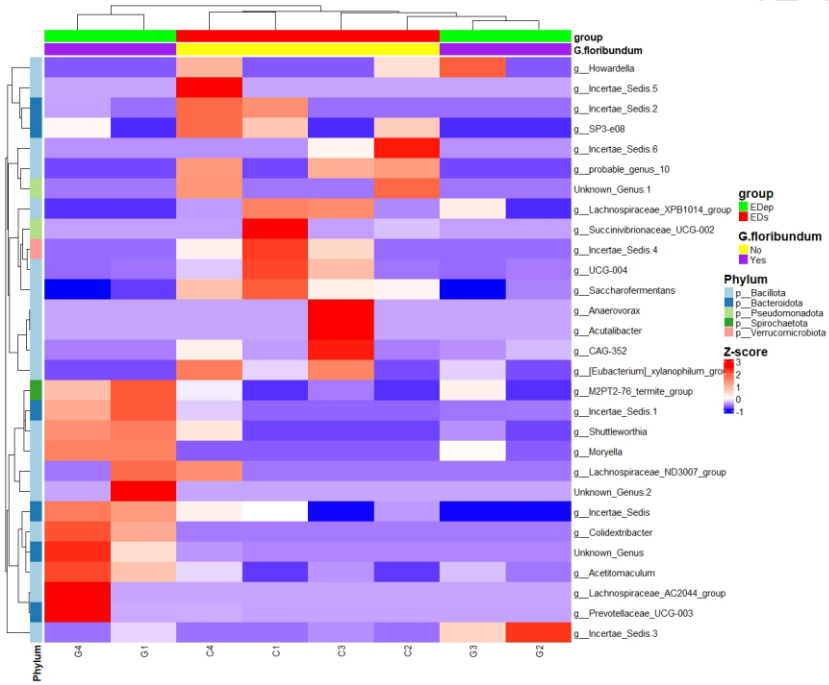

Figure 4. Heatmap showing differential abundance at genus level for the EDs and EDep periods.

The dendrogram on the left shows the phylum to which the identified genera belong, while the top dendrogram shows the samples from each group.
ED – experimental diet, s – stabilization period, ep – experimental period

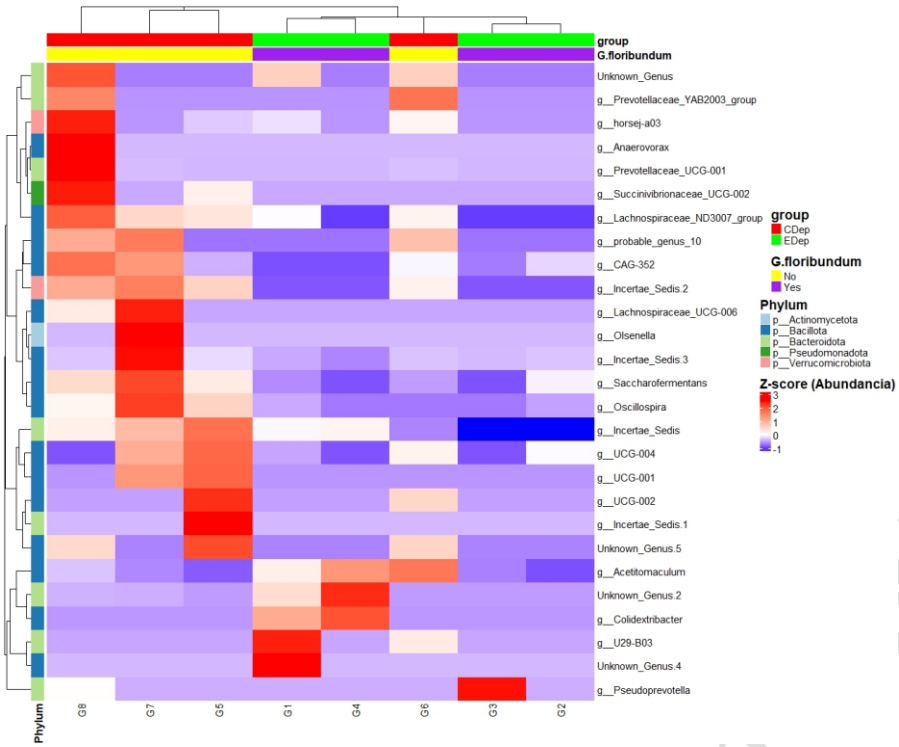

Figure 5. Heatmap showing differential abundance at genus level for the CDep and EDep groups.

The dendrogram on the left shows the phylum to which the identified genera belong, while the top dendrogram shows the samples in each group.
CD – control diet, ED – experimental diet, ep – experimental period

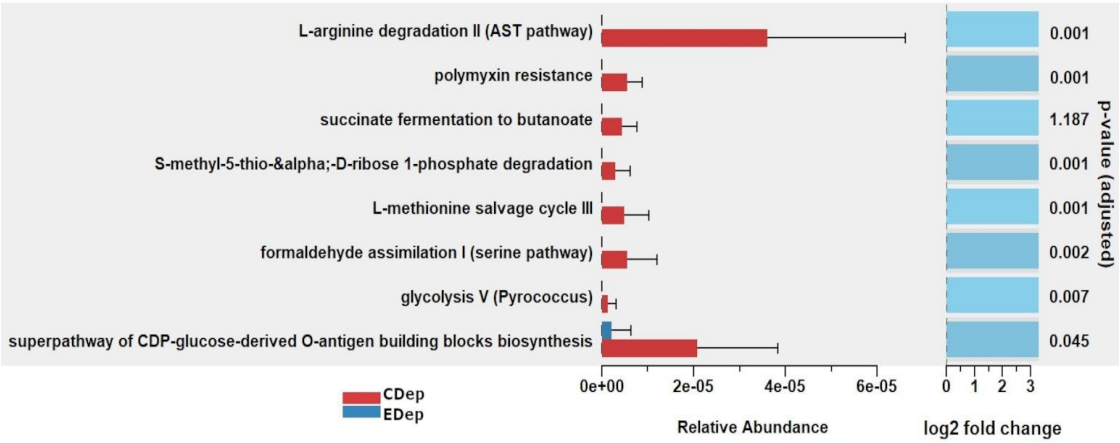

Figure 6. Differential metabolic pathways between the CDep and EDep groups.

The x-axis shows the level of relative abundance, and the y-axis shows the enriched metabolic pathways. The *P*-value is on the far right, and the fold change is on the left.
CD – control diet, ED – experimental diet, ep – experimental period