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Original Research Article

Saccharomyces cerevisiae and *Kluyveromyces marxianus* yeast co-cultures modulate the ruminal microbiome and metabolite availability to enhance rumen barrier function and growth performance in weaned lambs

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ABSTRACT

In lambs, weaning imposes stress that can contribute to impaired rumen epithelial barrier functionality and immunological dysregulation. In this study, the effects of a yeast co-culture consisting of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (NM) on rumen health in lambs was evaluated, with a focus on parameters including growth performance, ruminal fermentation, and epithelial barrier integrity, ruminal metabolic function, and the composition of the ruminal bacteria. In total, 24 lambs were grouped into four groups of six lambs including a control (C) group fed a basal diet, and N, M, and NM groups in which lambs were fed the basal diet respectively supplemented with *S. cerevisiae* yeast cultures (30 g/d per head), *K. marxianus* yeast cultures (30 g/d per head), and co-cultures of both yeasts (30 g/d per head), the experiment lasted for 42 d. Subsequent analyses revealed that relative to the C group, the average daily gain (ADG) of lambs in the NM group was significantly greater and exhibited significant increases in a range of mRNA relative expression including monocarboxylate transporter 1 (*MCT1*), (Na^+)/hydrogen (H^+) exchanger 1 (*NHE1*), (Na^+)/hydrogen (H^+) exchanger 3 (*NHE3*), proton-coupled amino acid transporter 1 (*PAT1*), vacuolar H^+-ATPase (*vH+ ATPase*), claudin-1, occludin in the rumen epithelium ($P < 0.05$). Compared with the C group, the pH of the rumen contents in the NM group was significantly decreased , and the concentrations of acetate, propionate, and butyrate were significantly increased ($P < 0.05$). Analysis of the rumen bacteria showed that the NM group exhibited increases in the relative abundance of *Prevotella*, *Treponema*, *Moryella*, *Fibrobacter*, *CF231* and *Ruminococcus* ($P < 0.05$). Metabolomics analyses revealed an increase in the relative content of phthalic acid and cinnamaldehyde in the NM group as compared to the C group ($P < 0.05$), together with the greater relative content of L-tyrosine, L-dopa, rosmarinic acid, and tyrosol generated by the tyrosine metabolic pathway ($P < 0.05$). Spearman's correlation analyses revealed relative abundance levels of *Fibrobacter* and *Ruminococcus* were positively correlated with the mRNA relative expression levels of *PAT1*, *NHE3*, and zonula occluden-1 (*ZO-1*), as well as with tyrosol, phthalic acid, and cinnamaldehyde levels ($P < 0.05$). Ultimately, these results suggest that dietary supplementation with NM has a wide range of beneficial effects on weaned lambs and is superior to single bacterial fermentation. These effects include improvements in daily gain and rumen epithelial barrier integrity, as well as improvements in the composition of the rumen microbiome, and alterations in tyrosine metabolic pathways.

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1. Introduction

In the meat sheep husbandry field, early weaning has conclusively been shown to improve ewe fertility while reducing the costs associated with feeding (Cloete et al., 2021). However, after weaning, some lambs exhibit diarrhea, reduced feed intake, slow

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growth, and impaired immune function (Cheng et al., 2021). While the addition of antibiotics to lamb feed has been effective as an approach to abrogating these undesirable outcomes (Estrada-Angulo et al., 2023), antibiotic administration can incur negative effects on the gastrointestinal homeostasis in recipient animals, resulting in harm while also contributing to environmental contamination (Kraemer et al., 2019). The ruminal environment in mammals has been shown to harbor a wide array of microbes that serve as essential regulators of immune function, metabolic activity, and the maintenance of the ruminal epithelial barrier (Liu et al., 2021). The use of dietary modulation to improve ruminal microbes and metabolites has demonstrated promising efficacy as a means of improving the health of animals (Redoy et al., 2020). In weaned lambs, yeast cultures have been demonstrated to influence their gastrointestinal microflora, stimulate growth, and improve immune activity. As they can be readily stored and offer a high degree of economic value, these cultures are commonly used in feed preparations (Amin and Mao, 2021). The impacts of different yeast strains on ruminal function, however, differs markedly (Pang et al., 2022). *Saccharomyces cerevisiae* cultures can reportedly contribute to increased ruminal microbial abundance and enhanced ruminal homeostasis (Suntara et al., 2021). Takemura et al., for example, conducted a study wherein the supplemental administration of *S. cerevisiae* cultures to calves fed an alfalfa hay diet resulted in an increase in the abundance of the dominant genera detected within ruminal contents after a feeding interval of 7 to 16 weeks (Takemura et al., 2020). Monteiro et al. further studied weaned lambs and found that the inclusion of *S. cerevisiae* cultures in their diets led to the successful mitigation of lactic acid accumulation in the rumen and an increase in pH consistent with the potential therapeutic value of this supplementation strategy (Monteiro et al., 2022). *Kluyveromyces marxianus* culture fermentation has been shown to produce a wide array of small peptides and organic acids (Leonel et al., 2021), and many organic acids have been shown to positively benefit ruminal regulation, including butyrate, propionate, and acetate (Zhen et al., 2023). The ability of animals to directly absorb small peptides produced by *K. marxianus* cultures can also modulate a wide array of metabolic and physiological activities that ultimately promotes greater ruminal health and animal growth, thus contributing to improved livestock-derived product quality (Cui et al., 2023; Intanoo et al., 2020).

In recent years, studies have demonstrated the benefits of co-cultured yeast cultures for animals, which includes the interactions between the fermentation strains, the production of organic acids and antimicrobial compounds, the regulation of immune function, and the ability to improve the integrity of the rumen epithelial barrier. Jia et al. determined that *Bacillus subtilis* and *S. cerevisiae* co-cultures were sufficient to increase the utilization of nitrogen by sheep ruminal microbes while altering fermentation patterns therein and increasing the relative abundance of members of the *Fibrobacter* genus (Jia et al., 2018). Xie et al. determined that *Lactobacillus acidophilus* and *B. subtilis* co-cultures were similarly capable of enhancing mucosal barrier integrity in weaned piglets via the modulation of intestinal microflora-derived short-chain fatty acids (Xie et al., 2022). Such co-cultures thus appear to offer a range of positive benefits to animal gastrointestinal health (Arowolo and He, 2018).

This study hypothesized that supplementation of *S. cerevisiae* and *K. marxianus* yeast co-cultures (NM) in the diets of weaned lambs may improve growth performance and improve rumen health by altering the rumen bacteria and metabolites. This information would have beneficial effects on the rumen homeostasis of weaned lambs. Therefore, the objective of this study was to evaluate the effects of dietary supplementation with NM on growth performance, rumen fermentation indices, epithelial barrier function, rumen bacteria, and metabolomics.

2. Materials and methods

2.1. Animal ethics statement

The procedures used in the study were approved by the Institutional Animal Protection and Utilization Committee of Inner Mongolia Agricultural University (202212006). These experiments were performed as per the guidelines established by the National Research Council (2022-6-10/SYXK 2022-0031).

2.2. Yeast culture preparation

Strains of *S. cerevisiae* and *K. marxianus* exhibiting good fermentation characteristics were isolated in the laboratory from naturally fermented horse milk, and pilot production was performed in Kehong Feed Co., Ltd. (Inner Mongolia, China). The base matrix contained 12% bran, 12% spraying corn bran, 10% corn, 10% rice bran, 10% cottonseed meal, 8% wheat shorts, 28% corn germ meal, and 10% soybean meal. *S. cerevisiae* (3×10^8 CFU/g), *K. marxianus* (3×10^8 CFU/g), and a 1:1 mixture of the two yeasts (3×10^8 CFU/g) were inoculated at a concentration of 8% per 1000 kg wet mixed matrix, with the addition of sterile water while stirring, resulting in a total moisture content in the system of 40%. Aerobic fermentation was then conducted for 72 h at 30 °C. The nutrient compositions of the yeast cultures are presented in Table 1.

2.3. Experimental design and diets

This study was conducted at Fuchuan Farm (Bayannur, Inner Mongolia, China). Using a complete randomized design, 24 weaned 2-month-old lambs (Dorper × Thin-tailed Han) in good health (23.5 ± 2.85 kg) were assigned at random into four equally sized groups ($n = 6$) such that the average weights of animals in all groups were comparable. These lambs were individually housed in areas separated by fences (approximately 2 m^2). Control animals (C group) were fed a basal diet, while animals in N group received the basal diet that had been supplemented with a 30 g/d per head culture of *S. cerevisiae*, animals in M group received the basal diet that had been supplemented with a 30 g/d per head culture of *K. maximus*, and NM group received the basal diet that had been supplemented with 30 g/d per head of *S. cerevisiae* and *K. maximus* co-cultured culture. These diets were used to feed animals in equal portions at 08:00 and 19:00 each day. The experiment lasted for 42 d, during which the first 7 d represented a period of adaptation. During this period, animals were allowed to freely access feed and water. Composition and nutrient levels of diets are presented in Table 2. Lambs were weighed at the beginning and end of the experiment before morning feeding and the average daily gain

Table 1
Nutrient composition of mixed feed and yeast culture (feed-based).

Item	MF	N	M	NM
Crude protein, %	19.23	20.35	20.55	20.39
Dry matter, %	92.04	93.23	92.58	93.21
Neutral detergent fiber, %	34.11	32.45	33.72	34.23
Acid detergent fiber, %	20.02	19.42	19.23	19.97
Live yeast cells, CFU/g		6.8×10^4	1.5×10^4	7.2×10^4
Lactic acid, mmol/kg		371.13	391.57	380.31

MF = mixed feed; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures.

Table 2
Composition and nutrient levels of diets (%, DM basis).

Item	Group			
	C	N	M	NM
Ingredients				
Peanut vine	9.30	9.30	9.30	9.30
Corn stalk	10.00	10.00	10.00	10.00
Sunflower seed skin	4.00	4.00	4.00	4.00
Alfalfa meal	10.00	10.00	10.00	10.00
Corn grain	29.00	29.00	29.00	29.00
Soybean meal	6.00	6.00	6.00	6.00
Germ meal	8.00	8.00	8.00	8.00
Cotton meal	6.00	6.00	6.00	6.00
Distillers dried grains with solubles	6.00	6.00	6.00	6.00
Sunflower cakes	7.50	7.50	7.50	7.50
NaCl	0.50	0.50	0.50	0.50
Limestone	0.50	0.50	0.50	0.50
$CaHPO_4$	0.50	0.50	0.50	0.50
Premix[1]	2.70	2.70	2.70	2.70
Total	100.00	100.00	100.00	100.00
Nutrient levels[2]				
Metabolizable energy, MJ/kg	10.19	10.21	10.18	10.22
Dry matter	89.77	88.25	88.13	87.75
Crude protein	15.62	16.23	15.98	16.85
Neutral detergent fiber	33.35	34.03	33.45	33.38
Acid detergent fiber	21.40	20.99	21.38	21.59

C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures.
[1] Premix provided the following per kilogram of diets: Fe 60 mg, Cu 12 mg, Zn 60 mg, Mn 45 mg, nicotinic acid 60 mg, I 0.6 mg, Se, 0.2 mg, vitamin A 3500 IU, vitamin D 1200 IU, vitamin E 20 IU, Ca 2 g, P 1 g, Co 20 mg, NaCl 5 g.
[2] Metabolizable energy was calculated as described (NRC, 2001), and the others were measured values.

(ADG) was calculated by dividing the weight gain (final weight − initial weight) by the number of days on feed. The daily feed intake of each lamb is continuously recorded to calculate the average daily feed intake (ADFI). The ratio of feed intake to body weight gain calculates the ratio of feed intake to body weight gain (F/G). Total mixed ration (TMR) was collected every two weeks and frozen for further analysis.

2.4. Sample collection and chemical analysis

The dry matter (DM) of feed samples was determined by drying at 105 °C for 24 h in a forced air oven (AOAC, 2000; method 930.15). The total nitrogen (N) content in the feed was determined by the micro-Kjeldahl method (K1100, Hanon instruments, Shandong, China) (AOAC, 2000; method 976.05), and crude protein (CP) was calculated as N × 6.25. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by an automatic fiber analyzer (Ankom Technology, Fairport, NY, USA) according to Van Soest et al. (1991), respectively. The lactic acid contents were evaluated using a commercial assay kit (Nanjing Jiancheng Bio Co., Nanjing, China), according to the manufacturer's instructions.

After slaughter, the epithelial tissue from the rumen was collected, frozen in liquid nitrogen, and subsequently stored at −80 °C for further analysis. The rumen contents were filtered using gauze, and ruminal pH were then measured with a portable pH meter (Ecoscan pH 5, Eutech Instruments, USA). Volatile fatty acids (VFA) were detected via gas chromatography (Agilent 8860 GC, USA). For VFA concentrations analyses, a capillary DB-FFAP column was used with a column temperature of 130 °C, a gasification temperature of 180 °C, and a detector temperature of 180 °C with a hydrogen ion flame detector. The carrier gas consisted of nitrogen (60 kPa), oxygen (50 kPa), and hydrogen (50 kPa). The sensitivity and attenuation values were 101 and 3.0, respectively. As an internal standard for these analyses, crotonic acid was selected. For metaphosphoric acid-crotonic acid solution preparation, deionized water was used to dissolve 25 g of metaphosphoric acid, followed by the addition of 0.6464 g of crotonic acid and adjustment to a final 100 mL volume.

2.5. Real-time quantitative PCR (RT-qPCR)

Trizol (TIANGEN., Ltd., Shanghai, China) was used as directed to extract RNA from rumen epithelial samples, after which a Nanodrop 2000 spectrophotometer (Agilent, Cary 60 UV-Vis, USA) was used to assess RNA quality, with an OD_{260}/OD_{280} ratio of 1.8 to 2.0 being indicative of RNA that was sufficiently pure for further use. An M5 Sprint Perfect RT Kit with gDNA Clean for qPCR II (RR037A, Takara Bio, Beijing, China) was used to prepare cDNA, and a LightCycler 480 Instrument (Roche, Basel, Beijing, China) was then used for RT-qPCR amplification with SYBR Premix Pro Taq HS qPCR Kit (CN830s, TaKaRa Bio Beijing, China). Each 15 μL reaction was composed of 7.5 μL of 2 × SYBR Green Pro Taq HS Premix, 1.5 μL of appropriate primers (F + R), 2 μL of cDNA, and 4.0 μL of RNase-free water. Thermocycler settings were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 15 s, 60 °C for 30 s. The $2^{-\triangle\triangle Ct}$ method was used to evaluate relative expression (Livak and Schmittgen, 2001), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a normalization control. Primer 3.0 (Applied Biosystems, CA, USA) was used to design primers, which were synthesized by Shenggong Biotech (Shanghai, China). All primers used for these analyses are presented in Table 3.

2.6. 16S rRNA sequencing

The OMEGA Soil DNA Kit (M5635-02) (OMEGA Bio-Tek, GA, USA) was used to extract DNA from 24 rumen content samples, after which a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and agarose gel electrophoresis were respectively used to assess DNA quantity and quality. The 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primer pair was used for the amplification of the V3–V4 region of the bacterial 16S rRNA gene, with primers having incorporated of sample-specific 7-bp barcodes. Each PCR reaction included 5 μL of 5 × buffer, 0.25 μL of Fast pfu DNA polymerase (5 U/μL), 2 μL of dNTPs (2.5 mmol/L), 1 μL each of forward and reverse primers (10 μmol/L), 1 μL of template DNA, and 14.75 μL of ddH_2O. Thermocycler settings were as follows: 98 °C for 5 min; 35 cycles of 98 °C for 30 s, 53 °C for 30 s, 72 °C for 45 s; 72 °C for 5 min. Vazyme VAHTSTM DNA Clean Beads (Vazyme, Shanghai, China) were used to purify PCR products, after which a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, CA, USA) was used for quantification. Equal amounts of each amplicon were pooled, and end-to-end 2 × 250 bp sequencing was then performed with the Illumina NovaSeq platform by Personalbio Technology Co, Ltd. (Shanghai, China) using the NovaSeq 6000 SP Reagent Kit (500 cycles). Raw reads have been uploaded to the NCBI Sequence Read Archive (SRA) database (entry number: PRJNA1029322).

2.7. Bioinformatics analyses

QIIME2 2019.4 (Bolyen et al., 2019) was used to analyze the ruminal bacteria based on a slightly modified version of the official tutorial (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequencing data underwent demultiplexing with the demux plug-in, after which cutadapt was used to remove primer sequences (Martin, 2011). Data were then filtered based on quality, de-noised, merged, and chimeric sequences were removed with the DADA2 plug-in (Callahan et al., 2016). Non-monadic amplification sequence variants (ASV) were aligned using matt, and fasttre2 was

Table 3
Primer sequences used for real-time quantitative PCR.

Gene	Primers (5′ to 3′)	Size, bp	GenBank accession number
PAT1	Forward: AGTGCAGTTTGAGGGCTCTTTG Reverse: CCTTTCGTAATCCTCCACATCA	199	XM_015101183.3
AE2	Forward: GGGAGATGAAATACCTGGATGC Reverse: TATACAGGCATGGGCATCTCGT	94	XM_027969012.2
DRA	Forward: AACAACACCCCGAACACCAAT Reverse: AACTTGCGGAAAAGGTGGTCA	212	NM_001280717.1
MCT1	Forward: TGGACATAAGCCTATTCAAGCAC Reverse: GCTAAGAAAGACCAGTGGTGTA	98	XM_042252642.1
MCT4	Forward: TTACCTGGTTTGGGCTCTGCT Reverse: ATAAGGCACCTGTCCAGTCATA	179	XM_042252144.1
vH$^+$ATPase	Forward: CTGGGTTTCTGCCACTTGATG Reverse: TTATCAACAGGGAAGTTCACAC	89	NM_001009360.1
NHE1	Forward: CATCAACAAGTTCCGCATCGT Reverse: AGGAACAGGTCACACATAGGGA	138	XM_004005085.5
NHE2	Forward: TTCTTTGTCGTGGGGATCGG ReverseR: GATCACGCGGATGTTGTGTG	96	NM_001009803
NHE3	Forward: GAAACAGCAGCATTCCCAACG Reverse: AACTCGATCCCGCCACTCAT	139	XM_042233997.1
Claudin-1	Forward: GGCATCCTGCTGGGACTAATA Reverse: ATCTTCTGTGCCTCGTCGTCTT	89	NM_001185016.1
Claudin-4	Forward: GCCTTCATCGGCAGCAACA Reverse: ACAACAGCACGCCAAACACG	193	NM_001185017.2
Occludin	Forward: CAGCGTTGTAAGGTCAGGCATA Reverse: TCTGTGAACTGTAGAGAGACCC	161	XM_015101255.3
ZO-1	Forward: CTGGTGAAGTCTCGGAAGAATG Reverse: TCAGGGACATTCAGCAGGGTA	254	XM_042235170.1
IL-1β	Forward: TGAAGTGATGGCTTGCTACAGTG Reverse: CACGATGACCGACACTGCCT	181	NM_001009465.2
INF-γ	Forward: AACCAGGTCATTCAAAGGAGCA Reverse: CCGGAATTTGAATCAGCCTTT	112	NM_001009803.1
TNF-α	Forward: CTTCCACCCCCTTGTTCCTC Reverse: TCCCTGGTAGATGGGTTCGT	155	NM_001024860.1
TGF-β1	Forward: CTGAGCCAGAGGCGGACTACTA Reverse: GCTGTGCGAGCTAGACTTCATTT	101	NM_001009400.2
IL-6	Forward: TCATGGAGTTGCAGAGCAGT Reverse: CAGCATGTCAGTGTGTGTGG	205	NM_001009392.1
IL-10	Forward: TGCTCTGTTGCCTGGTCTTCC Reverse: TGCTGTTCAGTTGGTCCTTCATT	174	NM_001009327.1
GAPDH	Forward: TGGCATCGTGGAGGGACTTA Reverse: CATCATACTTGGCAGGTTTCTCC	266	NM_001190390.1

PAT1 = proton-coupled amino acid transporter 1; *AE2* = anion exchanger 2; *DRA* = down-regulated in adenoma; *MCT1* = monocarboxylat transporter 1; *MCT4* = monocarboxylate transporter 4; *vH$^+$ATPase* = vacuolar H$^+$-ATPase; *NHE1* = (Na$^+$)/hydrogen (H$^+$) exchanger 1; *NHE2* = (Na$^+$)/hydrogen (H$^+$) exchanger 2; *NHE3* = (Na$^+$)/hydrogen (H$^+$) exchanger 3; *ZO-1* = zonula occluden-1; *IL-1β* = interleukin-1β; *INF-γ* = interferon-γ; *TNF-α* = tumor necrosis factor-α; *TGF-β1* = transforming growth factor-β1; *IL-6* = interleukin-6; *IL-10* = interleukin-10; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

used to construct a phylogenetic tree based on these results (Price et al., 2009). The RDP Classified algorithm was used to classify and analyze 16S rRNA gene sequences, and the Silva 132 database was used for comparisons which were conducted at a 70% comparison threshold.

2.8. Rumen metabolomics analyses

Samples of ruminal contents (about 45 mg) were combined with 500 mL of cold 70% (v/v) methanol containing 2-chlorophenylalanine (1 mg/mL) as an internal standard. The solution was then swirled for 3 min, followed by ultrasonication for 10 min in ice water. Samples were then centrifuged for 10 min at 12,000 × *g*, and supernatants were transferred into an automatic sampler for UPLC-MS/MS analyses (metware) performed with an appropriate UPLC-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM A system; MS, QTRAP® system). Samples were analyzed in positive and negative ESI modes at a 0.4 mL/min flow rate using solvent A (0.04% acetate in water) and B (0.04% acetic acetonitrile) with the following gradient settings: 95:5 v/v at 0 min, 5:95 v/v at 11 min, and 95:5 v/v at 12 min (A:B) v/v, . The ESI source settings included a source temperature of 500 °C and an ion spray voltage of 5500 V or −4500 V. Source gas I, gas II, and curtain gas pressures were respectively set at 55, 60, and 25 psi. Collison gas (CAD) was high.

2.9. Statistical analyses

One-way ANOVA were used to analyze ruminal fermentation parameters and the expression of cytokines and other genes. Data analyses were conducted with SPSS 22.0, and the results were presented using GraphPad Prism (v 8.0). $P < 0.05$ was considered indicative of a significant difference, whereas $0.05 \leq P < 0.10$ was considered indicative of a significant trend. Analyses of microbial communities were performed at the ASV level. Wilcoxon rank sum tests were used to compute richness estimates (ACE and Chao1 indexes) and alpha diversity indexes. Taxa abundance at the genus and phylum levels was compared with python 2.8. Differences in the most abundant genera among groups were identified with the Wilcoxon rank sum test. *P*-values were false discovery rate (FDR)-corrected. Analyst 1.6.3 was used to analyze and process mass spectrum data. Peaks extracted from rumen samples during metabolomics analyses were analyzed with principal component analysis (PCA) and orthogonal projection latent structure discriminant analysis (OPLS-DA) approaches, selecting those metabolites

that differed significantly in abundance among these groups based on a variable importance in projections (VIP) ≥ 1.00 and a fold-change (FC)≥1.2 or ≤0.5 between groups. Differences in the enrichment of different metabolic pathway modules were evaluated via online Kyoto Encyclopedia of Genes and Genomes (KEGG) database annotation. Spearman's correlation analyses were performed in R (v 3.2.1) to detect correlations among ruminal fermentation, inflammatory cytokine gene expression, barrier-related gene expression, and rumen microbiome-related metabolites in these experimental lambs.

3. Results

3.1. Growth performance

The growth performance of the lambs is shown in Table 4. The ADG of lambs in the NM group was significantly higher than that in the C group ($P = 0.044$). However, there were no significant differences in the other growth performance indicators ($P > 0.05$).

3.2. Analysis of ruminal fermentation and epithelial gene expression

Analyses of the ruminal fermentation parameters in these experimental animals revealed a significant decrease in pH in the NM group ($P = 0.024$). While acetate concentration was significantly lower in C group as compared to the M and NM groups ($P < 0.001$), propionate concentration was significantly higher in the N, M, and NM groups ($P < 0.001$) as compared to C group. Moreover, C group exhibited butyrate concentration significantly lower than that in the M and NM groups ($P < 0.001$) (Table 5).

The results on the permeability of the rumen epithelium are shown in Fig. 1. The mRNA relative expression levels of monocarboxylat transporter 1 (*MCT1*) in M group ($P < 0.05$) and NM group ($P < 0.01$) differed significantly from those in C group. The mRNA relative expression levels of (Na^+)/hydrogen (H^+) exchanger 1 (*NHE1*) was significantly higher in the NM group as compared to the C ($P < 0.001$), N ($P < 0.05$), and M groups ($P < 0.01$). The NM group also exhibited significant increases in the mRNA relative expression levels of (Na^+)/hydrogen (H^+) exchanger 3 (*NHE3*) ($P < 0.01$), proton-coupled amino acid transporter 1 (*PAT1*) ($P < 0.01$), and vacuolar H^+-ATPase (*vH$^+$ATPase*) ($P < 0.05$) compared to C group, whereas no significant differences in mRNA relative expression levels of anion exchanger 2 (*AE2*), down-regulated in adenoma (*DRA*), monocarboxylate transporter 4 (*MCT4*), or (Na^+)/hydrogen (H^+) exchanger 2 (*NHE2*) were observed among these four groups ($P > 0.05$).

Table 4
Effects of dietary supplementation of N, M, and NM yeast cultures on the growth performance of lambs.[1]

Item	Group				SEM	*P*-value
	C	N	M	NM		
Initial weight, kg	22.02	21.95	22.18	21.77	0.331	0.984
Final weight, kg	33.28	34.05	34.48	34.77	0.353	0.521
ADG, kg/d	0.32^{b}	0.35^{ab}	0.35^{ab}	0.37^{a}	0.011	0.044
ADFI, kg/d	1.52	1.58	1.65	1.64	0.620	0.871
F/G	4.74	4.62	4.78	4.46	0.924	0.679

ADG = average daily gain; ADFI = average daily feed intake; F/G = the ratio of feed intake to body weight gain; SEM = standard error of the mean.
[a,b] Within a row, values with different letter superscripts differ significantly at the $P < 0.05$ level.
[1] C = basal diet; N=*Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. Data are expressed as means and SEM, $n = 6$/group.

Table 5
Effects of dietary supplementation of N, M, and NM yeast cultures on rumen fermentation in lambs.[1]

Item	Group				SEM	*P*-value
	C	N	M	NM		
pH	7.36^{a}	6.76^{ab}	6.75^{ab}	6.21^{b}	0.332	0.024
Total VFA, mmol/L	112.75	120.57	128.97	131.36	8.213	0.521
Acetate, mmol/L	56.88^{b}	59.68^{ab}	62.61^{a}	65.69^{a}	4.834	<0.001
Propionate, mmol/L	16.75^{b}	19.66^{a}	21.55^{a}	21.61^{a}	1.111	<0.001
Butyrate, mmol/L	13.44^{b}	14.08^{ab}	16.93^{a}	16.18^{a}	0.970	<0.001
Valerate, mmol/L	2.92	2.83	3.02	3.06	0.532	0.694
Isobutyrate, mmol/L	19.83	21.29	21.54	21.61	1.481	0.762
Isovalerate, mmol/L	2.93	3.03	3.32	3.21	1.232	0.571

VFA = volatile fatty acids; SEM = standard error of the mean.
[a,b] Within a row, values with different letter superscripts differ significantly at the $P < 0.05$ level.
[1] C = basal diet; N=*Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. Data are expressed as means and SEM, $n = 6$/group.

3.3. Analysis of tight junction protein and cytokine mRNA relative expression levels in the rumen epithelium

To determine the effects of co-cultured yeast on the rumen epithelium, the mRNA relative expression of tight junction proteins and cytokines were analyzed in the samples. The mRNA relative expression levels of genes encoding claudin-1 ($P < 0.01$), zonula occluden-1 (*ZO-1*) ($P < 0.01$), and interferon-γ (*IFN-γ*) ($P < 0.05$) were significantly elevated in the N group compared to C group, while the opposite was true for the gene encoding interleukin-6 (*IL-6*) ($P < 0.01$). Samples from M group exhibited significantly higher claudin-1 mRNA relative expression level ($P < 0.01$) and significantly lower *IL*-6 mRNA relative expression levels ($P < 0.05$), while the NM group exhibited significantly elevated mRNA relative expression levels encoding claudin-1 ($P < 0.001$) and occludin ($P < 0.05$) together with significantly lower levels of mRNA relative expression encoding transforming growth factor-β1 (*TGF-β1*) ($P < 0.05$) and *IL-6* ($P < 0.01$). No significant changes in the levels of mRNA relative expression encoding claudin-4, *IL-1β*, interleukin-10 (*IL-10*), or tumor necrosis factor-α (*TNF-α*) were observed when comparing these four experimental groups (Fig. 2A and B). Increased mRNA relative expression of claudin-1, *ZO-1*, *IFN-γ*, and occludin is indicative of increases in rumen epithelial barrier function and cytokine production. N and NM groups showed better effects in regulating rumen epithelial barrier function and rumen immunity of weaned lambs.

3.4. 16S rRNA sequencing analyses of the ruminal bacteria

To explore the potential effects of co-cultured yeast cultures on the ruminal microbiome in experimental lambs, 16s rRNA gene sequencing was performed using primers specific for the V3–V4 region. In total 2,077,312 effective sequences were obtained across 24 samples (average: 86,555 sequences/sample), leading to the identification of 253 ASV at the 97% nucleotide sequence identity level (Fig. 3A). In total, 82 ASV were represented across samples in different groups, while the C, N, M, and NM groups respectively harbored 42 (16.6%), 24 (9.49%), 22 (8.70%), and 20 (7.91%) unique ASV. Alpha diversity indexes revealed that the Observed_species index and Chao1 index in C group were significantly higher than those in the N, M, and NM groups ($P < 0.05$), while the Shannon and Simpson indexes in M group significantly exceeded those for C, N, and NM groups ($P < 0.05$) (Table 6).

Analyses of β-diversity were also conducted to assess structural differences among these microbial communities in different groups of experimental lambs. Discrete distributions of samples were

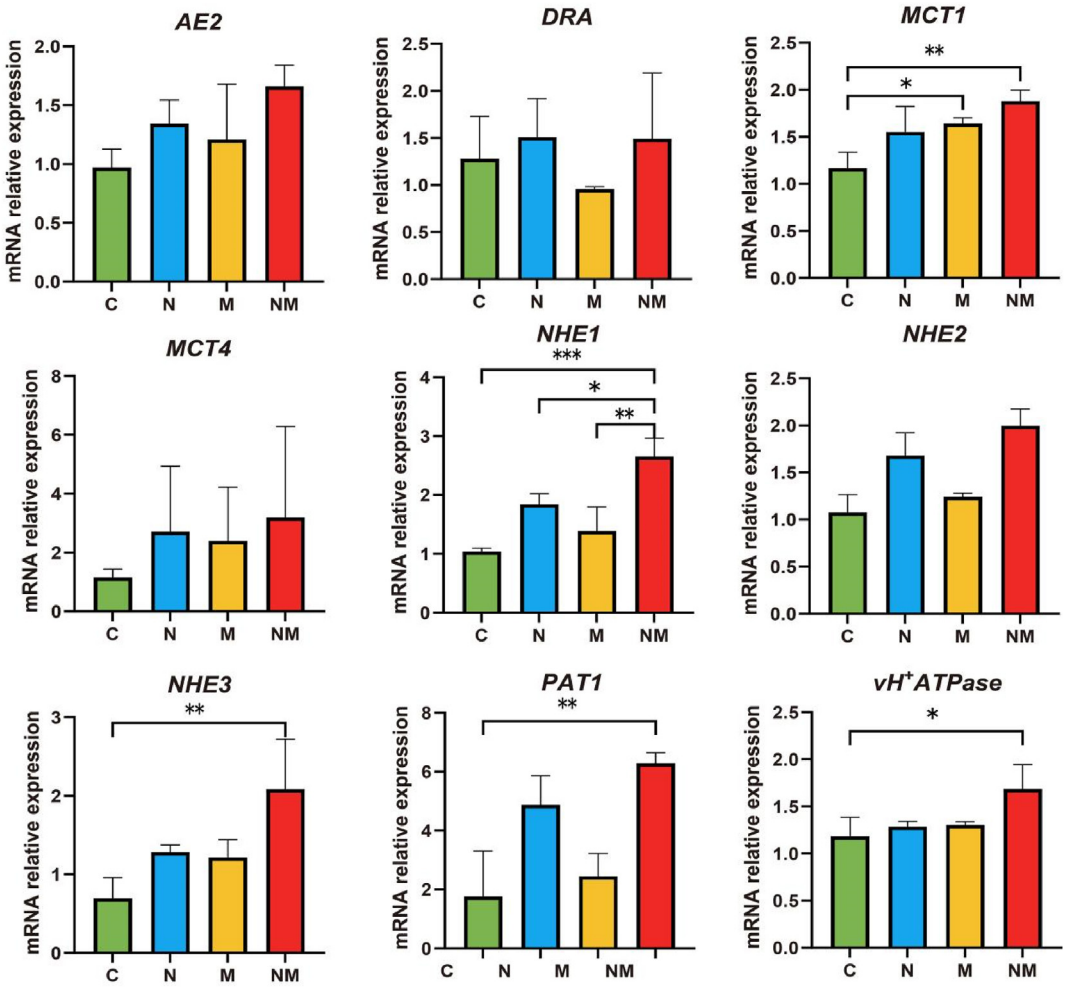

Fig. 1. Real-time quantitative PCR analysis of mRNA relative expressions of anion exchanger 2 (*AE2*), down-regulated in adenoma (*DRA*), monocarboxylate transporter 1 (*MCT1*), monocarboxylate transporter 4 (*MCT4*), (Na^+)/hydrogen (H^+) exchanger 1 (*NHE1*), (Na^+)/hydrogen (H^+) exchanger 2 (*NHE2*), (Na^+)/hydrogen (H^+) exchanger 3 (*NHE3*), proton-coupled amino acid transporter 1 (*PAT1*), vacuolar H^+-ATPase (*vH^+ATPase*). C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. n = 6/group; *P < 0.05, **P < 0.01, ***P < 0.001.

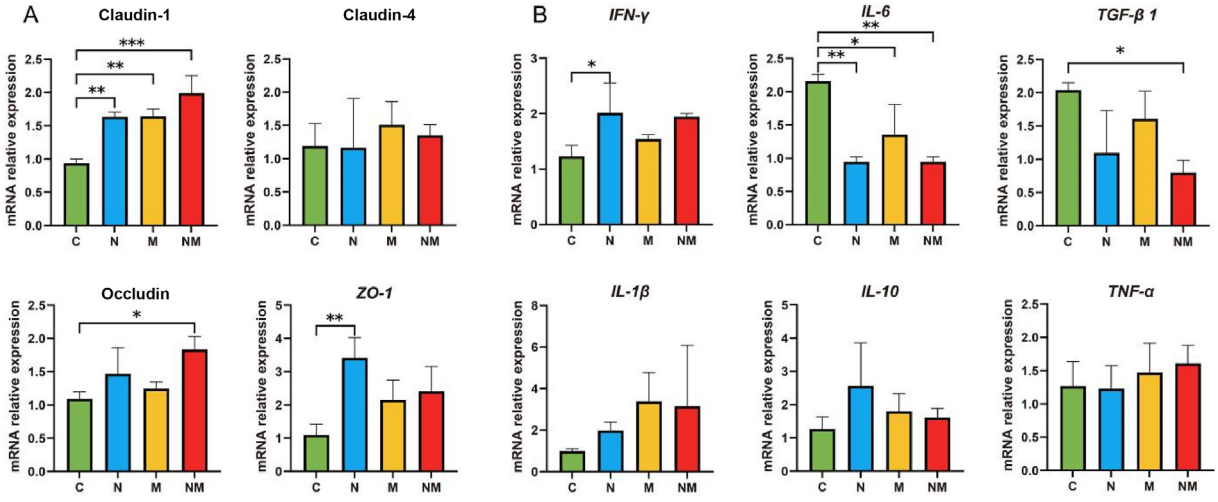

Fig. 2. Effects of dietary supplementation of N, M, and NM yeast cultures on the mRNA relative expression of tight junction proteins and cytokines in the rumen epithelium of lambs. (A) Real-time quantitative PCR was used to analyze the mRNA relative expression of claudin-1, occludin, claudin-4, and zonula occludens protein-1 (*ZO-1*). (B) Real-time quantitative PCR was used to analyze the mRNA relative expression of interferon-γ (*INF-γ*), interleukin-6 (*IL-6*), interleukin-10 (*IL-10*), transforming growth factor-β1 (*TGF-β1*), interleukin-1β (*IL-1β*), and tumor necrosis factor-α (*TNF-α*). C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. n = 6/group, *P < 0.05, **P < 0.01, ***P < 0.001.

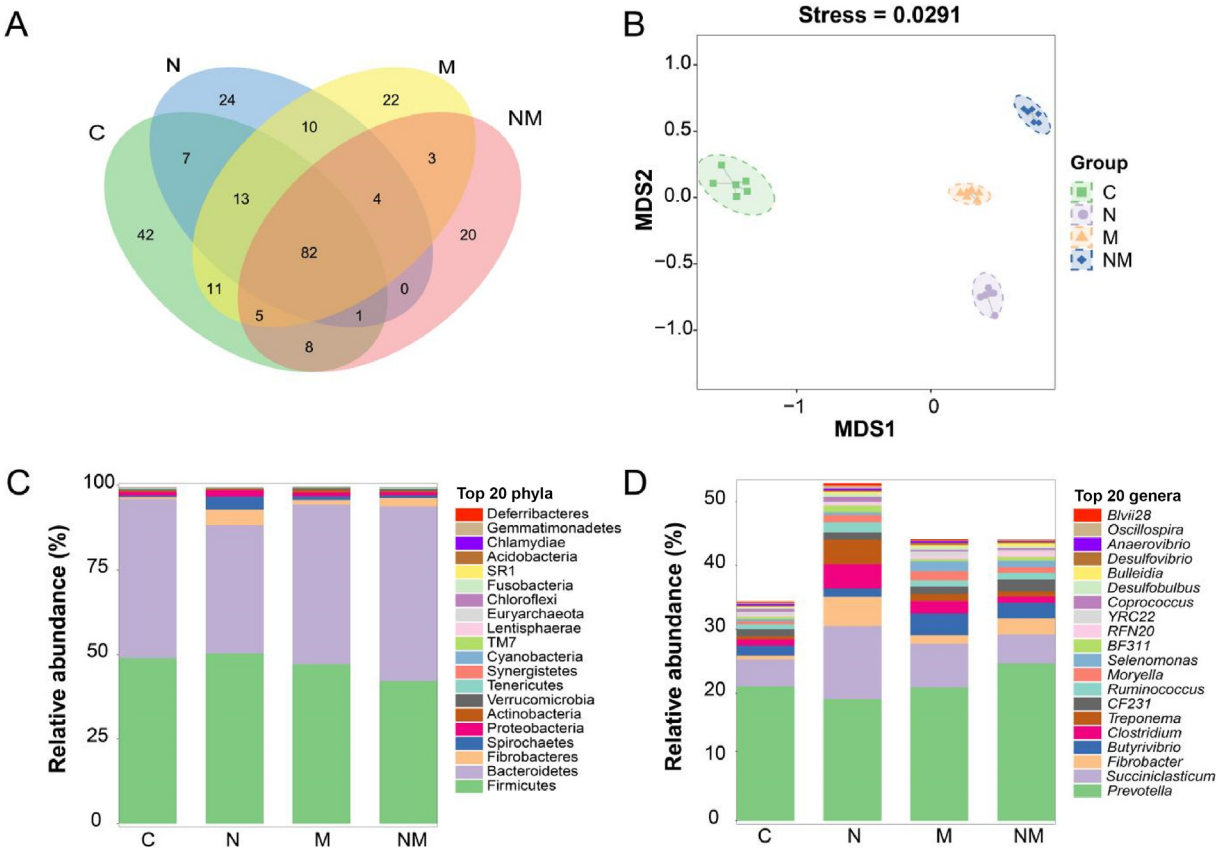

Fig. 3. Microbial diversity in the lamb rumen. (A) Venn diagram highlighting the overlapping amplicon sequence variant (ASV) when comparing ruminal bacteria composition among groups. (B) An non-metric multidimensional scaling (NMDS) plot. (C, D) The composition of the ruminal bacteria in the indicated groups at the phylum (C) and genus (D) levels. C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. $n = 6$/group.

observed in these four groups (Fig. 3B), with C group being spatially separated from the three other groups. Non-metric multidimensional scaling (NMDS) plots generated via the Jaccard method highlighted differences among groups in terms of bacteria composition. Firmicutes, Bacteroidetes, and Fibrobacteres were the predominant phyla in these samples (Fig. 3C), while the predominant genera were *Prevotella*, *Succiniclasticum*, and *Fibrobacter* (Fig. 3D). Compare to C group, N group exhibited significant increases in relative abundance of *Succiniclasticum, Clostridium,* and *Selenomonas* at the genus level ($P < 0.05$), while M group exhibited significantly increased relative abundance of *Succiniclasticum*, *Butyrivibrio*, and *Clostridium* ($P < 0.05$), and NM group exhibited significantly increased relative abundance of *Prevotella* and *CF231* ($P < 0.05$). Significant increases in relative abundance of *Fibrobacter, Treponema, Ruminococcus*, and *Moryella* were evident in the N, M, and NM groups ($P < 0.05$) (Table 7). In general, the changes in the dominant genera were more significant in NM group relative to the other experimental groups.

3.5. Analyses of correlations between ruminal bacterial, barrier function, and inflammatory gene expression

Correlations between rumen barrier function, inflammatory cytokine gene expression, and the dominant genera detected in ruminal samples were next assessed. The relative abundance of *Fibrobacter* ($P < 0.01$), *Succiniclasticum* ($P < 0.001$), *Treponema* ($P < 0.001$), *Ruminococcus* ($P < 0.01$), and *Clostridium* ($P < 0.01$) were all found to be positively correlated with the mRNA relative expression of *ZO-1*, whereas the relative abundance of *Succiniclasticum* ($P < 0.05$) and *Treponema* ($P < 0.05$) were positively

Table 6
Effects of dietary supplementation of N, M, and NM yeast cultures on rumen bacteria alpha diversity indexes in lambs.[1]

Item	Group				SEM	*P*-value
	C	N	M	NM		
Observed_species index	3763.53[a]	2972.97[c]	3302.40[b]	3064.77[c]	197.082	<0.001
Chao1 index	4534.25[a]	3596.90[d]	4185.55[b]	3838.55[c]	194.294	<0.001
Simpson index	0.991[d]	0.995[b]	0.996[a]	0.994[c]	0.0005	<0.001
Shannon index	9.33[b]	9.24[c]	9.47[a]	9.11[d]	0.061	<0.001

SEM = standard error of the mean.
[a-d] Within a row, values with different letter superscripts differ significantly at the $P < 0.05$ level.
[1] C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. Data are expressed as means and SEM, $n = 6$/group.

Table 7
Ruminal bacteria relative abundance (%) at the genus level in lambs.[1]

Item	Group				SEM	*P*-value
	C	N	M	NM		
Prevotella	0.211[b]	0.191[c]	0.209[c]	0.246[a]	0.0122	<0.001
Succiniclasticum	0.042[c]	0.114[a]	0.068[b]	0.045[c]	0.0171	<0.001
Fibrobacter	0.006[d]	0.046[a]	0.014[c]	0.025[b]	0.0093	<0.001
Butyrivibrio	0.016[c]	0.013[d]	0.034[a]	0.025[b]	0.0052	<0.001
Clostridium	0.010[c]	0.038[a]	0.019[b]	0.010[c]	0.0071	<0.001
Treponema	0.005[d]	0.039[a]	0.011[b]	0.008[c]	0.0084	<0.001
CF231	0.011[b]	0.011[b]	0.012[b]	0.018[a]	0.0022	<0.001
Ruminococcus	0.008[c]	0.016[a]	0.010[b]	0.011[b]	0.0021	<0.001
Moryella	0.005[d]	0.011[b]	0.015[a]	0.009[c]	0.0022	<0.001
Selenomonas	0.003[d]	0.004[c]	0.015[a]	0.010[b]	0.0033	<0.001

SEM = standard error of the mean.
[a-d] Within a row, values with different letter superscripts differ significantly at the $P < 0.05$ level.
[1] C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. Data are expressed as means and SEM, $n = 6$/group.

correlated with the mRNA relative expression of *IL-10*. In addition, *NHE3* mRNA relative expression was positively correlated with the relative abundance of *Fibrobacter* ($P < 0.01$) and *Ruminococcus* ($P < 0.05$) abundance, whereas the mRNA relative expression of *MCT4* was positively correlated with *Ruminococcus* relative abundance ($P < 0.05$) (Fig. 4). These data indicate that co-culture supplementation and associated modulation of the ruminal microbiome may contribute to improvements in rumen function.

3.6. Ruminal metabolic profiling

Changes in ruminal metabolic profiles in weaned lambs were analyzed using a UPLC-MS/MS approach. In total, 710 metabolites were detected as presented in the PCA score plots shown in Fig. 5A. The OPLS-DA score plots revealed good separation among the four groups, and a random permutation test confirmed that this model exhibited satisfactory accuracy (R2X = 0.272, R2Y = 0.995, Q2 = 0.914, Fig. 5B), enabling screening for differentially abundant metabolites (Fig. 6). Comparisons of the C and N groups, showed significantly higher relative contents of phthalic acid, tyrosol, rosmarinic acid, and cinnamaldehyde in the N group with a corresponding reduction in L-tyrosine acid ($P = 0.005$); when comparing the C and M groups, significantly higher relative contents of L-tyrosine, phthalic acid, and rosmarinic acid were present in the M group, whereas tyrosol and L-dopa relative contents were significantly decreased ($P < 0.05$); a comparison of the C and NM groups showed significantly increased relative contents of L-tyrosine, rosmarinic acid, phthalic acid, cinnamaldehyde, L-dopa, and tyrosol ($P < 0.05$) in the NM group; L-glutamine and phenylethylamine relative contents were found to be significantly lower in all

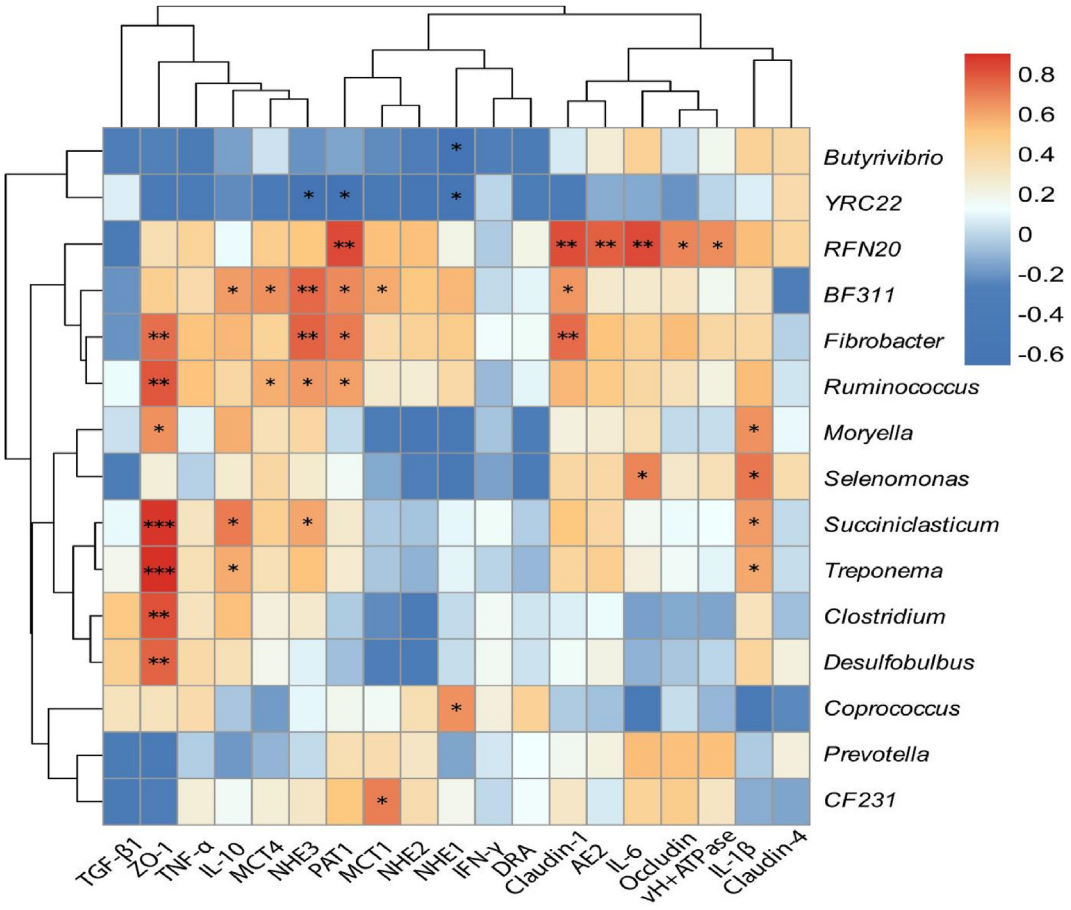

Fig. 4. Thermogram showing Spearman correlations of the relationships between rumen bacteria, the rumen epithelial barrier, and expression of cytokine genes. Positive and negative correlations are shown in red and blue, respectively. *MCT1* = monocarboxylate transporter 1; *NHE1*= (Na^+)/hydrogen (H^+) exchanger 1; *NHE2* = (Na^+)/hydrogen (H^+) exchanger 2; *NHE3* = (Na^+)/hydrogen (H^+) exchanger 3; *PAT1* = proton-coupled amino acid transporter 1; *vH$^+$ATPase* = vacuolar H^+-ATPase; *AE2* = anion exchanger 2; *DRA* = down-regulated in adenoma; *MCT4* = monocarboxylate transporter 4; *ZO-1* = zonula occludens protein-1; *IL-6* = interleukin-6; *TGF-β1* = transforming growth factor-β 1; *INF-γ* = interferon-γ; *IL-10* = interleukin-10; *IL-1β* = interleukin-1β; *TNF-α* = tumor necrosis factor-α. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $n = 6$/group.

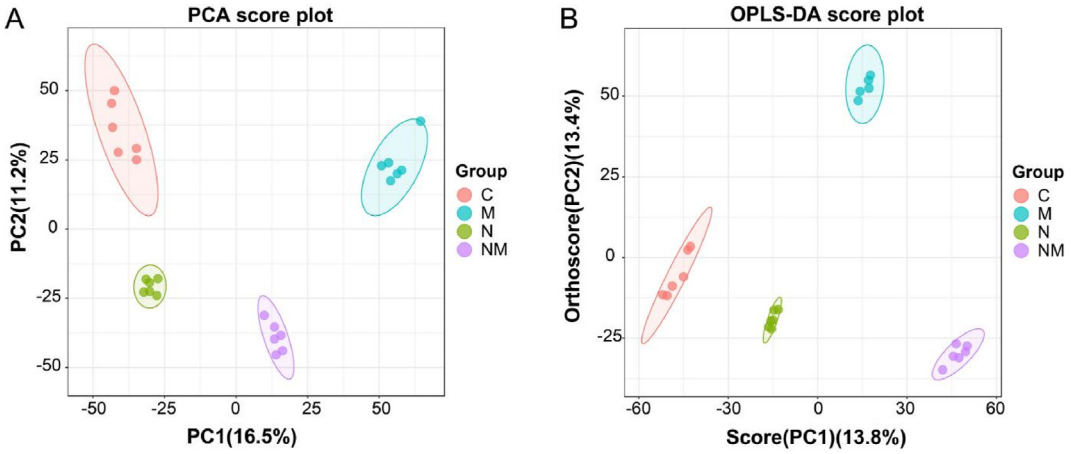

Fig. 5. Metabolomic profiling of the ruminal contents of weaned lambs fed C, N, M, and NM diets. (A) Principal component analysis (PCA) plot developed based on the ruminal metabolites found in the four treatment groups. (B) Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots revealing significant separation among the clusters for the four treatment groups. $n = 6$/group. C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures.

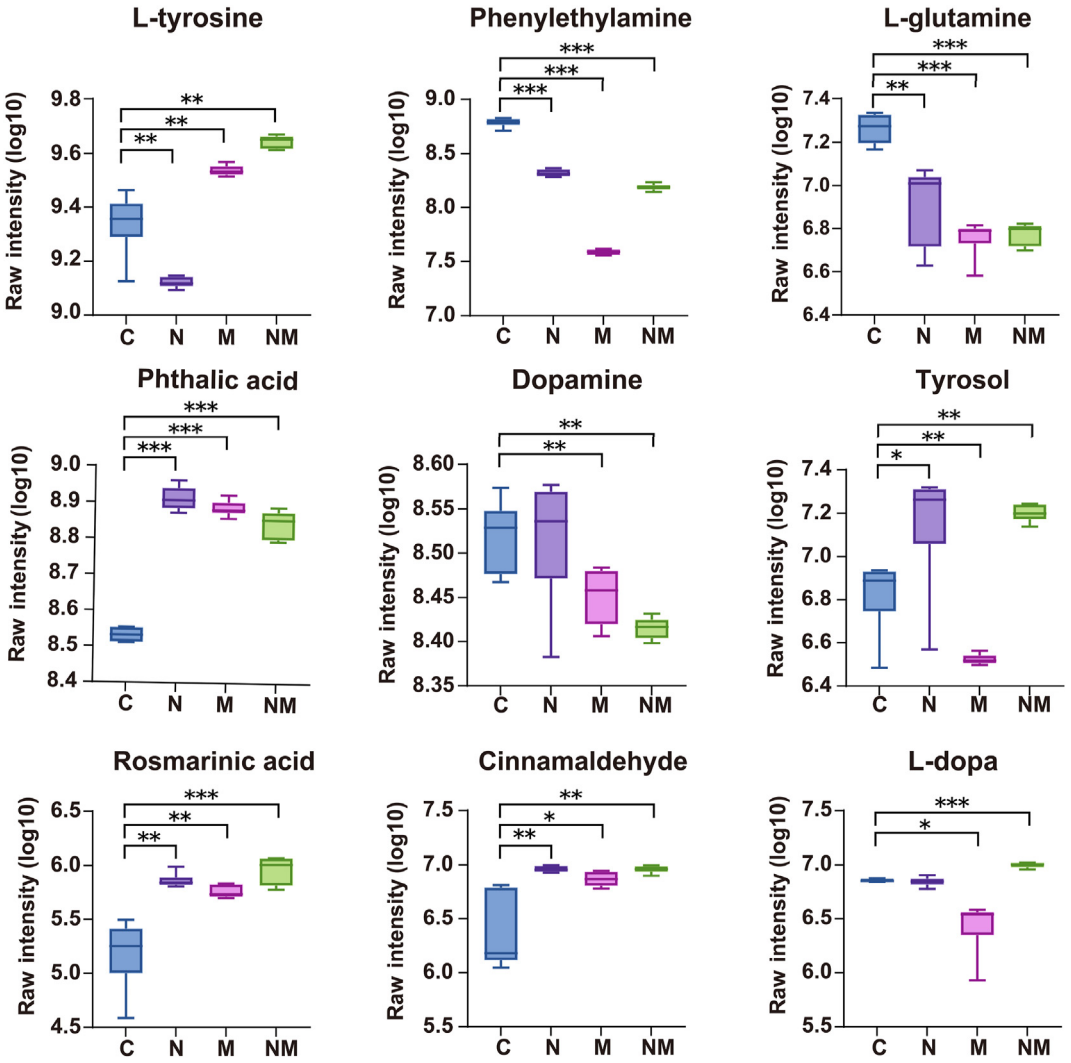

Fig. 6. Box-plot of each significantly different metabolite peak area (log10 transformed) among treatments. C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. Data was presented as median and whiskers represented the Tukey. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

experimental groups relative content to C group ($P < 0.05$) (Table 8). Pathway analyses suggested that relative to C group, the weaned lambs that underwent N and M dietary supplementation primarily exhibited changes in the biosynthesis of plant secondary metabolites and tyrosine metabolism, while animals in the NM group exhibited significant changes in tyrosine metabolism (Table 9). Dietary supplementation with co-cultured yeast cultures may thus significantly influence tyrosine metabolism.

3.7. Correlations between the bacteria and ruminal metabolites

Spearman correlation coefficients were used to explore relationships among ruminal microbes and differentially abundant metabolites. When comparing the C and N groups, significant positive correlations were observed between the relative abundance of *Ruminococcus* and *Fibrobacter* and the levels of phthalic acid, rosmarinic acid, cinnamaldehyde, and tyrosol ($P < 0.05$). Significant positive correlations were observed between the relative abundance of *Coprococcus* and the levels of phenylethylamine, naringin, tyrosol, and L-glutamine, whereas it was negatively correlated with L-tyrosine abundance ($P < 0.05$) (Fig. 7A). A comparison of the C and M groups also demonstrated positive correlations between the relative abundance of *Ruminococcus* and *Fibrobacter* and the levels of phthalic acid, rosmarinic acid, and tyrosol. Significant positive correlations were additionally detected between the relative abundance of *Coprococcus* and the levels of phenylethylamine, thymidine, pantothenic acid, tyrosol, and L-glutamine ($P < 0.05$) (Fig. 7B). Similarly, comparisons of the C and NM groups revealed significant positive correlations between the relative abundance of *Ruminococcus* and *Fibrobacter* and the ruminal levels of tyrosol, phthalic acid, and cinnamaldehyde. There was a significant positive correlation between the relative abundance of *Coprococcus* and the levels of phenylethylamine, isocitric acid, tyrosol, and L-glutamine, with a negative correlation with levels of L-tyrosine and creatine ($P < 0.05$) also shown (Fig. 7C).

Table 8
Metabolomic analysis of differentially expressed metabolites in rumen contents[1].

Name	Fold change	Variable importance in the projection	*P*-value	Type
C vs N				
L-tyrosine	0.49	1.69	0.005	Down
Phenylethylamine	0.34	2.02	<0.001	Down
L-glutamine	0.47	1.66	0.004	Down
Phthalic acid	2.4	1.82	<0.001	Up
Tyrosol	2.31	1.23	0.037	Up
Rosmarinic acid	3.99	1.54	0.003	Up
Cinnamaldehyde	3.04	1.64	0.008	Up
C vs M				
Tyrosol	0.47	1.48	0.007	Down
Phenylethylamine	0.06	1.84	<0.001	Down
L-glutamine	0.31	1.78	<0.001	Down
Dopamine	0.45	1.33	0.008	Down
L-dopa	0.43	1.32	0.010	Down
L-tyrosine	1.53	1.46	0.008	Up
Phthalic acid	2.26	1.67	<0.001	Up
Rosmarinic acid	3.12	1.34	0.007	Up
Cinnamaldehyde	2.47	1.38	0.014	Up
C vs NM				
Phenylethylamine	0.25	1.91	<0.001	Down
L-glutamine	0.32	1.88	<0.001	Down
Dopamine	0.78	1.71	0.001	Down
L-dopa	1.4	1.70	<0.001	Up
L-tyrosine	1.96	1.72	0.001	Up
Phthalic acid	2.05	1.72	<0.001	Up
Tyrosol	2.25	1.64	0.003	Up
Rosmarinic acid	5.11	1.51	<0.001	Up
Cinnamaldehyde	3.02	1.54	0.008	Up

[1] C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. $n = 6$/group.

4. Discussion

Levels of interest in the application of yeast cultures as an approach to improving growth performance, enhancing ruminal fermentation, modulating the ruminal microbiota, and alleviating adverse stress-related outcomes in post-weaned lambs have been rising in recent years (Ban and Guan, 2021). The strains most commonly used in this context include *S. cerevisiae* (Suntara et al., 2021), *Candida utilis* (Yang et al., 2021), and *Lactobacillus* (Karamad et al., 2020). However, there have been no prior reports examining the effects of *S. cerevisiae* and *K. marxianus* yeast co-cultures on ruminal dynamics and related processes in weaned lambs. In this study, NM yeast cultures increased the ADG in the lambs. Analyzing ruminal pH can provide an invaluable index for the assessment of the internal rumen microenvironment (Zhao et al., 2023). A pH of 6.0 to 6.5 is generally favorable for ruminal microbe proliferation, in

Table 9
Pathway analysis showing enriched pathways of differentially expressed metabolites in the lamb rumen.[1]

Pathway	Hits[2]	*P*-value[3]	-ln(*P*)	Impact[4]
C vs N				
Pyrimidine metabolism	8	0.0014	2.859	0.1231
Central carbon metabolism in cancer	6	0.0013	2.884	0.1622
Lysine degradation	7	0.0013	2.892	0.14
cAMP signaling pathway	5	0.0013	2.901	0.2
Purine metabolism	10	0.0012	2.907	0.1053
Biosynthesis of cofactors	22	0.0012	2.922	0.0679
Biosynthesis of plant hormones	9	0.0004	3.391	0.1324
Biosynthesis of amino acids	13	0.0003	3.473	0.1016
Tyrosine metabolism	10	0.0003	3.596	0.1282
Biosynthesis of plant secondary metabolites	18	0.000001	6.040	0.1277
C vs M				
Alcoholism	4	0.0005	3.339	0.4
Amphetamine addiction	4	0.0003	3.547	0.4444
Parkinson disease	6	0.0001	3.866	0.2857
Phenylalanine metabolism	10	0.0001	3.897	0.1667
Cocaine addiction	4	0.0001	4.075	0.5714
Plant hormone signal transduction	5	0.0001	4.179	0.4167
cAMP signaling pathway	7	0.00004	4.370	0.28
Taste transduction	9	0.000003	5.496	0.2812
Tyrosine metabolism	14	0.000002	5.642	0.1795
Biosynthesis of plant secondary metabolites	20	0.000001	6.092	0.1418
C vs NM				
Pyrimidine metabolism	9	0.00079	3.100	0.1385
Prolactin signaling pathway	4	0.00058	3.238	0.3636
Gap junction	4	0.00058	3.238	0.3636
Alcoholism	4	0.00038	3.421	0.4
cAMP signaling pathway	6	0.00030	3.526	0.24
Amphetamine addiction	4	0.00023	3.629	0.4444
Parkinson disease	6	0.00010	3.983	0.2857
Cocaine addiction	4	0.00007	4.159	0.5714
Dopaminergic synapse	5	0.000052	4.281	0.4167
Tyrosine metabolism	15	0.000001	6.691	0.1923

[1] C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. $n = 6$/group.
[2] Hits is the number of significantly increased or decreased metabolites in a single pathway.
[3] *P*-values calculated and corrected from the pathway enrichment analysis.
[4] Impact represents impact value in the pathway topology analysis.

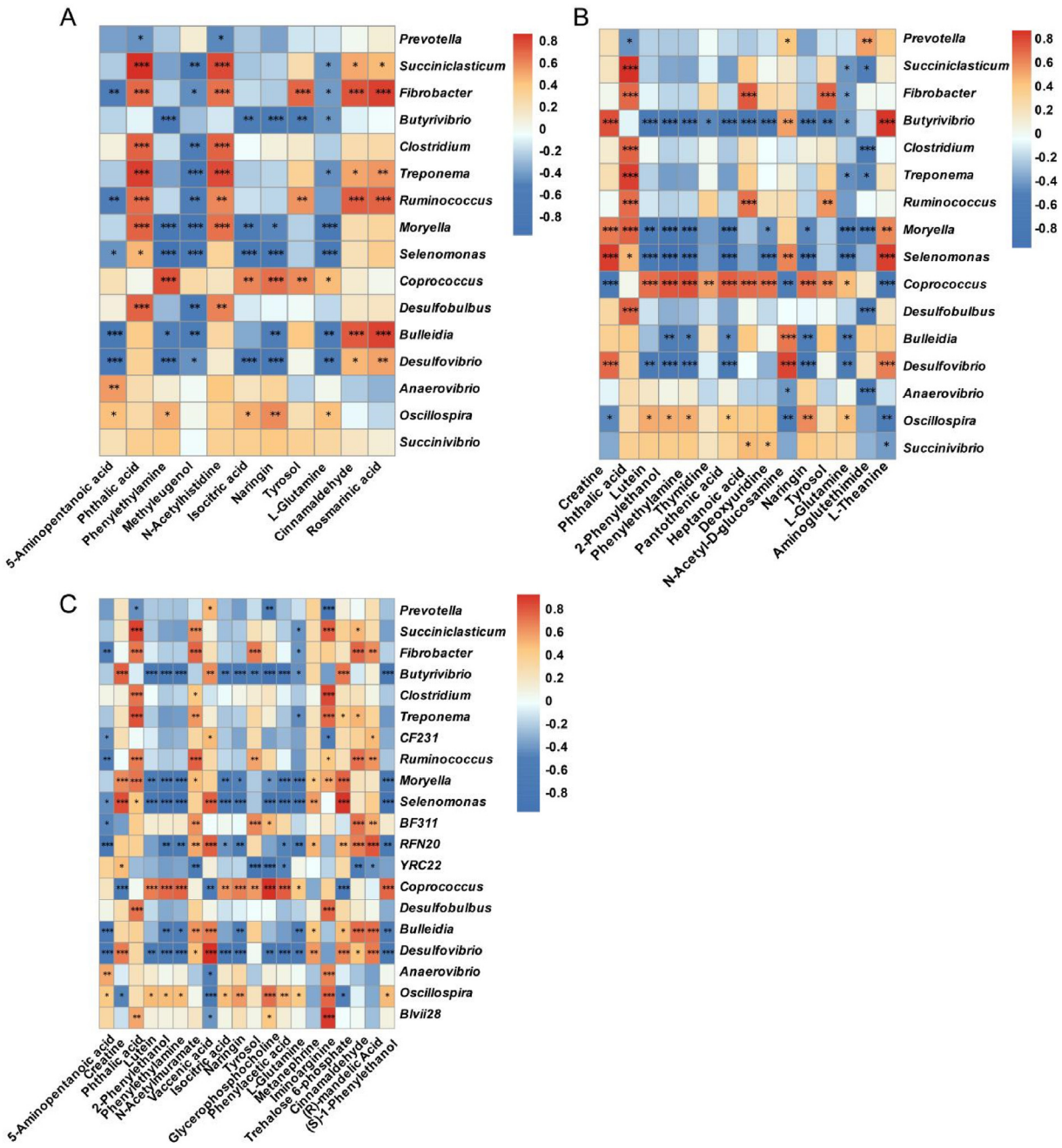

Fig. 7. Analyses of correlations between microbiome composition and metabolite levels in rumen contents. (A) C group vs. N group. (B) C group vs. M group. (C) C group vs. NM group. C = basal diet; N = *Saccharomyces cerevisiae* yeast cultures; M = *Kluyveromyces marxianus* yeast cultures; NM = *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* co-cultures yeast cultures. Significant positive and negative correlations are shown in red and blue, respectively. $n = 6$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

turn contributing to the production of ample VFA that can meet the energy requirements of the host (Matthews et al., 2019). In this study, NM group supplementation was associated with significantly reduced ruminal pH in the post-weaned lambs, which may be a consequence of the rapid ruminal nutrient fermentation observed following co-culture supplementation, leading to the production of organic acids and a marked drop in the pH in the rumen. Shifts in rumen pH can affect the microbial communities present therein and the fermentation products that they generate (Therion et al., 1982). In prior research focused on weaned calves and lambs, yeast cultures were found to enhance both ruminal fermentation activity and VFA uptake (Hassan et al., 2020). Rackwitz et al. analyzed the permeability of the rumen epithelium and found that adding yeast cultures led to an increase in butyrate uptake per unit of epithelial area (Bertens et al., 2023). Here, significantly increased propionate concentrations were detected in the three experimental groups, with the M and NM groups showing markely higher increases in the levels of acetate and butyrate. Acetate, propionate, and butyrate are substrates for the synthesis of primary nutrients in sheep and are also related to the energy balance (Hartinger and Zebeli, 2021). These results suggest that the addition of co-cultured yeast cultures improved the growth performance and decreased rumen pH, the addition of *K. marxianus* cultures and co-cultured yeast cultures to post-weaning diets in

lambs can improve the ruminal concentrations of acetate and propionate, thereby enhancing the function of the rumen.

In addition, an investigation of SCFA-related transporters has shown that the addition of yeast culture could promote the expression of *MCT1* in sheep rumen epithelium (Kuzinski and Röntgen, 2011). Here, dietary supplementation with the M and NM cultures was associated with an increase in *MCT1* expression, thus enhancing rumen epithelium-mediated butyrate uptake. The vH^+ATPase transporter is expressed within the rumen epithelium where it is responsible for 30% of total H^+ transport, thus shaping the pH within the rumen (Etschmann et al., 2006; Hu et al., 2021). Higher *vH^+ATPase* expression in the NM group may be related to the synergistic enhancement of microbe-mediated secretion of active nutrients and substances that are fermented in the rumen, reducing H^+ concentrations therein and maintaining the local pH balance via regulating *vH^+ATPase* expression. The cell membrane NHE1 and NHE3 proteins are responsible for the extracellular excretion of most H^+ from rumen epithelial cells, with these respective transporters accounting for 50% and 20%, of all transported H^+ (Etschmann et al., 2006; Hu et al., 2021). All experimental groups in this study exhibited significant differences in *NHE1* expression in the rumen epithelium as compared to control lambs, while significant increases in *NHE3* and *PAT1* expression were also observed in the NM group. Lamb dietary supplementation with yeast co-cultures may thus lead to at least some enhancement of the permeability of the rumen epithelium while promoting epithelial development in this following weaning.

Rumen epithelial barrier integrity in the lambs in the NM group in the present study was significantly enhanced. ZO-1, occludin, claudin-1, and claudin-4 are currently regarded as key tight junction proteins responsible for shaping the composition and function of the intercellular barrier within the rumen (Kuo et al., 2022; Wang et al., 2022). Lambs in the NM group exhibited the upregulation of *Claudin-1* and *Occludin* at the mRNA level. The integrity of the rumen epithelium is closely associated with inflammatory factors, and yeast cultures can inhibit rumen *IL-1β*, *TNF-α*, and *IFN-γ* expression by modulating signaling through TLR4-mediated pathways (Fang et al., 2017). TLR signaling activation can also stimulate NF-κB signaling, in turn leading to a reduction in the expression of pro-inflammatory mediators including IL-6, IL-1β, and TNF-α (Bu et al., 2019; Conrad et al., 2014). Yeast cultures were previously found to suppress ruminal *TNF-α* and *IL-6* expression in weaned lambs (Izuddin et al., 2019). Here, NM supplementation was associated with a drop in *TGF-β1* and *IL-6* gene expression. Corresponding decreases in *IL-6* expression were also evident in the N and M groups. Overall, the addition of the NM yeast culture could enhance the function of the rumen epithelial barrier.

To better understand how adding NM yeast cultures to the diet of weaned lambs can influence the rumen bacteria, 16S rRNA sequencing was performed. This approach revealed that the rumen microbial communities in these experimental lambs were dominated by *Bacteroidetes* and *Firmicutes*, in line with prior reports (Mizrahi et al., 2021). Significantly higher *Fibrobacterium* relative abundance was observed in the N, M, and NM groups as compared to the C group. In the gut of ruminants, *Fibrobacter* is known to serve as an efficient lignocellulose degrader (Ransom-Jones et al., 2012). Dietary yeast culture supplementation can contribute to improvements in the abundance of ruminal bacterial with the ability to degrade cellulose and starch as well as the ability to utilize lactate (Amin and Mao, 2021). Most notably, the NM group exhibited increases in the relative abundance of certain beneficial bacteria including *Prevotella*, *Fibrobacter*, *Ruminococcus*, and *Butyrivibrio.* Certain *Ruminococcus* strains have been identified as natural probiotics capable of promoting rumen health in the context of animal production (Hsieh et al., 2023; Mamuad et al., 2019; Yu et al., 2020). Consistently, in the present study, a positive correlation was noted between *Ruminococcus* relative abundance and the mRNA relative expression levels of *PAT1*, *NHE3*, *MCT4*, and *ZO-1*. Moreover, the relative abundance of *Fibrobacter* was positively associated with the expression levels of mRNAs encoding claudin-1, *PAT1*, *NHE3*, and *ZO-1*. These data thus indicate that the changes in rumen bacteria composition in these lambs fed an NM-supplemented diet may be associated with the enhancement of rumen epithelial barrier function and nutrient absorption.

Metabolomics analysis showed significantly higher cinnamaldehyde levels in the N and NM groups relative to those in C group. Cinnamaldehyde exhibits promising antibacterial efficacy and is also capable of exerting antioxidant and anti-inflammatory effects to alleviate external stress-related effects (Doyle et al., 2019). Significant reductions of the amino acid metabolism-related metabolite phenylethylamine were also detected. Phenylethylamine can be generated by the *Ruminococcus gnavus*-mediated dietary catabolism of phenylalanine, facilitating 5-hydroxytryptamine (5-HT) biosynthesis within the intestines via trace amine-associated receptor 1 (TAAR1) activation and thereby provoking irritable bowel syndrome-like symptoms including diarrhea (Zhai et al., 2023). Here, the observed reduction in the relative content of phenylethylamine may be indicative of altered ruminal digestive function and reduced incidence of diarrhea in weaned lambs. Tyrosine metabolism is another relevant process that was enriched in the metabolites that were differentially abundant in the N, M, and NM groups. As an essential amino acid, tyrosine is used by microbes and their hosts as a precursor to synthesize various metabolites (Schenck and Maeda, 2018). The NM group also presented with significant tyrosine-related metabolite enrichment, including elevated levels of rosmarinic acid, tyrosol, L-tyrosine, and L-dopa. Rosmarinic acid exhibits robust bioactivity, including reported antimicrobial, anti-inflammatory, and antioxidant properties (Zhao et al., 2022). Tyrosol can reportedly drive significant increases in the expression of Nrf2 anti-inflammatory pathway-related genes at the mRNA level while also contributing to increases in the relative abundance of beneficial thick-walled bacterial phyla within the small intestines of sheep, which is consistent with the results of the present study. Tyrosine hydroxylase catalyzes the processing of tyrosine to generate L-dopa, which is further processed by L-dopa decarboxylase to generate dopamine (Güvenç et al., 2019). Dopamine serves as a regulator of immunoinflammatory activity within the rumen through its ability to inhibit NLRP3 inflammasome activity and downstream inflammation via DRD1 signaling. Alternative tyrosine metabolism detected in this study may thus positively impact the ruminal health of weaned lambs (Bueno-Carrasco et al., 2022; Yan et al., 2015). Spearman correlation analyses revealed that *Coprococcus* relative abundance was significantly positively correlated with the levels of phenethylamine, tyrosol, and L-glutamine, whereas it was negatively correlated with L-tyrosine levels. Moreover, *Fibrobacter* and *Ruminococcus* relative abundance was found to be positively correlated with the levels of phthalic acid, cinnamaldehyde, and tyrosol. The tyrosine metabolism detected in the NM group may thus be associated with altered *Coprococcus, Fibrobacter,* and *Ruminococcus* relative abundance.

5. Conclusion

In summary, these results suggest that dietary supplementation with co-cultured yeast cultures can increase the average daily gain in lambs, alter the composition and metabolite profiles of the ruminal microbiome, promoting *Prevotella, Coprococcus Fibrobacter, CF231*, *Butyrivibrio*, and *Ruminococcus* enrichment while increasing the levels of metabolites including phthalic acid, L-dopa, L-tyrosine,

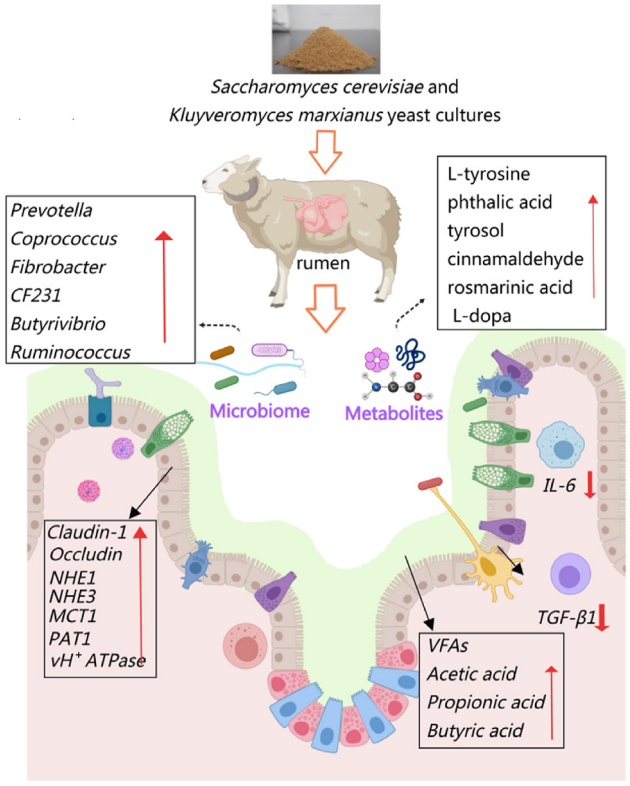

Fig. 8. Dietary supplementation with co-cultured yeast cultures in weaned lambs modulates the composition and metabolite profile of the ruminal microbiome, enhancing the nutrient absorption and barrier function of the rumen epithelium. *MCT1* = monocarboxylate transporter1; *NHE1* = (Na^+)/hydrogen (H^+) exchanger 1; *NHE3* = (Na^+)/hydrogen (H^+) exchanger 3; *PAT1* = proton-coupled amino acid transporter 1; *vH⁺ATPase* = vacuolar H^+-ATPase; *IL-6* = interleukin-6; *TGF-β1* = transforming growth factor-β1.

tyrosol, cinnamaldehyde, and rosmarinic acid. These shifts in the makeup of the ruminal microflora may enhance rumen epithelial barrier function and nutrient uptake changes. These findings offer insights that can support future efforts to establish antibiotic alternative strategies aimed at fostering greater rumen health in weaned lambs (Fig. 8).

Credit Author Statement

Dacheng Liu, Hui Chen and **Lan Yang** designed the research; **Zixuan Xu, Xiongshi Liu** and **Songjian Li** conducted the research; **Xiao Li, Xueqiang Li** and **Zixuan Xu** analyzed the data; **Zixuan Xu** wrote the original draft; **Chun Ying** and **Rui Du** had primary responsibility for the final content. All authors read and approved the final manuscript.

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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