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Original Research Article

Enhancing nutrient efficiency through optimizing protein levels in lambs: Involvement of gastrointestinal microbiota

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ABSTRACT

Improving the nutrient utilization efficiency of ruminants is of utmost significance for both economic and environmental benefits. Optimizing dietary protein levels represents a key nutritional strategy to enhance ruminant growth performance and reduce nitrogen emissions. In a 63-day experiment, 24 healthy Hulunbuir lambs (initial weight 17.1 ± 2.0 kg, 2.5 months old) were subjected to three treatments: a low-protein diet (LP; crude protein of 78.4 g/kg dry matter [DM]), a medium-protein diet (MP; crude protein of 112.0 g/kg DM), and a high-protein diet (HP; crude protein of 145.6 g/kg DM), with 8 lambs in each treatment (4 males and 4 females). Lambs in the MP treatment presented greater daily weight gain and feed conversion ratio than those in the HP treatment ($P < 0.05$, quadratically). Compared with the LP treatment, the MP treatment resulted in greater crude protein digestibility ($P < 0.001$, quadratically) and acid detergent fiber digestibility ($P = 0.022$, quadratically). In the serum, the urea nitrogen level increased quadratically with increasing dietary protein levels ($P < 0.001$), while the LP treatment exerted the highest concentrations of glutamate, glycine, alanine, and histidine ($P < 0.05$, quadratically). The ammonia nitrogen concentrations in the rumen and colon increased quadratically with increase in dietary protein levels ($P < 0.05$). The HP treatment increased the molar concentrations of isobutyrate and isovalerate in the rumen and colon ($P < 0.05$, quadratically). In contrast, the LP treatment decreased the molar proportion of acetate ($P = 0.007$, quadratically) and increased the molar proportion of butyrate ($P < 0.001$, quadratically) in the colon. The microbial diversity and structure were significantly altered by dietary protein level intervention across all gastrointestinal regions. The rumen of the MP treatment was enriched with fiber-degrading bacteria *Fibrobacter_succeinogenes* and starch-degrading bacteria *Selenomonas_ruminantium*. The colon in the LP treatment harbored microbial biomarkers including *Escherichia* spp. and *Lactobacillus amylovorus*, and the colon in the MP treatment was characterized by the enrichment of *Solibacillus_cecembensis*. These findings suggest that the MP diet with a crude protein content of 112.0 g/kg DM improved the growth performance and nutrient efficiency of lambs, which was achieved via the involvement of the gastrointestinal microbiota.
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1. Introduction

With the rapid development of animal husbandry, there is an urgent need for a global protein feed supply (Kim et al., 2019). In China, more than 50% of the protein feed supply relies on imports, and the high price of imported feed undoubtedly increases the cost of animal production (National Bureau of Statistics of China, 2022). Pressure from government and consumers has forced the livestock industry to reduce its nitrogen (N) emissions, which threatens both human health and the environment (Ershadi et al.,

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2020). Ruminant livestock possess the ability to generate superior products by utilizing low-value crude protein (CP) feed and providing high-quality CP sources for human consumption (Broderick, 2018). Hence, there is an urgent need to develop nutritional strategies to enhance the protein utilization efficiency of ruminants for economic and environmental benefits (Guyader et al., 2016).

Nitrogen (N) metabolism is one of the most important metabolic pathways for ruminants to maintain normal physiological functions, such as nutrient absorption, immune homeostasis and body growth (Hristov et al., 2019). Dietary proteins are inevitably degraded into ammonia by the ruminal microbiota (Bach et al., 2005). These microorganisms subsequently convert ammonia to microbial protein (MCP) through ammonia assimilation processes involving a myriad of microbial enzymes such as glutamine synthetase, glutamate synthetase, glutamate dehydrogenase, alanine dehydrogenase, and asparagine synthetase (Bach et al., 2005; Wang and Tan, 2013). Meanwhile, a proportion of ammonia can enter the liver and be converted to urea via the ornithine cycle (Bach et al., 2005). Some of the urea will re-enter the gastrointestinal tract (GIT), providing a N source for MCP synthesis (Hailemariam et al., 2021). The remaining urea is primarily eliminated in the urine via the kidneys (Hristov et al., 2019). This complex host–microbiota interaction as a crucial element in maintaining the N metabolic ecosystem for safeguarding the overall health and functionality of ruminant animals warrants further exploration.

A variety of internal and external factors affect the N metabolism of ruminants, particularly the diet composition (Estrada-Angulo et al., 2018; Firkins et al., 2007; Vosooghi-poostindoz et al., 2014; Wang et al., 2021a,b). For instance, in Hu lambs, fecal N excretion was greater, whereas urinary N excretion tended to be lower, when soybean meal was replaced by dried distillers' grains with solubles (DDGS) (Shen et al., 2018). In dairy cows, partial substitution of rapeseed meal with *Spirulina platensis* microalgae decreased the N utilization efficiency, whereas partial substitution of faba bean with *S. platensis* microalgae increased the N utilization efficiency (Lamminen et al., 2019). The use of low-protein diets is generally accepted as an effective nutritional strategy to reduce N emissions from ruminants (El-Kadi et al., 2006; Prima et al., 2019; Zhang et al., 2023), and there is a need to explore how ruminants respond to various dietary protein levels to establish the appropriate protein requirements. Several studies have pioneered the investigation of the effects of dietary protein levels on rumen fermentation and microbial diversity (Cui et al., 2019; Li et al., 2020; Lv et al., 2020; Saro et al., 2020), but these studies are not sufficient to reflect the overall gastrointestinal N metabolism due to the regional heterogeneity of the microbiota (Jiao et al., 2024).

To fill this knowledge gap, we conducted a dietary intervention study with three different protein levels in lambs and speculated that balancing protein levels could optimize N efficiency through modulating gastrointestinal microorganisms. To test our hypothesis, we investigated the GIT microbes via full-length amplicon sequencing and integrated the data to illustrate their effects on nutrient metabolism.

2. Materials and methods

2.1. Animal ethics statement

The animal experiments were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (permission No. CAS2022060), and this work was performed following the ARRIVE guidelines.

2.2. Diets and experimental design

The animal trial was conducted in the sheep yard at the Grass and Animal Husbandry Engineering Ecological Laboratory, Chinese Academy of Sciences (Hulunbuir City, Inner Mongolia Autonomous Region, China). Twenty-four healthy Hulunbuir lambs (initial weight 17.1 ± 2.0 kg, 2.5 months old) were randomly allocated to 1 of 3 treatments: a medium-protein diet (MP), a low-protein diet (LP) or a high-protein diet (HP), with 8 lambs in each treatment (4 males and 4 females). The experimental diets (Table 1) were formulated based on the Feeding Standard of Meat-Producing Sheep and Goats (NY/T 816–2021, China). The lambs were fed a MP diet (CP of 112.0 g/kg DM, the recommended CP level), a LP diet (CP of 78.4 g/kg DM, CP content reduced by 30% compared to MP) or a HP diet (CP of 145.6 g/kg DM, CP content increased by 30% compared to MP) in individual pens (1.5 m × 0.8 m × 1.5 m). The total experimental period lasted for 63 d, including an adaptation period of 7 d and a trial period of 56 d. The lambs were fed twice a day at 08:00 and 16:00 in amounts to ensure less than 10% orts and feed refusal was recorded daily. All the lambs had free access to water throughout the experimental period. The feed was provided in individual removable steel box feeders, and daily feed intake was obtained by calculating the weight difference between the remaining feed and the provided feed. The body weight (BW) of each lamb was determined every two weeks using a weighbridge scale (XK3190-A12, Shanghai Yaohua Weighing System Co., Ltd., China). The body length (BL) and body height (BH) of each lamb were measured every two weeks via a tape measure (HMK3m9S3X, Partikelmess- und Analysesysteme, Germany). Average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were calculated per pen during the total feeding period using the data of BW and feed consumption. All the measurements were carried out in a sheep yard in Hulunbuir City of China.

Table 1
Component and nutrient levels of diets at different protein levels (DM basis, %).

Item	Treatments[1]		
	LP	MP	HP
Ingredients			
Alfalfa	15.00	25.00	30.00
Oat grass	20.00	10.00	5.00
Corn	50.20	40.60	27.40
Wheat bran	10.60	7.60	7.60
Soybean meal		12.00	25.00
Fat powder		0.60	0.80
$CaCO_3$	0.50	0.50	0.50
$CaHPO_4$	1.00	1.00	1.00
Premix[2]	2.00	2.00	2.00
NaCl	0.50	0.50	0.50
MgO	0.20	0.20	0.20
Total	100.00	100.00	100.00
Nutrient levels[3]			
DM	94.90	94.90	95.23
CP	7.84	11.20	14.56
Starch	41.67	33.93	26.84
NDF	40.13	35.65	34.70
ADF	17.97	18.03	18.38
GE, MJ/kg	17.81	17.26	16.71

DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; GE = gross energy.
[1] LP = low-protein diet; MP = medium-protein diet; HP = high-protein diet.
[2] Premix provided the following per kilogram of the diets: Fe 221.31 mg, Cu 8.12 mg, Zn 71.31 mg, Mn 71.31 mg, I 1.23 mg, Co 0.54 mg, Se 0.17 mg, VA 3250 IU, VD 2000 IU, VE 40 IU.
[3] Nutrient levels were measured values.

2.3. Sample collection

The floor drain type fecal collector was set under the feeding pens to ensure that feces would not fall to the ground, and fecal samples were collected from each lamb from d 55 to 61, and mixed to obtain a homogeneous sample. Blood from the jugular vein of each lamb was collected into 5-mL vacuum collection tubes (Changsha Zhifan Biotechnology Co., Ltd., Hunan, China) before the morning feeding on d 63. The serum was separated via centrifugation at 3000 × *g* for 15 min at 4 °C.

The lambs were slaughtered at the end of the experiment, with a final weight of 24.7 ± 0.7 kg. After slaughter, ruminal digesta samples were collected from five locations in the rumen (the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral), and combined to represent a homogenous sample (Jiao et al., 2017). The ileal and colonic digesta samples were collected from the middle region of the respective intestine (Jiao et al., 2024). All the digesta samples were immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.4. Nutrient analysis

Approximately 200 g of each fecal and feed sample was taken to dry at 65 °C for 72 h, and ground into powder by a disintegrator with a 40-mesh sieve (ZX-1000Y, ZUNXI, China). Determination of DM (method 930.15) and ash (method 942.05) was conducted according to the Association of Official Analytical Chemists procedures (AOAC, 2006), and the CP content was determined based on the N concentration with a flow injection apparatus (AA3, Seal Analytical, Germany) according to the method 984.13 (AOAC, 2006). The acid-detergent fiber (ADF) and neutral detergent fiber (NDF) contents were analyzed by a fiber analyzer (FT12, Gerhardt Analytical Systems, Germany) according to Van Soest et al. (1991). The starch content was analyzed via an amylase assay kit (BC0705, Beijing Solarbio Technology Co., Ltd., China). The digestibility of each nutrient component was calculated using acid-insoluble ash (AIA) as an indicator, and its content was measured following the method of Furuichi and Takahashi (1981). The gross energy (GE) content was analyzed using an isothermal automatic calorimeter (5E-AC 8018, Changsha Kaiyuan Instruments Co., Ltd., Changsha, China).

2.5. Serum biochemical indicators

The concentrations of serum biochemical indicators were assayed according to the procedure described by Wang et al. (2021b), using a fully automated biochemical analyzer (Cobas c311, Roche, Switzerland).

2.6. Serum free amino acid (AA) profile

Frozen serum samples (1 mL) were thawed at 4 °C, and 1 mL of 8% sulfosalicylic acid was added. After being incubated at room temperature for 15 min, the samples were centrifuged at 14,000 × *g* for 10 min. The concentrations of free AAs in the supernatant were determined according to the method of Wu et al. (2022), using a fully automated amino acid analyzer (L8900, Hitachi, Japan).

2.7. Gastrointestinal fermentation parameters

The concentrations of volatile fatty acids (VFAs) in the digesta from the rumen, ileum, and colon were determined using a liquid injection gas chromatograph (7890A, Agilent, Santa Clara, USA) as detailed by Jiao et al. (2015b). Ammonia nitrogen levels in the digesta were assayed with the phenol-hypochlorite method via a multifunctional enzyme labeling instrument (Infinite M200 PRO, TECAN, Switzerland) at a wavelength of 625 nm (Jiao et al., 2014). Yeast RNA was used as an RNA standard to determine the nucleic acid purine bases, and the MCP concentration was calculated based on the purine-to-nitrogen ratio (Ushida et al., 1985).

2.8. DNA extraction, amplicon sequencing, and bioinformatics analysis

Microbial DNA was extracted from the digesta samples using the bead-beating method as described previously (Jiao et al., 2015a). The DNA yield and integrity were assessed using a Qubit 2.0 fluorometer (Thermo Scientific, MA, USA). The V1–V9 region of the full-length 16S ribosomal RNA gene was amplified using primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′). The PCR conditions were listed as follows: 95 °C for 2 min; 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 5 min. Amplicons were purified via the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.), and subsequently mixed at equal molar ratios prior to PacBio sequencing (San Francisco, CA, U.S.).

PacBio raw reads were processed via the SMRT Link Analysis software (version 9.0) to obtain demultiplexed circular consensus sequence (CCS) reads, and sequences with lengths <800 bp or >2500 bp were filtered. After quality control, the unoise3 method in USEARCH (v. 10.0.240) software was used to denoise the sequence (Edgar and Flyvbjerg, 2015), and VSEARCH (v. 2.22.1) was used to obtain the amplicon sequence variants (ASVs) with 97% similarity (Rognes et al., 2016). The taxonomic classification of each ASVs was assigned against the latest Greengenes2 database with a 0.80 confidence threshold (McDonald et al., 2024), and the community composition of each sample was counted at the genus and species levels. Alpha and beta diversities were analyzed via the vegan package in R software (version 2.6.4) (Oksanen et al., 2013). The “alpha_div” command was applied to calculate alpha diversity indices. The “adonis” function was used to implement PERMANOVA analysis of beta diversity, with the Euclidean distance matrix and 999 permutation tests. The random forest analysis was conducted using the microeco package (Liu et al., 2021). The ggplot2 package was used to create all the figures (Cao et al., 2023).

2.9. Statistical analysis

All the statistical analyses were conducted using the SPSS software (version 27.0.1). The normality of the data distribution was checked via the Shapiro–Wilk test. Initially, data of growth performance, nutrient digestibility, serum biochemical indicators and AAs, and fermentation parameters were analyzed with a two-way ANOVA with the fixed effects of sex, treatment, and sex × treatment interaction, and results showed that they were affected neither by sex × treatment interaction nor by sex. Herein, comparisons among the three dietary protein level treatments were made by one-way ANOVA followed by Duncan's multiple range tests, via the following model:

$$Y_{jk} = \mu + CP_k + Lamb_{j:k} + e_{jk}$$

where Y_{jk} was the dependent variable, μ was the least squares mean, CP_k was the fixed effect of the kth treatment ($k = 78.4, 112.0, 145.6$). $Lamb_{j:k}$ was the random effect of the jth lamb ($j = 1$ to 24) in the kth diet treatment, and the e_{jk} is the error residual. Orthogonal polynomial contrasts were employed to assess the linear and quadratic effects of dietary protein level treatments. A significant difference was considered at $P < 0.05$.

3. Results

3.1. Growth performance and nutrient digestibility

Lambs fed the MP diet had greater total weight gain and average daily gain compared to those fed the HP diet ($P < 0.05$, quadratically), whereas a similar average daily feed intake was noted across the three treatments ($P = 0.917$, Table 2). Accordingly, lambs fed the MP diet presented improvements in feed conversion ratio compared with those fed the HP diet ($P = 0.049$, quadratically). In addition, the LP treatment exhibited a decrease in CP digestibility compared with that of the other two groups ($P < 0.001$, quadratically). ADF digestibility in the MP treatment was greater than that in the LP treatment ($P = 0.022$, quadratically). Dietary protein levels did not affect the digestibility of DM ($P = 0.106$), starch ($P = 0.060$), or NDF ($P = 0.078$).

3.2. Serum biochemical indicators and AA profiles

Most of the serum biochemical indicators were similar among the three treatments, including energy-related, lipid-related, and liver function-related metabolites ($P > 0.05$, Table 3). The only exception was noted for serum urea nitrogen, which exhibited a quadratic increase with the increase in dietary protein levels ($P < 0.001$). In terms of AA profile, LP treatment improved the concentrations of glutamate, glycine, alanine, and histidine in the serum compared to those of the HP treatment ($P < 0.05$, quadratically, Table 4).

3.3. Fermentation parameters in the gastrointestinal tract

The ammonia nitrogen concentration in the digesta of the rumen and colon increased quadratically with the increase indietary protein levels ($P < 0.05$, Table 5). The LP treatment markedly increased the ruminal MCP concentration compared to the HP treatment ($P = 0.043$, quadratically). Regarding the VFA profile, the HP treatment increased the molar concentrations of isobutyrate, valerate, and isovalerate in the rumen as compared to the LP treatment ($P < 0.05$, quadratically). Similarly, the molar concentrations of isobutyrate and isovalerate in the colon of the HP treatment were greater than those in the other two treatments

Table 2
Effects of diets with different protein levels on growth performance and nutrient digestibility of lambs.

Item	Treatments[1]			SEM	*P*-value[2]		
	LP	MP	HP		ANOVA	L	Q
Growth performance							
Total weight gain, kg	7.61^{ab}	8.58^{a}	7.00^{b}	0.245	0.018	0.318	0.022
Total length gain, cm	6.25	6.63	5.75	0.658	0.873	0.764	0.873
Total height gain, cm	4.63	4.75	3.50	0.568	0.513	0.342	0.513
Average daily feed intake, g/d	857.88	863.50	854.50	8.878	0.917	0.881	0.923
Average daily gain, g/d	135.97^{ab}	153.13^{a}	125.00^{b}	4.373	0.018	0.316	0.022
Feed conversion ratio	6.46^{ab}	5.75^{b}	6.87^{a}	0.195	0.044	0.403	0.049
Nutrient digestibility, %							
DM digestibility	75.47	78.05	74.61	0.694	0.106	0.626	0.106
CP digestibility	57.61^{b}	76.90^{a}	79.76^{a}	2.245	<0.001	<0.001	<0.001
Starch digestibility	96.11	96.47	94.34	0.333	0.060	0.026	0.013
NDF digestibility	72.31	76.23	71.06	0.988	0.078	0.615	0.078
ADF digestibility	65.67^{b}	73.73^{a}	68.44^{ab}	1.263	0.022	0.383	0.022

DM = dry matter; CP = crude fiber; NDF = neutral detergent fiber; ADF = acid detergent fiber.
$^{a, b}$ Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] LP = low-protein diet (CP, of 78.4 g/kg DM); MP = medium-protein diet (CP of 112.0 g/kg DM); HP = high-protein diet (CP of 145.6 g/kg DM).
[2] ANOVA means the *P*-value of three treatments based on one-way ANOVA; L means *P*-value based on linear effect of three treatments; Q means *P*-value based on quadratic effect of three treatments.

Table 3
Effects of diets with different protein levels on biochemical indicators in the serum.

Item	Treatments[1]			SEM	*P*-value[2]		
	LP	MP	HP		ANOVA	L	Q
Total protein, g/L	70.51	71.86	76.66	1.749	0.335	0.155	0.335
Albumin, g/L	31.74	31.61	33.59	0.936	0.648	0.432	0.648
Urea nitrogen, mmol/L	4.31^{b}	6.68^{ab}	10.16^{a}	0.535	<0.001	<0.001	<0.001
Blood ammonia, μmol/L	78.55	80.49	84.21	2.693	0.702	0.403	0.702
Creatinine, μmol/L	64.88	64.63	63.00	1.688	0.886	0.660	0.895
Glucose, mmol/L	4.53	4.64	4.85	0.116	0.532	0.263	0.532
Total bilirubin, μmol/L	1.36	1.29	1.41	0.073	0.796	0.787	0.796
Alanine transaminase, U/L	19.63	18.74	18.13	1.373	0.912	0.665	0.912
Glutamic oxaloacetic transaminase, U/L	117.75	117.63	114.88	3.184	0.988	0.721	0.924
Alkaline phosphatase, U/L	299.75	307.75	282.33	15.529	0.800	0.211	0.297
Lactate dehydrogenase, U/L	592.88	569.63	556.88	15.007	0.631	0.338	0.631
Total triglycerides, mmol/L	0.28	0.30	0.31	0.015	0.690	0.384	0.690
Total cholesterol, mmol/L	1.83	1.54	1.53	0.063	0.082	0.051	0.082
Free fatty acids, mmol/L	0.08	0.11	0.11	0.008	0.233	0.951	0.998
Low-density lipoprotein, mmol/L	0.65	0.56	0.58	0.029	0.489	0.367	0.489
High-density lipoprotein, mmol/L	1.29	1.05	1.05	0.043	0.092	0.019	0.026

$^{a, b}$ Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] LP = low-protein diet (CP of 78.4 g/kg DM); MP = medium-protein diet (CP of 112.0 g/kg DM); HP = high-protein diet (CP of 145.6 g/kg DM).
[2] ANOVA means the *P*-value of three treatments based on one-way ANOVA; L means *P*-value based on linear effect of three treatments; Q means *P*-value based on quadratic effect of three treatments.

Table 4
Effects of diets with different protein levels on serum free amino acid concentrations (μg/mL).

Item	Treatments[1]			SEM	*P*-value[2]		
	LP	MP	HP		ANOVA	L	Q
Aspartic acid	3.14	3.25	2.45	0.183	0.152	0.123	0.152
Glutamate	10.71^{a}	7.72^{b}	7.83^{b}	0.447	0.007	0.006	0.003
Threonine	12.10	11.50	11.68	0.606	0.924	0.781	0.924
Serine	5.81	4.88	4.85	0.261	0.245	0.139	0.245
Proline	6.08	6.12	5.02	0.288	0.214	0.133	0.214
Glycine	25.35^{a}	21.22^{ab}	20.47^{b}	0.813	0.023	0.011	0.023
Alanine	11.17^{a}	9.84^{ab}	8.50^{b}	0.435	0.035	0.009	0.035
Valine	13.76	12.36	13.36	0.639	0.674	0.806	0.674
Methionine	2.16	1.97	1.61	0.103	0.084	0.026	0.084
Isoleucine	5.68	5.51	5.45	0.227	0.914	0.681	0.914
Leucine	8.41	8.27	7.80	0.452	0.857	0.592	0.857
Tyrosine	9.76	8.30	7.78	0.419	0.135	0.051	0.135
Phenylalanine	5.16	5.09	4.28	0.230	0.233	0.124	0.233
Lysine	15.32	11.66	13.22	0.787	0.165	0.287	0.165
Histidine	5.33^{a}	4.95^{ab}	4.16^{b}	0.172	0.011	0.003	0.011
Arginine	16.90	17.27	17.63	0.556	0.876	0.602	0.876
Total amino acids	156.84	139.91	136.08	4.809	0.175	0.077	0.175

$^{a, b}$ Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] LP = low-protein diet (CP, of 78.4 g/kg DM); MP = medium-protein diet (CP, of 112.0 g/kg DM); HP = high-protein diet (CP of 145.6 g/kg DM).
[2] ANOVA means the *P*-value of three treatments based on one-way ANOVA; L means *P*-value based on linear effect of three treatments; Q means *P*-value based on quadratic effect of three treatments.

($P < 0.05$, quadratically). In addition, the LP treatment decreased the acetate molar proportion and increased the butyrate molar proportion in the colon when compared to the MP and HP treatments ($P < 0.05$, quadratically).

3.4. Microbial composition in the gastrointestinal tract

As shown in Fig. 1, there were no significant variations in the ACE or Chao 1 indexes of the GIT microbiota ($P > 0.05$). Analysis of beta diversity revealed that the GIT region and protein level significantly affected the microbial community ($P < 0.001$, Fig. 2A). Specifically, microbial diversity was distinguished by dietary protein levels ($P < 0.05$) in the rumen (Fig. 2B), ileum (Fig. 2C) and colon (Fig. 2D).

To further screen microorganisms that respond differently to various dietary protein levels, we employed random forest analysis to assess the relative importance of each feature via the Mean Decrease Gini value. We identified 18, 13, and 20 genus-level microbial biomarkers (Fig. 3A), and 20, 9, and 20 species-level microbial biomarkers (Fig. 3B) in the rumen, ileum, and colon, respectively. In the rumen, *Prevotella_sp900318625*, *Prevotella_sp90277665*, *Selenomonas_A_ruminantium_42743* and *Fibrobacter_succinogenes_779654* were selected as featured biomarkers in the MP treatment, and the relative abundances of *Prevotella_sp001553265* and *Limosilactobacillus_mucosae* were greater in the LP treatment. *Bac-11_sp902778855*, *Paraburkholderia_fungorum* and *Alistipes_A_871400_sp002362235* were selected as featured biomarkers in the HP treatment. In the ileum, *UBA1067_sp004551905* and *UBA11452_sp002069785* were enriched in the LP treatment; *Mesomycoplasma_moatsii* and *Phyllobacterium_myrsinacearum* were enriched in the MP treatment, and *Parafannyhessea_umbonata_A* and *Syntrophococcus_sucromutans* were enriched in the HP treatment. In the colon, *Frisingicoccus_caecimuris*, *Lactobacillus_amylovorus*, and *Prevotella_ruminicola* were enriched in the LP treatment; *Solibacillus_cecembensis* and *Mesomycoplasma_moatsii* were enriched in the MP treatment; while *Paraprevotella_sp003477995* was selected as a biomarker for the HP treatment.

Table 5
Effects of diets with different protein levels on fermentation characteristics in gastrointestinal tract.

Item	Treatments[1]			SEM	*P*-value[2]		
	LP	MP	HP		ANOVA	L	Q
Rumen							
Ammonia nitrogen, mmol/kg	2.54^{b}	4.69^{a}	5.38^{a}	0.061	<0.001	0.017	<0.001
Microbial protein, mg/g	4.16^{a}	3.23^{ab}	2.92^{b}	0.215	0.043	0.016	0.043
Total VFA, mmol/kg	133.93	125.43	108.21	6.600	0.280	0.113	0.280
VFA percentage, mol/100 mol							
Acetate	62.55	62.56	65.13	0.740	0.350	0.159	0.270
Propionate	19.21	19.42	16.67	1.004	0.520	0.313	0.482
Isobutyrate	0.88^{b}	1.16^{ab}	1.44^{a}	0.070	<0.001	<0.001	0.001
Butyrate	14.61	13.49	12.84	0.612	0.510	0.246	0.511
Isovalerate	1.88^{b}	2.24^{ab}	2.70^{a}	0.101	0.001	<0.001	0.001
Valerate	0.88^{b}	1.13^{a}	1.22^{a}	0.047	0.001	0.001	0.004
Ileum							
Ammonia nitrogen, mmol/kg	1.54	1.80	1.94	0.147	0.556	0.038	0.121
Microbial protein, mg/g	1.05	0.86	0.85	0.077	0.379	0.218	0.379
Total VFA, mmol/kg	4.89	3.61	2.80	0.374	0.054	0.019	0.065
Colon							
Ammonia nitrogen, mmol/kg	1.89^{b}	3.04^{a}	3.09^{a}	0.151	<0.001	0.017	<0.001
Microbial protein, mg/g	3.30	4.05	3.76	0.193	0.293	0.349	0.293
Total VFA, mmol/kg	58.04	54.90	48.02	2.180	0.150	0.058	0.160
VFA percentage, mol/100 mol							
Acetate	75.72^{b}	79.26^{a}	78.70^{a}	0.525	0.008	0.017	0.007
Propionate	14.33	13.54	14.43	0.393	0.619	0.920	0.619
Isobutyrate	0.58^{b}	0.81^{b}	1.35^{a}	0.098	0.001	0.017	0.001
Butyrate	8.04^{a}	4.89^{b}	3.49^{b}	0.456	<0.001	0.017	<0.001
Isovalerate	0.43^{b}	0.60^{b}	1.04^{a}	0.083	0.003	0.001	0.004
Valerate	0.99	0.92	1.00	0.057	0.804	0.546	0.804

VFA = volatile fatty acid.
$^{a, b}$ Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] LP = low-protein diet (CP, of 78.4 g/kg DM); MP = medium-protein diet (CP, of 112.0 g/kg DM); HP = high-protein diet (CP, of 145.6 g/kg DM).
[2] ANOVA means the *P*-value of three treatments based on one-way ANOVA; L means *P*-value based on linear effect of three treatments; Q means *P*-value based on quadratic effect of three treatments.

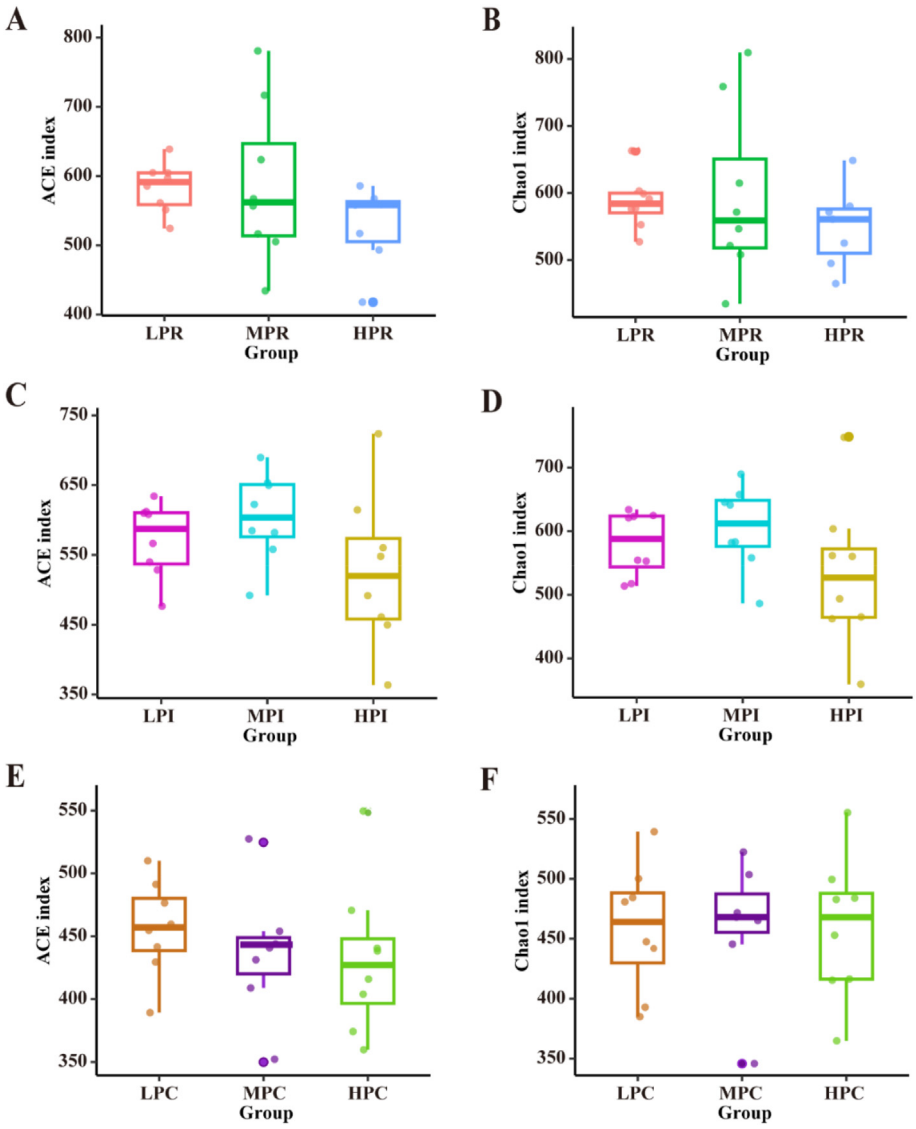

Fig. 1. The alpha-diversity indexes of microbial community in the gastrointestinal tract. ACE index (A) and Chao 1 index (B) in the rumen. ACE index (C) and Chao 1 index (D) in the ileum. ACE index (E) and Chao 1 index (F) in the colon. LPR, MPR, HPR = rumen of lambs fed with a low-protein (crude protein of 78.4 g/kg dry matter [DM]), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet; LPI, MPI, HPI = ileum of lambs fed with a low-protein (crude protein of 78.4 g/kg DM), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet; LPC, MPC, HPC = colon of lambs fed with a low-protein (crude protein of 78.4 g/kg DM), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet. $n = 8$.

4. Discussion

In animal husbandry, a suitable dietary protein level is sought to balance economic and environmental benefits (Guyader et al., 2016). In this study, the LP and MP treatments promoted weight gain by more than 10% when compared with the HP treatment. Notably, the LP treatment exhibited lower CP digestibility, similar to a previous study by Sileshi et al. (2021). This might be due to variations in protein sources (Ipharraguerre and Clark, 2005), with low-quality corn and wheat bran in the LP diet, whereas high-quality soybean meal in the MP and HP diets. Moreover, the MP treatment had greater ADF digestibility and similar NDF digestibility to the other two groups, implying a greater capacity to digest hemicellulose, which might be attributed to the variation in dietary fiber composition (La Rosa et al., 2022). In summary, the MP treatment improved the overall nutrient digestibility in lambs.

Carbohydrates are broken down into monosaccharides by GIT microorganisms in ruminants. These monosaccharides are further converted into pyruvate to synthesize VFAs, thus providing 70% of the energy requirements for ruminants (Jiao et al., 2015c). As anticipated, different dietary protein levels changed the GIT microbial fermentation patterns in this study. Notably, the LP treatment decreased the proportions of isobutyrate, valerate, and isovalerate compared to the HP treatment in the rumen. As these branched-chain VFAs are produced from the catabolism of amino acids and the decarboxylation and reduction of α-Keto acid (Oliphant and Allen-Vercoe, 2019), their decreased proportion might be due to both the lower CP content and digestibility in the LP treatment. Furthermore, LP treatment enhanced colonic butyrate production, which might be linked to the greater dietary carbohydrate level (Cui et al., 2019; Lv et al., 2020). Since butyrate is the preferred energy source for intestinal epithelial cells (IECs) (Oliphant and Allen-Vercoe, 2019), its increased production can

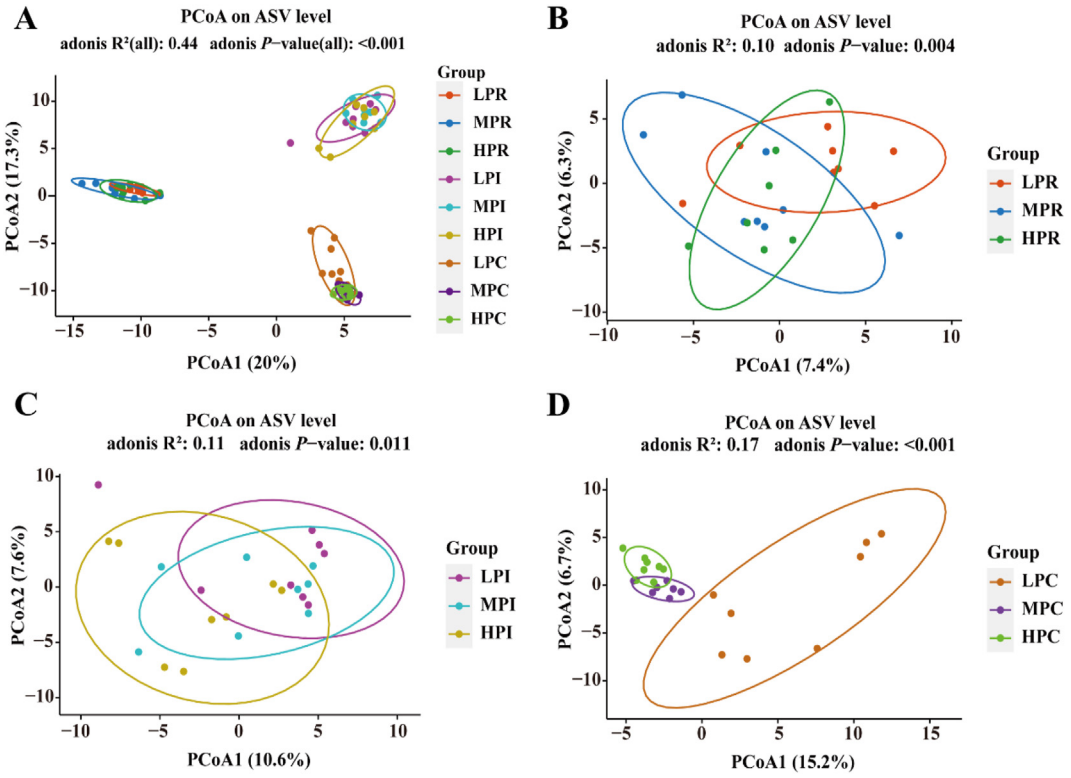

Fig. 2. Principal coordinate analysis (PCoA) of microbial community in the gastrointestinal tracts. (A) PCoA of microbial community in the rumen, ileum and colon. (B) PCoA of microbial community in the rumen. (C) PCoA of microbial community in the ileum. (D) PCoA of microbial community in the colon. LPR, MPR, HPR = rumen of lambs fed with a low-protein (crude protein of 78.4 g/kg dry matter [DM]), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet; LPI, MPI, HPI = ileum of lambs fed with a low-protein (crude protein of 78.4 g/kg DM), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet; LPC, MPC, HPC = colon of lambs fed with a low-protein (crude protein of 78.4 g/kg DM), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet. $n = 8$.

stimulate the proliferation of IECs and promote the absorption capacity of nutrients, thereby improving weight gain in lambs fed the LP diet compared with those fed the HP diet.

In ruminants, a large amount of dietary CP is rapidly decomposed in the rumen with the involvement of a myriad of microbial enzymes, with ammonia as the end product. Excess ammonia then enters the liver and is converted into urea (Firkins et al., 2007; Hailemariam et al., 2021). Hence, the GIT ammonia content and serum urea nitrogen level of lambs fed the HP diet were greater than those fed the LP diet. Similarly, as an index reflecting the amount of nitrogen absorbed and metabolized by animals, the urea nitrogen concentration is positively correlated with dietary protein intake (Vasconcelos et al., 2009). Intriguingly, the MCP production in the rumen of lambs in the LP treatment was greater than that of lambs in the HP treatment. A plausible explanation may be that LP treatment enhances urea nitrogen recycling to the rumen epithelium, resulting in more urea entering the rumen as a nitrogen source for the growth of resident microorganisms (Zhang et al., 2023). Furthermore, the concentrations of glutamate, glycine, alanine, and histidine were increased in the serum of LP lambs. These AAs are amino donors and auxiliaries in the urea cycle and might be derived from the liver of LP lambs with greater urea recycling capacity (Morris, 2002).

The gastrointestinal microbiota is an important modulator of the utilization of dietary nutrients, and changes in its composition may affect the growth performance and overall health of animals (Jiao et al., 2024). In this study, we deciphered the role of microorganisms implicated in nutrient utilization efficiency from a whole-gastrointestinal perspective. As anticipated, the microbial diversity and structure were altered by dietary CP level intervention across all GIT regions, similar to previous observations in the rumen of lambs (Cui et al., 2019). Intriguingly, the rumen of the MP treatment was enriched with the fiber-degrading bacteria *Fibrobacter_succeinogenes* and the starch-degrading bacteria *Selenomonas_ruminantium* (Fernando et al., 2010; Yeoman et al., 2021), which might contribute to the greater hemicellulose decomposition capacity as mentioned above (Cui et al., 2019). Notably, some species of *Prevotella* in the rumen respond inconsistently to various dietary protein levels (Lv et al., 2020). As a numerically predominant microorganism in the rumen, the diversified *Prevotella* spp. are capable of utilizing starches, other noncellulosic polysaccharides, and simple sugars as energy sources, and are proficient producers of VFAs (Tett et al., 2021). The diversity and prevalence of *Prevotella* species and strains in the rumen microbiota and their metabolic versatility in response to dietary protein levels remain to be elucidated. In the ileal microbiota, a surge of *UBA11452* spp. was noticed for the LP treatment. This genus, which belongs to the class *Vitallales*, increases in abundance under protein-deficient dietary conditions and consequently leads to a decrease in beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (Rinninella et al., 2019). Regarding the colonic microbiota, the LP diet harbored selected microbial biomarkers such as *Escherichia* spp. and *Lactobacillus_amylovorus*. *Escherichia* spp. have been reported to be involved in both AA synthesis and catabolism (Portune et al., 2016), whereas *Lactobacillus_amylovorus* efficiently decomposes peptides (Jing et al., 2022). The enrichment of these microorganisms in the LP treatment might be linked to the increased concentrations of AAs, particularly glutamate, glycine, alanine, and histidine in the

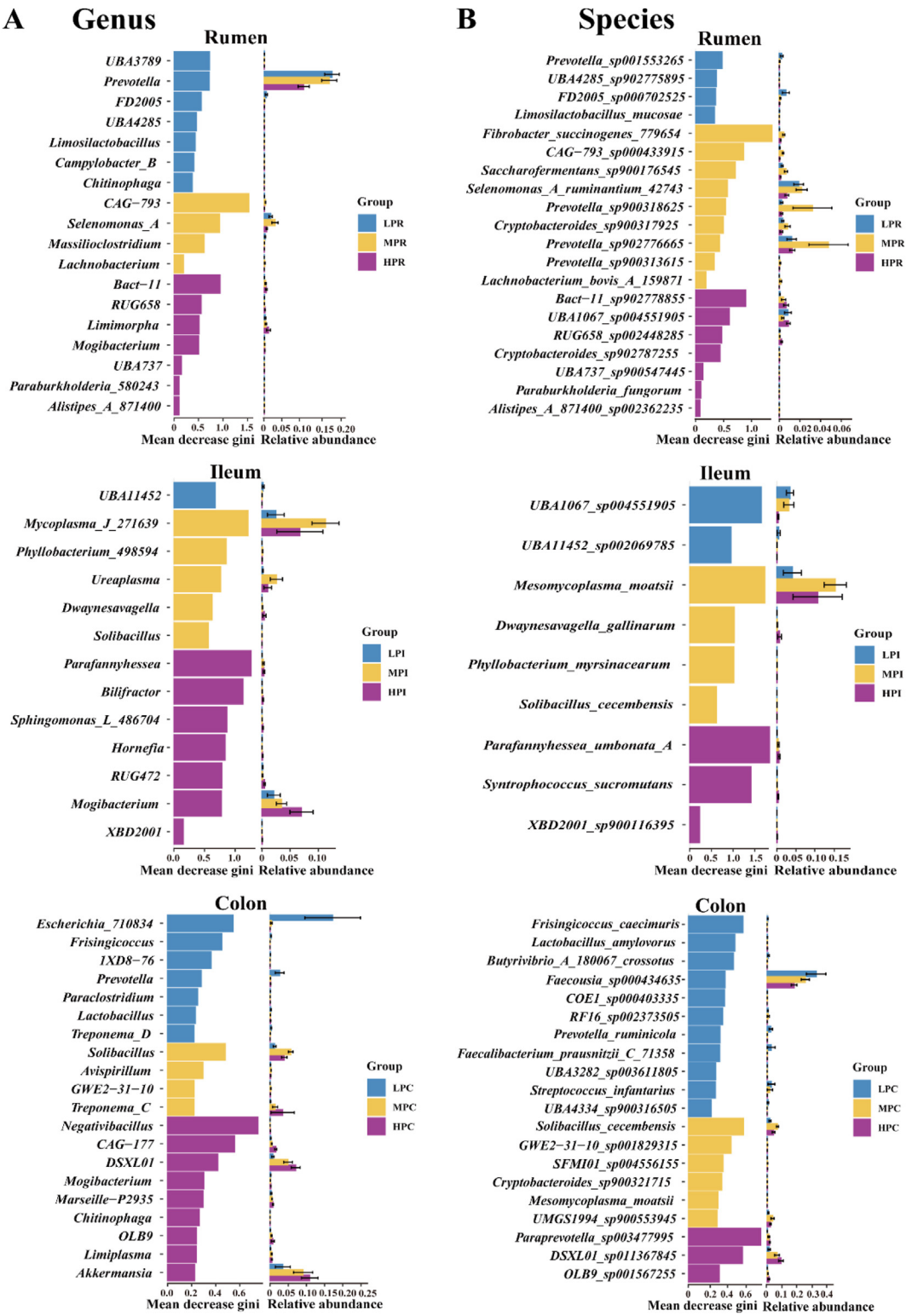

Fig. 3. Random forest results for gastrointestinal microbiota at genus level (A) and species level (B). LPR, MPR, HPR = rumen of lambs fed with a low-protein (crude protein of 78.4 g/kg dry matter [DM]), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet; LPI, MPI, HPI = ileum of lambs fed with a low-protein (crude protein of 78.4 g/kg DM), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet; LPC, MPC, HPC = colon of lambs fed with a low-protein (crude protein of 78.4 g/kg DM), medium-protein (crude protein of 112.0 g/kg DM) or high-protein (crude protein of 145.6 g/kg DM) diet. $n = 8$.

gut and serum, as mentioned above. Concurrently, LP treatment led to the amplification of *Paraburkholderia_fungorum* and *Mogibacterium* spp., both of which were implicated in nitrogen metabolism and intestinal inflammation (Liu et al., 2023; Wang et al., 2019), which might negatively affect the growth and health of lambs. In contrast, the colonic microbiota of the MP treatment was characterized by the enrichment of *Solibacillus_cecembensis*, a species that may drive efficient fiber fermentation and acetate production

(Duan et al., 2023). In summary, the enhanced nutrient efficiency through moderate protein levels was achieved by the joint involvement of the gastrointestinal microbiota.

5. Conclusion

Lambs fed the MP diet had greater weight gain and greater utilization efficiency of dietary nutrients when compared with those fed the LP and HP diets. Therefore, a MP diet with a CP content of 112.0 g/kg DM was optimal for lambs. The fermentation profile and microbial diversity were remarkably altered by dietary protein level intervention across all GIT regions. The MP treatment increased the abundance of fiber-degrading bacteria such as *Fibrobacter_succeinogenes* and *Solibacillus_cecembensis* in the rumen and colon, thereby improving the nutrient efficiency and growth performance of lambs.

CRediT authorship contribution statement

Zhibin Luo: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Huimin Ou:** Investigation, Formal analysis. **Christopher S. McSweeney:** Writing – review & editing. **Zhiliang Tan:** Writing – review & editing. **Jinzhen Jiao:** Writing – original draft, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Data availability statement

The amplicon sequencing data have been deposited in the China National GenBank Database with accession number CNP0006141.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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