

Dietary thymol supplementation improves growth performance and reproductive endocrine status in growing Texel × Kazakh rams and remodels the rumen microbiota and metabolome

Qiming Zheng

Xinjiang Agricultural University

Shuhao Zong

Xinjiang Agricultural University

Hai Li

Bureau of Agriculture and Rural Affairs of Zhaosu County

Haifeng Deng

Bureau of Agriculture and Rural Affairs of Zhaosu County

Pengshun Liu

Xinjiang Agricultural University

Xingnuo Feng

Xinjiang Agricultural University

Xiaobin B. Li

172387243@qq.com

Xinjiang Agricultural University

Research Article

Keywords: growth performance, lambs, metabolomics, reproductive hormones, rumen microbiota, Thymol

Posted Date: August 20th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10231190/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Background

Thymol is a natural monoterpene phenolic compound with antimicrobial, antioxidant, and anti-inflammatory activities and has been reported to modulate rumen fermentation and nutrient utilization in ruminants. However, its effects on growing rams, particularly on reproductive endocrine status and rumen microbiota–metabolome interactions, remain unclear. This study evaluated the effects of dietary thymol supplementation on growth performance and serum reproductive hormone levels in growing Texel × Kazakh rams and explored the associated changes in rumen fermentation, microbial community structure, and metabolite profiles.

Results

Thirty 13 ± 0.5 -month-old Texel × Kazakh crossbred rams with similar body weight were randomly assigned to a control group fed a basal total mixed ration or a thymol group fed the basal diet supplemented with 30 mg/kg thymol on a dry matter basis, with 15 rams per group. The experiment lasted 72 days. Thymol supplementation increased total weight gain and average daily gain and decreased the feed-to-gain ratio ($P < 0.01$), without affecting average daily feed intake. Apparent digestibility of crude protein, ether extract, neutral detergent fiber, and acid detergent fiber, as well as nitrogen and phosphorus retention efficiency, was improved in the thymol group ($P < 0.05$). Serum total cholesterol and high-density lipoprotein cholesterol concentrations were decreased ($P < 0.01$), whereas testosterone and estrone concentrations were increased ($P < 0.01$), and estradiol and corticosterone concentrations were also increased ($P < 0.05$). Ruminal pH was elevated ($P < 0.05$), while ammonia nitrogen concentration was reduced ($P < 0.01$). 16S rRNA gene sequencing showed that thymol increased rumen microbial richness and evenness and altered microbial community structure. Several fibrolytic or carbohydrate-utilizing genera, including Rikenellaceae_RC9_gut_group, NK4A214_group, Christensenellaceae_R-7_group, and Treponema, were enriched in the thymol group, whereas Prevotella and Selenomonas were reduced or tended to be reduced. Untargeted metabolomics identified 569 differential metabolites, which were mainly enriched in the cAMP signaling pathway, purine metabolism, nucleotide metabolism, cGMP-PKG signaling pathway, and glutathione metabolism. Representative metabolites, including adenosine monophosphate, 3-hydroxybutyric acid, inosine, and adenosine, were significantly correlated with average daily gain, rumen fermentation parameters, and serum reproductive hormone levels.

Conclusions

Dietary supplementation with 30 mg/kg thymol improved growth performance, feed conversion efficiency, nutrient digestibility, nitrogen and phosphorus retention, and serum reproductive hormone levels in growing Texel × Kazakh rams. These effects were accompanied by increased rumen microbial

diversity, enrichment of fibrolytic bacterial taxa, reduced ruminal ammonia nitrogen, and shifts in rumen metabolites related to purine metabolism, cAMP signaling, and glutathione metabolism. These findings suggest that thymol may serve as a natural feed additive for improving growth and reproductive endocrine status in growing rams, potentially through coordinated modulation of the rumen microbiota and metabolome.

Introduction

Thymol ($C_{10}H_{14}O$) is a natural monoterpene phenolic compound widely present in the volatile oils of Lamiaceae plants, such as thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*). It possesses a range of biological activities, including broad-spectrum antimicrobial, antioxidant, and anti-inflammatory properties, and has been regarded as a promising plant-derived feed additive in ruminant production [1–4]. In ruminants, the regulatory effects of thymol have been preliminarily demonstrated. Previous studies have shown that thymol can affect growth performance, nutrient digestibility, ruminal microbial protein synthesis, total volatile fatty acid concentrations, ammonia nitrogen production, and plasma glucose levels in sheep [5, 6]. In vitro studies further indicated that thymol can alter the relative abundances of major rumen bacterial phyla, including Firmicutes and Bacteroidetes, thereby modulating rumen fermentation patterns [7]. In addition, thymol has been reported to up-regulate the mRNA expression of tight junction proteins, such as CLDN-1, OCLDN, and ZO-1, and to alleviate rumen inflammation by suppressing the overexpression of pro-inflammatory cytokines such as IL-6 [8]. These findings provide an important basis for the application of thymol in ruminant nutrition. However, most previous studies have focused on fattening sheep or general production traits, whereas the effects of thymol in growing rams remain insufficiently understood.

The growing stage of rams is a critical period during which rapid somatic growth occurs alongside the continued development of the reproductive endocrine system [9, 10]. Nutritional interventions during this period may therefore influence not only growth performance but also reproductive endocrine status. In recent years, the functional connection between gastrointestinal microbiota and host reproductive physiology has received increasing attention. The concept of a gut–reproductive axis has recently been proposed in livestock [11]. Studies in sheep have also suggested that gut or rumen microbiota and their metabolites may vary with reproductive stage and may be associated with sperm motility and reproductive hormone levels [12, 13]. These findings suggest that rumen microbial metabolites may represent a potential link between dietary nutrition and reproductive endocrine function in growing rams.

Although thymol has been shown to modulate rumen microbial communities, previous studies have rarely integrated rumen microbiota profiling with rumen metabolomics to characterize the metabolic responses associated with thymol supplementation. Moreover, whether thymol can influence reproductive hormone levels in growing rams, and whether such changes are associated with alterations in the rumen microbiota and metabolome, remains unclear. Therefore, the present study was conducted using 13 ± 0.5 -month-old Texel $\times$ Kazakh crossbred growing rams supplemented with 30 mg/kg thymol on a dry matter basis. Growth performance, nutrient apparent digestibility, blood biochemical

parameters, serum reproductive hormones, rumen fermentation parameters, rumen microbial community structure, and rumen metabolite profiles were comprehensively assessed. This study aimed to evaluate the effects of dietary thymol supplementation on growth performance and reproductive endocrine indicators in growing rams and to characterize the associated changes in rumen microbiota and metabolome.

Materials and methods

Ethics approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Xinjiang Agricultural University (approval No. 2020022). The experiment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and complied with the ARRIVE guidelines.

Animals, diets, and experimental design

The feeding trial was conducted from March to May 2025 at the Suffolk Sheep Farm in the Ili Kazakh Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China. Thirty male third-generation Texel × Kazakh crossbred growing rams, aged 13 ± 0.5 months and with similar initial body weight, were selected for the study. Before the start of the trial, all rams were clinically examined and confirmed to be healthy, with no signs of digestive or metabolic disorders.

The rams were randomly allocated to two groups with 15 animals per group: a control group (CON), which received a basal total mixed ration (TMR), and a thymol group (THY), which received the same basal TMR supplemented with thymol. The THY group was supplemented with 30 mg/kg thymol on a dry matter (DM) basis, calculated as active thymol. The commercial thymol product contained 90% active thymol; therefore, it was included in the diet at 33.3 mg/kg DM to provide 30 mg/kg DM active thymol. The thymol product was purchased from Qingdao Mude Biotechnology Co., Ltd. (Qingdao, China) and was thoroughly mixed into the TMR during feed preparation to ensure uniform distribution.

The experiment lasted 72 days, including a 12-day adaptation period and a 60-day main experimental period. The basal TMR was formulated according to the nutrient requirements of sheep recommended by the NRC [14]. The ingredient composition and nutrient levels of the basal TMR are shown in Table 1. All rams were housed individually under the same management conditions, with free access to feed and water. Feed was offered twice daily at 08:00 and 18:30. The sheep barn was cleaned and disinfected regularly, and routine deworming and vaccination procedures were conducted according to farm management practices.

Table 1

Ingredient composition and nutrient levels of the basal TMR for Texel × Kazakh crossbred growing rams

Feed Component	Content (%)	Nutritional Level ¹	Content
Mountain Grass	36.00	Dry Matter DM (%)	92.30
Alfalfa	24.00	Organic Matter OM (%)	93.08
Corn	17.50	Digestible Energy DE(MJ/kg)	16.34
Soybean Meal	7.50	Crude Protein CP (%)	13.64
Wheat middlings	5.60	Crude Fat EE (%)	2.92
Wheat bran	5.00	Neutral Detergent Fiber NDF (%)	43.69
Cotton Seed Meal	2.00	Acid Detergent Fiber ADF (%)	30.66
Premix ²	2.00	Calcium Ca (%)	0.90
Sodium bicarbonate	0.40	Phosphorus P (%)	0.49
Total	100.00		
¹ Digestible energy is a calculated value; all other nutrient levels are laboratory-measured values. Dry matter content is measured on a naturally air-dried basis; other nutrients are on a dry matter basis.			
² The premix provides per kilogram of concentrate: Iron 127.50 mg, Zinc 293.25 mg, Manganese 190.80 mg, Copper 13.05 mg, Selenium 1.18 mg; Vitamin A 16,000 IU, Vitamin D ₃ 4,080 IU, Vitamin E 82 mg, Niacinamide 32 mg, Biotin 0.08 mg.			

Growth performance and feed intake

Body weight was measured on days 0, 30, and 60 of the main experimental period before morning feeding after overnight fasting. Each ram was weighed using a floor scale with an accuracy of 0.01 kg. Feed offered and refusals were recorded daily for each ram to calculate average daily feed intake (ADFI). Average daily gain (ADG), total weight gain, and feed-to-gain ratio (F/G) were calculated as follows:

$ADG = (\text{final body weight} - \text{initial body weight}) / \text{number of experimental days}$.

Total weight gain = final body weight – initial body weight.

$F/G = ADFI / ADG$.

Digestion and metabolism trial

A 5-day digestion and metabolism trial was conducted from days 46 to 50 of the main experimental period. During this period, each ram was housed in an individual professional metabolic cage equipped with a feces–urine separation device. Feed intake, feed refusals, fecal output, and urine output were

recorded daily for each animal. The collection system was designed to prevent fecal contamination of urine and to minimize sample loss.

Feed and refusal samples were collected daily before morning feeding using the quartering method. Total feces from each ram were collected, mixed thoroughly, and weighed daily. Approximately 10% of the daily fecal output was sampled and stored at -20°C for subsequent analysis. In addition, approximately 100 g of fresh feces was mixed with 10 mL of 10% sulfuric acid for nitrogen fixation and stored at -20°C for crude protein determination. Urine was collected in containers preloaded with 10% sulfuric acid at approximately 30 mL/L to maintain pH below 3. After daily volume recording, 10% of the urine was sampled and stored at -20°C for analysis of nitrogen, calcium, phosphorus, and energy. At the end of the collection period, fecal and urine samples from each ram were pooled in proportion to daily output to obtain representative samples.

The contents of DM, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), calcium (Ca), and phosphorus (P) in feed and fecal samples were determined according to the relevant national standard methods of the People's Republic of China. All analyses were performed in triplicate, and results were expressed on a DM basis. Apparent nutrient digestibility and nutrient retention were calculated using the total collection method [15, 16].

Rumen fluid collection and fermentation analysis

Rumen fluid was collected from all rams before morning feeding on the third day before the end of the main experimental period. Samples were collected using an oral stomach tube. To reduce the risk of saliva contamination, the first 100 mL of rumen fluid was discarded, and approximately 50 mL of rumen fluid was then collected from each animal. The rumen fluid was filtered immediately through four layers of sterile cheesecloth.

The pH of rumen fluid was measured immediately using a laboratory pH meter (PHS-25, Shanghai Leici Instrument Co., Ltd., Shanghai, China). Ammonia nitrogen ($\text{NH}_3\text{-N}$) concentration was determined using the phenol–sodium hypochlorite colorimetric method. Concentrations of volatile fatty acids (VFAs), including acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate, were measured using a high-performance liquid chromatography system (Waters Baseline 520, Waters Corporation, Milford, MA, USA) equipped with a C18 column and UV detection at 214 nm. Total VFA concentration was calculated as the sum of individual VFA components.

Additional aliquots of rumen fluid were placed in sterile nuclease-free cryovials, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent microbial DNA extraction and untargeted metabolomic analysis. Phenotypic data, including growth performance, nutrient digestibility, nutrient retention, blood biochemical parameters, serum hormones, and rumen fermentation parameters, were collected from all 30 experimental rams. For 16S rRNA gene sequencing and untargeted metabolomic analyses, rumen fluid samples from 10 randomly selected rams per group were used.

Blood collection and biochemical and hormone analyses

Blood samples were collected from the jugular vein of each ram on days 0, 30, and 60 of the main experimental period before morning feeding. For each sampling time, 10 mL of blood was collected from each ram, including 5 mL into a heparinized vacuum tube for plasma preparation and 5 mL into a non-anticoagulant vacuum tube for serum preparation. Blood samples were centrifuged at $1,500 \times g$ for 15 min at 4°C. Plasma and serum aliquots were then stored at -80°C until analysis.

Plasma biochemical parameters, including total protein (TP), albumin (ALB), urea (UREA), uric acid (UA), glucose (GLU), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), total bilirubin (T-Bil), direct bilirubin (D-Bil), aspartate aminotransferase (AST), γ -glutamyltransferase (γ -GT), lactate dehydrogenase (LDH), and creatine kinase (CK), were measured using a fully automated biochemical analyzer (BS-240VET, Shenzhen Mindray Medical Electronics Co., Ltd., Shenzhen, China) with corresponding commercial reagent kits according to the manufacturer's instructions.

Serum samples collected on day 60 of the main experimental period were used for hormone analysis. Serum concentrations of testosterone, dihydrotestosterone (DHT), estradiol (E2), estrone (E1), dehydroepiandrosterone (DHEA), androstenedione, aldosterone, cortisol, and corticosterone were determined using commercial competitive enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. Absorbance was measured at 450 nm using a microplate reader, and hormone concentrations were calculated from standard curves generated for each assay. All samples were analyzed in duplicate, and samples from the CON and THY groups were balanced across assay plates to minimize batch effects. The intra-assay and inter-assay coefficients of variation were maintained below 10%. Detailed information on the ELISA kits, including manufacturers, catalogue numbers, detection ranges, sensitivities, and assay precision, is provided in Supplementary Table S1.

Rumen microbial profiling

Total microbial genomic DNA was extracted from rumen fluid samples using the CTAB method. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA integrity was checked by agarose gel electrophoresis. The V3–V4 region of the bacterial 16S rRNA gene was amplified using the primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3').

Purified amplicons were sequenced using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with paired-end 250-bp sequencing. Raw sequences were processed using the QIIME2 pipeline, version 2022.8. After demultiplexing and quality filtering, paired-end reads were denoised, dereplicated, and chimera-filtered to generate amplicon sequence variants (ASVs). Taxonomic assignment of ASVs was performed against the SILVA 138 database.

Alpha diversity indices, including Chao1, Shannon, Simpson, and Pielou evenness, were calculated to evaluate microbial richness and evenness. Beta diversity was assessed using weighted and unweighted UniFrac distances and visualized by principal coordinate analysis (PCoA). Differences in microbial community structure between groups were tested using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. Linear discriminant analysis effect size (LEfSe) analysis was performed to identify differentially abundant taxa between groups, with an LDA score threshold of > 2.0.

Rumen fluid untargeted metabolomics

Untargeted metabolomic analysis of rumen fluid samples was performed by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Rumen fluid samples were extracted using methanol–water (4:1, v/v). The extracts were analyzed using an ultra-high-performance liquid chromatography–tandem mass spectrometry system coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in both positive and negative ionization modes.

Raw data were processed for peak detection, peak alignment, peak filtering, and normalization using Progenesis Q1 software, version 3.0 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Metabolites were annotated by matching accurate mass, MS/MS fragmentation patterns, and retention time against the Majorbio Database, the Human Metabolome Database (HMDB, version 5.0), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The annotation criteria included a mass deviation of < 10 ppm and an MS/MS spectral matching score > 60.

Quality control (QC) samples were prepared by pooling equal aliquots from all rumen fluid samples and were used to monitor instrument stability and analytical reproducibility throughout the analysis. QC samples were injected at regular intervals during the LC-MS/MS sequence. Metabolic features with poor reproducibility in QC samples were removed before downstream analysis. Principal component analysis (PCA) was used to evaluate overall data quality and sample distribution. Partial least squares discriminant analysis (PLS-DA) was performed to evaluate metabolic differences between the CON and THY groups, and the reliability of the PLS-DA model was assessed using permutation testing.

Differential metabolites were screened using variable importance in projection (VIP) > 1.0 and false discovery rate (FDR)-adjusted $P < 0.05$. P values were adjusted using the Benjamini–Hochberg method to control the FDR. KEGG pathway enrichment analysis was performed to identify metabolic pathways affected by thymol supplementation.

Statistical analysis

Data were organized using Microsoft Excel and analyzed using SPSS 27.0 (IBM Corp., Armonk, NY, USA). Data are presented as means $\pm$ standard error of the mean (SEM). The individual ram was considered the experimental unit. Normality and homogeneity of variance were assessed before statistical analysis. For single-time-point variables, differences between the CON and THY groups were analyzed using an

independent-samples t-test; when assumptions were not met, the Mann–Whitney U test was used. Blood biochemical parameters measured over time were analyzed using a repeated-measures mixed model, with treatment, time, and their interaction as fixed effects and individual ram as a random effect.

Microbiome and metabolomic data were analyzed as described in the corresponding sections. Spearman’s rank correlation analysis was performed to evaluate associations among differentially abundant rumen bacterial genera, representative differential metabolites, and host phenotypic indicators, including growth performance, nutrient digestibility, nutrient retention, rumen fermentation parameters, blood biochemical parameters, and serum reproductive hormone levels. A value of $P < 0.05$ was considered statistically significant, $P < 0.01$ was considered highly significant, and $0.05 \leq P < 0.10$ was considered a trend. Figures and tables were generated using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) and R software.

Results

Growth Performance

The effects of dietary thymol supplementation on the growth performance of Texel × Kazakh crossbred growing rams are presented in Table 2. Initial body weight, final body weight, and average daily feed intake (ADFI) did not differ significantly between the CON and THY groups ($P > 0.05$). Compared with the CON group, the THY group had higher total weight gain (14.84 vs. 12.28 kg, $P = 0.001$) and average daily gain (ADG; 247.31 vs. 204.67 g/d, $P = 0.001$). The feed-to-gain ratio (F/G) was lower in the THY group than in the CON group (8.27 vs. 9.92, $P = 0.002$).

Table 2
Effects of dietary thymol supplementation on growth performance of Texel × Kazakh crossbred growing rams

Item	CON	THY	SEM	P-value
Initial BW (kg)	49.79	48.37	1.23	0.256
Final BW (kg)	62.07	63.21	1.05	0.289
Total weight gain (kg)	12.28	14.84	0.71	0.001
ADG (g/d)	204.67	247.31	8.9	0.001
ADFI (kg/d)	1.99	2.01	0.03	0.483
F/G (kg feed/kg gain)	9.92	8.27	0.49	0.002
CON = control group (basal TMR); THY = thymol group (basal TMR + 30 mg/kg thymol on a DM basis). Data are presented as means, n = 15. Final BW was measured on day 60.				
BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F/G = feed-to-gain ratio.				

Nutrient apparent digestibility and metabolism

The effects of dietary thymol supplementation on nutrient apparent digestibility are shown in Table 3. Compared with the CON group, the THY group had higher apparent digestibility of crude protein (CP; 74.85% vs. 72.21%, $P = 0.025$), ether extract (EE; 81.48% vs. 77.88%, $P = 0.002$), neutral detergent fiber (NDF; 57.10% vs. 53.31%, $P = 0.025$), and acid detergent fiber (ADF; 51.51% vs. 47.48%, $P = 0.024$). Digestible energy was also greater in the THY group than in the CON group (10.99 vs. 10.36 MJ/kg DM, $P = 0.004$). No significant differences were observed in dry matter, organic matter, or calcium digestibility between groups ($P > 0.05$).

Table 3

Effects of dietary thymol supplementation on nutrient apparent digestibility of Texel × Kazakh crossbred growing rams

Item	CON	THY	SEM	P-value
DM (%)	66.00	68.06	1.32	0.131
OM (%)	69.11	70.13	1.38	0.468
CP (%)	72.21	74.85	1.12	0.025
EE (%)	77.88	81.48	1.05	0.002
Ca (%)	42.87	45.91	1.90	0.121
NDF (%)	53.31	57.10	1.60	0.025
ADF (%)	47.48	51.51	1.68	0.024
DE (MJ/kg DM)	10.36	10.99	0.20	0.004
CON = control group (basal TMR); THY = thymol group (basal TMR + 30 mg/kg thymol on a DM basis). Data are presented as means, n = 15 .				
DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; Ca = calcium; NDF = neutral detergent fiber; ADF = acid detergent fiber; DE = digestible energy.				

The effects of thymol supplementation on nutrient metabolism are presented in Table 4. Nitrogen retention was significantly higher in the THY group than in the CON group (17.35 vs. 14.76 g/d, $P = 0.009$), and the nitrogen retention rate was also increased (41.30% vs. 35.45%, $P = 0.005$). Phosphorus retention and phosphorus retention rate were higher in the THY group than in the CON group (3.82 vs. 3.33 g/d, $P = 0.028$; 39.77% vs. 35.36%, $P = 0.035$, respectively). Metabolizable energy intake, calcium retention, and calcium retention rate were not significantly affected by thymol supplementation ($P > 0.05$).

Table 4
Effects of dietary thymol supplementation on nutrient retention and energy metabolism of Texel × Kazakh crossbred growing rams

Items	CON	THY	SEM	P-value
ME (MJ/d)	16.80	17.60	0.80	0.324
N retention (g/d)	14.76	17.35	0.92	0.009
N retention rate (%)	35.45	41.30	1.93	0.005
Ca retention (g/d)	6.99	7.59	0.39	0.129
Ca retention rate (%)	41.72	44.93	1.94	0.109
P retention (g/d)	3.33	3.82	0.21	0.028
P retention rate (%)	35.36	39.77	1.99	0.035
CON = control group (basal TMR); THY = thymol group (basal TMR + 30 mg/kg thymol on a DM basis). Data are presented as means, n = 15 .				
ME = metabolizable energy; N = nitrogen; Ca = calcium; P = phosphorus.				

Blood Biochemical Parameters

The effects of dietary thymol supplementation on blood biochemical parameters are shown in Table 5. Significant treatment effects were observed for several indices related to protein, energy, lipid, bilirubin, and enzyme metabolism. Compared with the CON group, the THY group had lower concentrations of total protein (TP; 58.60 vs. 60.20 g/L, $P < 0.001$) and albumin (ALB; 22.54 vs. 24.35 g/L, $P = 0.008$). Urea concentration was not significantly different between groups ($P > 0.05$), whereas uric acid tended to be lower in the THY group ($P = 0.075$). For energy and lipid metabolism, glucose (GLU), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were lower in the THY group than in the CON group ($P < 0.01$). Significant treatment × time interactions were observed for GLU, TC, and HDL-C, indicating that the effects of thymol on these parameters varied over the experimental period. For bilirubin and enzyme-related parameters, total bilirubin (T-Bil), direct bilirubin (D-Bil), aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), and lactate dehydrogenase (LDH) were lower in the THY group than in the CON group ($P < 0.01$). Treatment × time interactions were observed for D-Bil and AST. Creatine kinase activity was not significantly affected by thymol supplementation ($P > 0.05$).

Table 5
Effects of dietary thymol supplementation on blood biochemical parameters of Texel × Kazakh crossbred growing rams

Item	CON	THY	SEM	P-value		
				TRT	Date	TRT × Date
TP (g/L)	60.20	58.60	2.30	< 0.001	< 0.001	0.402
ALB (g/L)	24.35	22.54	0.47	0.008	< 0.001	0.014
UREA (mmol/L)	3.35	4.96	0.79	0.153	0.658	0.186
UA (μmol/L)	3.79	3.39	0.16	0.075	< 0.001	0.507
GLU (mmol/L)	4.78	3.69	0.11	< 0.001	0.067	< 0.001
TC (mmol/L)	1.39	1.27	0.25	0.001	0.014	0.002
HDL-C (mmol/L)	0.84	0.77	0.14	< 0.001	0.073	0.001
T-Bil (μmol/L)	0.67	0.51	0.03	< 0.001	< 0.001	0.224
D-Bil (μmol/L)	0.20	0.12	0.02	0.001	< 0.001	< 0.001
AST (U/L)	120.10	107.15	2.93	0.002	< 0.001	0.001
γ-GT (U/L)	59.84	46.46	1.29	< 0.001	0.034	0.064
LDH (U/L)	472.55	409.40	9.26	< 0.001	< 0.001	0.064
CK (U/L)	256.34	216.97	18.67	0.140	< 0.001	0.080
CON = control group (basal TMR); THY = thymol group (basal TMR + 30 mg/kg thymol on a DM basis). Values are means, n = 15. TRT = treatment effect; Date = time effect;						
TRT × Date = treatment × time interaction effect.						
TP = total protein; ALB = albumin; UREA = urea; UA = uric acid; GLU = glucose; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; T-Bil = total bilirubin; D-Bil = direct bilirubin; AST = aspartate aminotransferase; γ-GT = γ-glutamyl transferase; LDH = lactate dehydrogenase; CK = creatine kinase; TRT = treatment effect; Date = time effect; TRT × Date = treatment × time interaction effect.						

Serum hormone levels

The effects of dietary thymol supplementation on serum hormone levels of Texel × Kazakh crossbred rams are presented in Table 6. Testosterone concentration was markedly higher in the THY group than in the CON group ($P < 0.01$), with an increase of approximately 25%. E1 concentration was also markedly higher in the THY group ($P < 0.01$), increasing by approximately 50%. E2 and CORT concentrations were significantly higher in the THY group than in the CON group ($P < 0.05$), with increases of approximately 13% and 23%, respectively. DHT tended to be higher in the THY group but did not reach statistical

significance ($P > 0.05$). DHEA, androstenedione, aldosterone, and cortisol levels did not differ significantly between the two groups ($P > 0.05$).

Table 6

Effects of dietary thymol supplementation on serum reproductive hormone concentrations of Texel × Kazakh crossbred growing rams

Item	CON	THY	SEM	P-value
Testosterone (ng/mL)	13.07	16.30	0.43	< 0.001
DHT (pg/mL)	376.99	463.20	24.27	0.075
E2 (pg/mL)	601.56	678.45	19.71	0.048
DHEA (ng/mL)	8.23	8.86	0.38	0.425
E1 (ng/mL)	1.87	2.80	0.14	< 0.001
Androstenedione (nmol/L)	3.63	4.24	0.34	0.389
Aldosterone (pg/mL)	582.22	610.12	37.66	0.722
Cortisol (ng/mL)	59.86	70.61	3.18	0.091
CORT (ng/mL)	5.08	6.23	0.28	0.038
CON = control group (basal TMR); THY = thymol group (basal TMR + 30 mg/kg thymol on a DM basis). Values are means, n = 15. Serum samples collected on day 60 were used for hormone analysis.				
Testosterone = testosterone; DHT = dihydrotestosterone; E2 = estradiol; DHEA = dehydroepiandrosterone; E1 = estrone; CORT = corticosterone.				

Rumen fermentation parameters

The effects of dietary thymol supplementation on rumen fermentation parameters of Texel × Kazakh crossbred rams are presented in Table 7. Rumen fluid pH was significantly higher in the THY group than in the CON group ($P < 0.05$), with both groups remaining within the normal physiological range. $\text{NH}_3\text{-N}$ concentration was markedly lower in the THY group than in the CON group ($P < 0.01$), decreasing by approximately 11%. TVFA concentration did not differ significantly between the two groups ($P > 0.05$), although the THY group showed numerically higher values. Among individual VFA components, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate concentrations did not differ significantly between groups ($P > 0.05$). However, propionate concentration tended to be higher in the THY group ($P = 0.05$), with an increase of approximately 27% compared with the CON group.

Table 7

Effects of dietary thymol supplementation on rumen fermentation parameters of Texel × Kazakh crossbred growing rams

Item	CON	THY	SEM	P-value
pH	6.357	6.430	0.026	0.011
TVFA (mmol/L)	48.401	60.231	4.040	0.148
Acetate (mmol/L)	33.326	41.979	6.078	0.172
Propionate (mmol/L)	8.671	11.019	1.115	0.05
Isobutyrate (mmol/L)	0.719	0.711	0.140	0.956
Butyrate (mmol/L)	4.885	5.751	0.570	0.146
Isovalerate (mmol/L)	0.501	0.456	0.085	0.607
Valerate (mmol/L)	0.301	0.312	0.042	0.757
NH ₃ -N (mg/L)	192.4	170.3	4.009	< 0.001
CON = control group (basal TMR); THY = thymol group (basal TMR + 30 mg/kg thymol on a DM basis). Values are means, n = 15 per group.				
TVFA = total volatile fatty acids; NH ₃ -N = ammonia nitrogen.				

Rumen bacterial diversity and community composition

To investigate the microbiological basis underlying the regulation of host phenotypes by thymol, the diversity, structure, and composition of the rumen bacterial community were analyzed using 16S rRNA gene sequencing. Compared with the CON group, thymol supplementation significantly enhanced the alpha diversity of the rumen microbial community. Specifically, the Chao1, Shannon, and Pielou_e indices were markedly higher in the THY group than in the CON group ($P < 0.001$), and the Simpson index was also significantly higher ($P < 0.01$) (Fig. 1A–D). Principal coordinates analysis (PCoA) based on weighted and unweighted UniFrac distances revealed a clear separation between the CON and THY groups in the ordination space (Fig. 1E–F), and PERMANOVA (999 permutations) further confirmed that the microbial community structure differed significantly between the two groups ($P < 0.001$). At the phylum level, Bacteroidota and Firmicutes were the dominant phyla in both groups, accounting for more than 80% of the total relative abundance (Fig. 2A). To identify the key microbial taxa responsible for the between-group differences, LEfSe analysis (LDA score > 2.0) was performed to screen for differentially abundant biomarkers. As shown in Fig. 2I, the LDA score histogram identified multiple genera with significant differences between groups. Among them, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Christensenellaceae_R-7_group*, and *Treponema* were enriched in the THY group, whereas *Prevotella* and *Selenomonas* were enriched in the CON group. The cladogram (Fig. 2J) further illustrated the phylogenetic distribution of these differentially abundant taxa, showing that multiple enriched lineages, from the class level (Clostridia) to the genus level, were present in the THY group. To quantitatively

validate the LEfSe results, the relative abundances of the key differentially abundant genera were further compared using box plots (Fig. 2C–H). These comparisons confirmed the LEfSe findings: the relative abundances of *Rikenellaceae_RC9_gut_group* and *NK4A214_group* were markedly higher in the THY group than in the CON group ($P < 0.01$); *Christensenellaceae_R-7_group* and *Treponema* were significantly higher in the THY group ($P < 0.05$); and *Selenomonas* was significantly higher in the CON group than in the THY group ($P < 0.05$). In addition, the relative abundance of *Prevotella* tended to be higher in the CON group than in the THY group ($P = 0.056$; Fig. 2H).

Rumen fluid untargeted metabolomics analysis

The rumen fluid samples from the CON and THY groups were analyzed using UHPLC-MS/MS-based untargeted metabolomics. The PLS-DA score plot revealed a clear separation between the two groups along the predictive component direction (Fig. 3A), and the permutation test (200 permutations) confirmed that the model was not overfitted (Q^2 intercept < 0 ; Fig. 3B). Using the criteria of $VIP > 1.0$ and FDR-adjusted $P < 0.05$, a total of 569 differential metabolites were identified, of which 264 were up-regulated and 305 were down-regulated in the THY group (Fig. 3C). KEGG pathway enrichment analysis showed that the differential metabolites were significantly enriched in the cAMP signaling pathway, purine metabolism, nucleotide metabolism, cGMP-PKG signaling pathway, glutathione metabolism, regulation of lipolysis in adipocytes, phenylalanine metabolism, tyrosine metabolism, tryptophan metabolism, and parathyroid hormone synthesis, secretion and action (Fig. 3D; Table S9). Based on VIP values and pathway representativeness, eight metabolites were selected for subsequent correlation analysis: adenosine monophosphate (AMP), 3-hydroxybutyric acid, inosine, L-pyroglutamic acid, trans-cinnamic acid, 3-indoleacetic acid, p-hydroxyphenylacetic acid, and adenosine.

Rumen microbiota–metabolite–host phenotype correlation analysis

To elucidate how the remodeled rumen microbiota and differential metabolites coordinately regulate host phenotypes, Spearman correlation analysis was performed among the differentially abundant genera at the genus level, representative differential metabolites selected from the significantly enriched KEGG pathways, and key host phenotypic indicators (Fig. 4). At the microbiota–phenotype level, several bacterial genera enriched in the THY group were positively associated with growth performance, nutrient utilization, rumen fermentation, and serum hormone levels. *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Christensenellaceae_R-7_group*, *Treponema*, *Fibrobacter*, *Saccharofermentans*, and *UCG-002* showed positive correlations with serum testosterone. *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Fibrobacter*, and *Saccharofermentans* were negatively correlated with ruminal $\text{NH}_3\text{-N}$ concentration. *Treponema* and *Saccharofermentans* were positively correlated with NDF digestibility. *Selenomonas* showed negative correlations with ADG, TVFA, and testosterone.

At the metabolite–phenotype level, 3-hydroxybutyric acid was positively correlated with ADG, NDF digestibility, TVFA, and propionate, and negatively correlated with F/G. AMP was positively correlated

with TVFA, propionate, and ruminal pH. AMP and inosine were positively correlated with testosterone, whereas inosine, p-hydroxyphenylacetic acid, and trans-cinnamic acid were negatively correlated with $\text{NH}_3\text{-N}$. Adenosine was positively correlated with testosterone and E2 and negatively correlated with $\text{NH}_3\text{-N}$ and TC. Trans-cinnamic acid was positively correlated with NDF digestibility, ruminal pH, and E2. These results indicate that thymol-associated changes in rumen bacterial taxa and metabolites were correlated with host growth performance, rumen fermentation, nutrient utilization, and reproductive hormone levels.

Discussion

Regulation of growth performance and nutrient utilization by thymol in growing rams

In the present study, dietary thymol supplementation significantly increased total weight gain and ADG and decreased F/G in Texel $\times$ Kazakh crossbred growing rams, whereas ADFI remained unchanged, indicating that the growth-promoting effect of thymol was attributable to improved feed conversion efficiency rather than increased feed intake. This finding is consistent with the observation of Ahmadibonakdar et al. [17] that thymol reduced residual feed intake in feed-restricted lambs. However, Biricik et al. [18] did not observe significant improvement in feed conversion rate when thymol was supplemented to Merino sheep under a high-concentrate diet. This discrepancy may be closely related to diet type and supplementation dose. The present study used a forage-based TMR with an NDF content of 43.69%, under which conditions the promoting effect of thymol on fiber digestion may be more pronounced. Furthermore, the supplementation dose used in this study was considerably lower than that in the aforementioned study and was closer to the optimal fermentation-promoting concentration determined by Yu et al. [7] in an in vitro study with goats, whereas high doses exerted inhibitory effects, further indicating that the effects of thymol are strictly dose-dependent.

The systematic improvement in nutrient apparent digestibility provides a key explanation for the enhanced growth performance. The digestibility of CP, EE, NDF, and ADF was significantly increased in the THY group, accompanied by a corresponding increase in DE. However, the magnitude of improvement in digestibility alone was insufficient to fully account for the enhancement in growth performance, suggesting that metabolic conversion efficiency beyond digestion was also improved. The increases in NDF and ADF digestibility are of particular significance for energy supply in forage-based diets, because fiber serves as the primary energy carrier in such diets. Greater degradation of structural carbohydrates into VFA provides an additional energy source for the animal. Zamani et al. [5] demonstrated that thymol reduced ruminal $\text{NH}_3\text{-N}$ concentration and increased plasma glucose levels in sheep, indicating simultaneous improvements in nitrogen and energy utilization. Liu et al. [6] reported that thymol significantly increased ruminal microbial protein and total VFA concentrations in sheep, and the present study further confirmed these effects in growing rams.

The improvement in nitrogen metabolism represents another key aspect of the enhanced nutrient utilization efficiency. Nitrogen retention and the nitrogen retention rate were significantly increased in the

THY group, whereas ruminal $\text{NH}_3\text{-N}$ concentration was significantly decreased, which is consistent with reduced ruminal deamination and/or improved microbial nitrogen capture. In vitro studies have confirmed that thymol significantly reduces ammonia production by ruminal hyper-ammonia-producing bacteria [19, 20]. Taken together with the significant increase in CP digestibility, these findings suggest that thymol may have reduced excessive ruminal deamination and ammonia nitrogen losses, thereby improving whole-body nitrogen utilization efficiency. This may have allowed a greater proportion of dietary nitrogen to be retained for tissue deposition; however, microbial protein synthesis, rumen-undegradable protein flow, and intestinal amino acid absorption were not directly measured in this study. The significant increase in nitrogen retention provided a material basis for skeletal muscle growth, and the increases in phosphorus retention rate and amount provided greater mineral security for bone mineralization.

Effects of thymol on blood biochemical parameters and reproductive hormones

In the present study, repeated-measures ANOVA revealed widespread effects of thymol treatment, sampling time, and their interaction on serum biochemical parameters in growing rams, indicating that the systemic metabolic regulation exerted by thymol exhibited pronounced temporal dynamics.

Regarding protein metabolism, TP and ALB concentrations were significantly decreased in the THY group. Considered together with the reduced ruminal $\text{NH}_3\text{-N}$ concentration and increased nitrogen retention rate, this change is more likely to reflect a metabolic redistribution of amino acids toward tissue deposition rather than impaired hepatic protein synthesis. Correspondingly, the coordinated decreases in AST, $\gamma\text{-GT}$, and LDH activities, as well as bilirubin levels, were consistent with a hepatoprotective effect [21].

With respect to energy and lipid metabolism, serum GLU concentration was markedly decreased, which may be related to the ameliorating effect of thymol on insulin resistance [22]. The changes in lipid metabolism exhibited more complex characteristics: serum TC and HDL-C concentrations were significantly decreased, whereas testosterone, E2, and E1 levels were significantly increased. Cholesterol is the common precursor of all steroid hormones, and the decrease in its plasma concentration may reflect a functional shift in lipid metabolic flow from plasma lipoproteins toward Leydig cells. Leydig cells take up cholesterol esters from plasma lipoproteins via LDL receptor-mediated endocytosis to provide substrates for steroidogenesis [23, 24]. Saber et al.[25] demonstrated in a rat model that thymol up-regulated the gene expression of key steroidogenic enzymes, including StAR, CYP11A1, $3\beta\text{-HSD}$, and $17\beta\text{-HSD}$, providing molecular corroboration for the metabolic changes observed in the present study. Nevertheless, the functional mobilization of cholesterol toward the testosterone synthesis pathway currently remains a reasonable inference based on metabolic phenotypes, and its certainty requires further verification using methods such as isotope tracing.

Serum testosterone, E2, and E1 levels were significantly increased in the THY group, and DHT also showed an increasing trend. The growing period is a critical window for HPG axis maturation and the programming of reproductive performance in rams [9]. The concurrent improvement in metabolic status and reproductive endocrine function induced by thymol during this stage has important practical implications for optimizing the production potential of growing rams. Thymol can up-regulate the gene expression of key steroidogenic enzymes in Leydig cells [25], and its structural analogue thymoquinone has been shown to restore HPG axis function and significantly up-regulate GnRH, FSH, and LH levels [26], suggesting that thymol-related compounds may act at both the central and peripheral levels of the HPG axis. However, GnRH, LH, and FSH were not directly measured in this study, and the regulatory level responsible for the increase in testosterone could not be distinguished.

Notably, serum corticosterone was also significantly increased. Although elevated corticosterone is generally regarded as an indicator of stress, the concurrent decreases in liver enzymes and bilirubin levels ruled out the possibility of a systemic stress state, and the significant improvement in growth performance also functionally negated the presence of adverse stress effects. Given that the effect of glucocorticoids on testosterone synthesis is concentration-dependent [27], the elevated corticosterone observed in this study is more likely to reflect physiological regulation rather than a stress response.

In summary, thymol was associated with improved metabolic status and elevated reproductive hormone levels in growing rams; however, direct testicular mechanisms require further verification.

Effects of thymol on rumen microbial community structure and its functional significance

In the present study, thymol supplementation significantly altered the diversity and overall structure of the rumen microbial community in growing rams. The increases in alpha diversity indices indicated that thymol increased species richness and evenness of the rumen microbial community under forage-based TMR conditions. Yu et al. [7] found in an in vitro study with goats that thymol significantly affected multiple genera of bacteria, archaea, and protozoa, and the present study further confirmed this finding under in vivo conditions in growing rams. Beta diversity analysis confirmed a significant separation in community structure between the two groups, indicating that thymol induced directional community remodeling. At the phylum level, Bacteroidota and Firmicutes remained the dominant phyla, and the overall dominant phylum pattern did not undergo dramatic changes, consistent with the report of Zhao et al. [28] that the dominant phyla remained stable after supplementation with *Allium mongolicum* Regel essential oil in sheep. However, LEfSe analysis revealed significant differential biomarkers at the genus level. The enriched taxa in the THY group—*Clostridia*, *Oscillospirales*, *Lachnospirales*, and *Rikenellaceae*—are all functional groups closely associated with fiber degradation and carbohydrate metabolism. The multi-level enrichment from class to genus indicates that the regulation of the rumen microbial community by thymol had systematic characteristics [29, 30].

At the genus level, the relative abundances of *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Christensenellaceae_R-7_group*, and *Treponema* were significantly increased in the THY group, whereas

those of *Prevotella* and *Selenomonas* showed a decreasing trend or were significantly decreased. *Rikenellaceae_RC9_gut_group* is a core fiber-degrading bacterium in the rumen, and its increased abundance was consistent with the improvements in NDF and ADF digestibility. *Treponema* synergistically participates in the degradation of structural carbohydrates [31]. *NK4A214_group* is a common colonizer of the rumen epithelium and may play an important role in fiber degradation and the maintenance of rumen epithelial health [32]. *Christensenellaceae_R-7_group* is closely associated with host metabolic health and short-chain fatty acid production [33–36]. The systematic enrichment of these fiber-degrading bacteria provides a microbiological explanation for the improved fiber digestibility observed in the THY group.

Prevotella is primarily responsible for peptide degradation and ammonia production in the rumen [37]. Its abundance showed a decreasing trend, consistent with the reduction in $\text{NH}_3\text{-N}$ concentration in the THY group. The in vitro study of Pal et al. [19] provides an in vitro reference for the association between the decreased abundance of *Prevotella* and the reduced $\text{NH}_3\text{-N}$ concentration. *Selenomonas ruminantium* participates in the conversion of lactate to propionate via the succinate pathway [38]. In addition, several genera that did not meet the stringent LEfSe significance threshold but showed enrichment trends in the THY group deserve attention. *Fibrobacter succinogenes*, *Ruminococcus*, and *Butyrivibrio* are major cellulolytic bacteria and butyrate producers in the rumen, respectively, and their coordinated enrichment may have further enhanced ruminal fiber degradation and SCFA production capacity. These genera exhibited multiple significant associations with host phenotypes in Spearman correlation analysis, suggesting that they may have played a synergistic role in the improvement of rumen fermentation induced by thymol.

In summary, thymol reshaped the rumen bacterial community in growing rams, characterized by the enrichment of several fibrolytic or carbohydrate-utilizing taxa and the reduction of taxa associated with protein degradation and lactate utilization. These microbial shifts were consistent with the improved fiber digestibility and reduced ruminal $\text{NH}_3\text{-N}$ concentration observed in the THY group.

Multi-level regulation of host phenotypes by remodeled rumen microbiota and altered metabolites

Spearman correlation analysis revealed significant statistical associations among the differentially abundant genera, differential metabolites, and host phenotypes. At the microbiota–phenotype level, fiber-degrading bacteria enriched in the THY group showed widespread positive correlations with host growth performance and nutrient digestibility. Among them, *Ruminococcus* and *Butyrivibrio* were significantly positively correlated with ADG and TVFA and significantly negatively correlated with F/G. *Ruminococcus* is a major fiber-degrading bacterium in the rumen, and *Butyrivibrio* is a major butyrate producer. The positive correlations of these two genera with growth performance suggest that the metabolic pathway of fiber degradation to SCFA production and subsequent energy supply may have contributed to the growth-promoting effect of thymol. *Treponema* and *Saccharofermentans* were significantly positively correlated with NDF digestibility.

Notably, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Christensenellaceae_R-7_group*, *Treponema*, *Fibrobacter*, *Saccharofermentans*, and *UCG-002* were all significantly positively correlated with serum testosterone levels, whereas *Selenomonas* enriched in the CON group was significantly negatively correlated with ADG, TVFA, and testosterone. This widespread and consistent association pattern between fiber-degrading bacteria and reproductive hormones echoes the gut–testis axis concept proposed by Wang et al.[12]. Regarding ammonia nitrogen metabolism, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Fibrobacter*, and *Saccharofermentans* were significantly negatively correlated with $\text{NH}_3\text{-N}$ concentration, whereas *Prevotella*, *Selenomonas*, and *Succiniclasicum* were significantly positively correlated with $\text{NH}_3\text{-N}$. The strong positive correlation of *Prevotella* with $\text{NH}_3\text{-N}$ is consistent with its functional role as a major protein-degrading and ammonia-producing bacterium in the rumen [37], and aligns with the inference that thymol improves dietary protein utilization efficiency by inhibiting protein-degrading bacteria and reducing ruminal ammonia nitrogen losses [5]. *Selenomonas* and *Succiniclasicum* are both key propionate-producing genera in the rumen[38, 39], and their significant positive correlations with $\text{NH}_3\text{-N}$ may be related to their similar ecological niches or metabolic synergy with *Prevotella*.

Untargeted metabolomic analysis detected widespread differences in metabolites between the two groups. KEGG pathway enrichment analysis showed that these differential metabolites were primarily mapped to the cAMP signaling pathway, purine metabolism, glutathione metabolism, phenylalanine metabolism, tyrosine metabolism, and tryptophan metabolism. At the energy metabolism level, the significant positive correlation of 3-hydroxybutyric acid with ADG and TVFA was directionally consistent with the metabolic pathway in which the enrichment of fiber-degrading bacteria promoted carbohydrate degradation and increased SCFA production. β -Hydroxybutyrate is not only an energy metabolite but also functions as a signaling molecule that regulates gene expression by inhibiting histone deacetylases [40]. AMP and inosine, as shared nodes of the cAMP signaling pathway and purine metabolism, were both significantly positively correlated with ADG, propionate, and testosterone levels, and significantly negatively correlated with $\text{NH}_3\text{-N}$ concentration. AMP is a core activator of the AMPK signaling pathway [41], and adenosine is a natural ligand of adenosine receptors [42]. The significant positive correlations of these two metabolites with testosterone and E2, as well as their significant negative correlations with $\text{NH}_3\text{-N}$ and TC, suggest that a statistical association may exist between the adenosine–cAMP signaling pathway and reproductive endocrine regulation, although whether this association reflects a functional regulatory role remains to be verified.

At the amino acid and antioxidant metabolism level, p-hydroxyphenylacetic acid and trans-cinnamic acid, which are involved in aromatic amino acid metabolism pathways, were significantly negatively correlated with $\text{NH}_3\text{-N}$ concentration, directionally consistent with the inference that ruminal protein degradation was reduced and more amino acids were retained for body protein deposition [43]. 3-Indoleacetic acid is a product of rumen microbial tryptophan metabolism, and its level changes may indirectly reflect the metabolic fate of tryptophan. L-Pyroglutamic acid belongs to the glutathione metabolism pathway, and its level changes indirectly reflect the metabolic state of the glutathione–antioxidant system [44], which is directionally consistent with the report that thymol can exert antioxidant effects by scavenging H_2O_2

and restoring GSH levels [27]. The enhancement of antioxidant capacity may have indirectly protected the steroidogenic function of Leydig cells from oxidative damage, providing another potential mechanistic explanation for the elevated testosterone levels observed in this study. However, antioxidant indices and testicular tissue markers were not measured in the present study; therefore, this explanation requires further verification.

Taken together, the multi-level changes observed in this study can be summarized as the following pathway: thymol remodeled the rumen microbial community by enriching fiber-degrading bacteria, such as *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Treponema*, and *Fibrobacter*, and inhibiting protein-degrading bacteria, such as *Prevotella*, and lactate-utilizing bacteria, such as *Selenomonas*. Accompanying this was a marked alteration in the rumen metabolite profile, with differential metabolites significantly enriched in the cAMP signaling pathway, purine metabolism, and glutathione metabolism, and elevated concentrations of key metabolites such as 3-hydroxybutyric acid, AMP, inosine, and adenosine in the THY group. These metabolite changes were significantly correlated with improvements in host energy metabolism and nitrogen utilization efficiency, as well as elevated reproductive hormone levels, including testosterone and E2. Given the consistent positive correlations of purine metabolism- and cAMP signaling pathway-related metabolites, including AMP, inosine, and adenosine, with testosterone levels, these pathways may be associated with the elevated reproductive hormone levels observed in the THY group. However, because Spearman correlation analysis cannot establish causal relationships, whether these rumen metabolites participate directly in host reproductive endocrine regulation requires further verification.

It should be emphasized that Spearman correlation analysis reflects co-variation trends among variables, and based on the data available in this study, the causal direction among microbiota remodeling, metabolite alterations, and reproductive hormone elevation cannot be determined. Furthermore, GnRH, LH, and FSH were not directly measured, and the regulatory level primarily responsible for the increase in testosterone—direct regulation at the testicular level or indirect activation at the hypothalamic–pituitary level—could not be distinguished. In addition, untargeted metabolomics is a discovery-based analysis, and the exact identities and functions of the differential metabolites still require verification by targeted metabolomics and isotope tracing. Future studies employing multiple dose gradients combined with causal verification experiments are needed to further examine the proposed regulatory pathway and to determine the optimal supplementation dose of thymol for growing rams.

Conclusion

Dietary supplementation with 30 mg/kg thymol improved growth performance, feed conversion efficiency, nutrient digestibility, and nitrogen and phosphorus retention in growing Texel × Kazakh crossbred rams. Thymol supplementation also altered circulating metabolic indicators and increased serum reproductive hormone levels, including testosterone, estradiol, and estrone. At the rumen level, thymol increased bacterial richness and evenness, enriched several fibrolytic or carbohydrate-utilizing taxa, improved ruminal nitrogen utilization as reflected by reduced $\text{NH}_3\text{-N}$ concentration, and altered

rumen metabolites mainly involved in purine metabolism, cAMP signaling, and glutathione metabolism. Representative metabolites, including adenosine monophosphate, 3-hydroxybutyric acid, and inosine, were positively correlated with ADG and testosterone levels. Overall, these findings suggest that 30 mg/kg thymol is a promising nutritional strategy for improving both growth performance and reproductive endocrine status in growing rams, with its effects closely linked to coordinated remodeling of the rumen microbiota and metabolome.

Abbreviations

ADF: Acid detergent fiber; ADFI: Average daily feed intake; ADG: Average daily gain; ASV: Amplicon sequence variant; CON: Control group; CP: Crude protein; DM: Dry matter; F/G: Feed-to-gain ratio; FDR: False discovery rate; KEGG: Kyoto Encyclopedia of Genes and Genomes; LDA: Linear discriminant analysis; LEfSe: Linear discriminant analysis effect size; NDF: Neutral detergent fiber; $\text{NH}_3\text{-N}$: Ammonia nitrogen; PCA: Principal component analysis; PCoA: Principal coordinates analysis; PERMANOVA: Permutational multivariate analysis of variance; PLS-DA: Partial least squares discriminant analysis; THY: Thymol group; TMR: Total mixed ration; TVFA: Total volatile fatty acids; VIP: Variable importance in projection.

Declarations

Ethics approval and consent to participate

All protocols were approved by the Animal Care and Use Committee of Xinjiang Agricultural University (permission number 2020022). Informed consent - Owners gave informed consent for their animals' inclusion in the study. All methods were carried out in accordance with relevant guidelines and regulations for the use of animal subjects. The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The raw 16S rRNA gene amplicon sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1502513. The data supporting the other findings of this study are included in this article and its supplementary information files. Additional data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Zhaosu County 2024 Grassland Animal Husbandry Transformation and Upgrading Project (Science and Technology Integration and Extension)—“Open Bidding for Selecting the Best Candidates” Industry–University–Research Science and Technology Project (Project No. 20241000L001); the Xinjiang Uygur Autonomous Region Key R&D Program—Prefecture–Department Linkage Project; and the Xinjiang Agricultural University Undergraduate Innovation Project (2026).

Authors' contributions

QZ and SZ contributed equally to this work and share first authorship. QZ and SZ contributed to the study design, animal feeding trial, experimental management, sample collection, laboratory analyses, data analysis, result interpretation, and manuscript drafting.

HL and HD contributed to project coordination, provided the experimental site, and offered guidance on animal feeding management during the trial.

PL and XF assisted with laboratory analyses, data organization, and figure preparation.

XL conceived and designed the study, supervised the project, acquired funding, and critically revised the manuscript for important intellectual content.

All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank the Zhaosu County Bureau of Agriculture and Rural Affairs (Xinjiang, China) for its assistance and support, and the Scientific Research Platform of Xinjiang Agricultural University for technical support.

References

1. Fachini-Queiroz FC, Kummer R, Estevão-Silva CF, Carvalho MD, Cunha JM, Grespan R et al. 2012. Effects of thymol and carvacrol, constituents of *Thymus vulgaris* L. essential oil, on the inflammatory response. *Evid Based Complement Alternat Med*. 2012: 657026. 10.1155/2012/657026
2. Liolios CC, Gortzi O, Lalas S, Tsaknis J, Chinou I. Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. *Food Chem*. 2009;112(1):77–83. 10.1016/j.foodchem.2008.05.060.
3. Shabnum S, Wagay MG. Essential oil composition of *Thymus vulgaris* L. and their uses. *J Res Dev*. 2011;11:83–94.
4. Pérez Gaudio D, Giantin M, Pauletto M, Dacasto M. Pharmacotoxicological aspects of thymol in veterinary medicine. A systematic review. *Front Vet Sci*. 2025;12:1562641.

- 10.3389/fvets.2025.1562641.
5. Zamani Z, Alipour D, Moghimi HR, Mortazavi SA, Zolhavarieh SM. Effect of free thymol and sustained release thymol on rumen fermentation and plasma metabolites in sheep. *Anim Prod Res.* 2015;3(4):75–88.
 6. Liu LS, Zhou R, Wu JP, Lang X, Wang CL. Effects of carvacrol and thymol on nutrient apparent digestibility, rumen fermentation characteristics, and number of fibrolytic bacteria in sheep (in Chinese). *Chin J Anim Nutr.* 2022;34(1):478–87. 10.3969/j.issn.1006-267x.2022.01.045.
 7. Yu J, Cai L, Zhang J, Yang A, Wang Y, Zhang L et al. 2020. Effects of thymol supplementation on goat rumen fermentation and rumen microbiota in vitro. *Microorganisms.* 8(8): 1160. 10.3390/microorganisms8081160
 8. Ahmadibonakdar Y, Vakili A, Javadmanesh A, Seradj AR, Rajaei-Sharifabadi H. Thymol modulates rumen barrier function and inflammation in feed-restricted lambs. *Anim Nutr.* 2026;25:19–29. 10.1016/j.aninu.2025.09.013.
 9. Yarney TA, Sanford LM. Pubertal development of ram lambs: Physical and endocrinological traits in combination as indices of postpubertal reproductive function. *Theriogenology.* 1993;40(4):735–44. 10.1016/0093-691X(93)90209-N.
 10. Lee VW, Cumming IA, de Kretser DM, Findlay JK, Hudson B, Keogh EJ. Regulation of gonadotrophin secretion in rams from birth to sexual maturity. I. Plasma LH, FSH and testosterone levels. *J Reprod Fertil.* 1976;46(1):1–6. 10.1530/jrf.0.0460001.
 11. Zhou X, Cao J, Feng G, Li Y, Liu D, Liu G. Commensal microbiota and reproductive health in livestock: mechanisms, cross-system crosstalk, and precision strategies. *Animals.* 2026;16(3):371. 10.3390/ani16030371.
 12. Wang M, Ren C, Wang P, Cheng X, Chen Y, Huang Y, et al. Microbiome–metabolome reveals the contribution of the gut–testis axis to sperm motility in sheep (*Ovis aries*). *Animals.* 2023;13(6):996. 10.3390/ani13060996.
 13. Sha Y, Liu X, Li X, Wang Z, Shao P, Jiao T, et al. Succession of rumen microbiota and metabolites across different reproductive periods in different sheep breeds and their impact on the growth and development of offspring lambs. *Microbiome.* 2024;12(1):172. 10.1186/s40168-024-01892-z.
 14. National Research Council. *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids.* Washington, DC: National Academies; 2007. 10.17226/11654.
 15. Hoffmann CA, Sarturi JO, Weindorf DC, Henry DD, Ramirez-Ramirez HA, Jackson S, et al. The use of portable X-ray fluorescence spectrometry to measure apparent total tract digestibility in beef cattle and sheep. *J Anim Sci.* 2020;98(3):skaa048. 10.1093/jas/skaa048.
 16. Ikusika OO, Mpendulo CT, Zindove TJ, Okoh AI. Effect of varying inclusion levels of fossil shell flour on growth performance, water intake, digestibility and N retention in Dohne-Merino wethers. *Animals.* 2019;9(8):565. 10.3390/ani9080565.
 17. Ahmadibonakdar Y, Vakili A, Javadmanesh A, Rajaei-Sharifabadi H. Effect of thymol on the efficiency of feed utilization and compensatory growth in severe feed-restricted lambs. *Trop Anim Health Prod.*

2024;56(7):248. 10.1007/s11250-024-04046-3.

18. Biricik H, Oral HH, Taluğ AM, Cengiz ŞŞ, Koyuncu M, Dikmen S. The effects of carvacrol and/or thymol on the performance, blood and rumen parameters, and carcass traits of Merino sheep. *Turk J Vet Anim Sci.* 2016;40:651–9. 10.3906/vet-1601-53.
19. Chanu YM, Paul SS, Dey A, Andonissamy J. Bioactive plant compounds reduce ammonia production in enrichment culture of ruminal hyper-ammonia producing bacteria. *Sci Rep.* 2026;16(1):8210. 10.1038/s41598-026-38231-1.
20. Lakes JE, Ramos LI, Cardenas ML, Mast NL, Flythe MD. Effects of phyto-phenolic compounds on ammonia production by select amino acid fermenting bacteria. *FEMS Microbiol Lett.* 2025;372:fna018. 10.1093/femsle/fna018.
21. Peirovy Y, Asle-Rousta M. Thymol and p-Cymene protect the liver by mitigating oxidative stress, suppressing TNF- α /NF- κ B, and enhancing Nrf2/HO-1 expression in immobilized rats. *Chem Biol Drug Des.* 2024;104(3):e14618. 10.1111/cbdd.14618.
22. Saravanan S, Pari L. Role of thymol on hyperglycemia and hyperlipidemia in high fat diet-induced type 2 diabetic C57BL/6J mice. *Eur J Pharmacol.* 2015;761:279–87. 10.1016/j.ejphar.2015.05.034.
23. Hu J, Zhang Z, Shen WJ, Azhar S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab (Lond).* 2010;7:47. 10.1186/1743-7075-7-47.
24. Zhou YX, Wei J, Deng G, Hu A, Sun PY, Zhao X, et al. Delivery of low-density lipoprotein from endocytic carriers to mitochondria supports steroidogenesis. *Nat Cell Biol.* 2023;25(7):937–49. 10.1038/s41556-023-01160-6.
25. Saber TM, Arisha AH, Abo-Elmaaty AMA, Abdelgawad FE, Metwally MMM, Saber T, Mansour MF. Thymol alleviates imidacloprid-induced testicular toxicity by modulating oxidative stress and expression of steroidogenesis and apoptosis-related genes in adult male rats. *Ecotoxicol Environ Saf.* 2021;221:112435. 10.1016/j.ecoenv.2021.112435.
26. Hassan E, El-Neweshy M, Hassan M, Noreldin A. Thymoquinone attenuates testicular and spermatotoxicity following subchronic lead exposure in male rats: possible mechanisms are involved. *Life Sci.* 2019;230:132–40. 10.1016/j.lfs.2019.05.067.
27. Hampl R, Stárka L. Glucocorticoids affect male testicular steroidogenesis. *Physiol Res.* 2020;69(Suppl 2):S205–10. 10.33549/physiolres.934508.
28. Zhao YX, Khas E, Bao ZB, Ao CJ, Bai C. Effects of *Allium mongolicum* Regel essential oil supplementation on growth performance, nutrient digestibility, rumen fermentation, and bacterial communities in sheep. *Front Vet Sci.* 2022;9:926721. 10.3389/fvets.2022.926721.
29. Du X, Cheng X, Ji K, Degen AA, Liang Y, Wu X, et al. Effects of supplementary thyme on immunity responses, antioxidant indices, rumen enzymes concentrations and rumen bacteria composition in Hu sheep. *Anim Feed Sci Technol.* 2023;306:115828. 10.1016/j.anifeedsci.2023.115828.
30. Peng W, Wang Y, Wei M, Liu S, Liu K, Xiao M, et al. Effects of changes in rumen microbial adaptability on rumen fermentation and nutrient digestion in Horqin beef cattle during different seasons of grazing and supplementary feeding. *BMC Microbiol.* 2025;25(1):556. 10.1186/s12866-025-04199-7.

31. Xie X, Yang C, Guan LL, Wang J, Xue M, Liu JX. Persistence of cellulolytic bacteria *Fibrobacter* and *Treponema* after short-term corn stover-based dietary intervention reveals the potential to improve rumen fibrolytic function. *Front Microbiol.* 2018;9:1363. 10.3389/fmicb.2018.01363.
32. Wang W, Wang Y, Cui Z, Yang Y, An X, Qi J. Fermented wheat bran polysaccharides intervention alters rumen bacterial community and promotes rumen development and growth performance in lambs. *Front Vet Sci.* 2022;9:841406. 10.3389/fvets.2022.841406.
33. Bian G, Yu S, Cheng C, Huang H, Liu J. Ruminal microbiota-host crosstalks promote ruminal epithelial development in neonatal lambs with alfalfa hay introduction. *mSystems.* 2024;9(2):e0103423. 10.1128/msystems.01034-23.
34. Waters JL, Ley RE. The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* 2019;17:83. 10.1186/s12915-019-0699-4.
35. Zhao J, Zhao X, Gao J, Bai B, Niu J, Yang Y, et al. Ensiled diet improved the growth performance of Tibetan sheep by regulating the rumen microbial community and rumen epithelial morphology. *J Anim Sci.* 2024;102:skae173. 10.1093/jas/skae173.
36. Cai Y, Zou J, Zhou Y, Yang J, Wang C, Mao H. Nitrogen utilization and ruminal microbiota of Hu lambs in response to varying dietary metabolizable protein levels. *Animals.* 2025;15(14):2147. 10.3390/ani15142147.
37. Wallace RJ. Ruminal microbial metabolism of peptides and amino acids. *J Nutr.* 1996;126(4 Suppl):S1326–34. 10.1093/jn/126.suppl_4.1326S.
38. Paynter MJB, Elsdon SR. Mechanism of propionate formation by *Selenomonas ruminantium*, a rumen micro-organism. *J Gen Microbiol.* 1970;61(1):1–7. 10.1099/00221287-61-1-1.
39. van Gylswyk NO. *Succiniclasticum ruminis* gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int J Syst Bacteriol.* 1995;45(2):297–300. 10.1099/00207713-45-2-297.
40. Newman JC, Verdin E. β -Hydroxybutyrate: a signaling metabolite. *Annu Rev Nutr.* 2017;37:51–76. 10.1146/annurev-nutr-071816-064916.
41. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol.* 2012;13(4):251–62. 10.1038/nrm3311.
42. Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov.* 2006;5(3):247–64. 10.1038/nrd1983.
43. Zheng X, Xie G, Zhao A, Zhao L, Yao C, Chiu NH, et al. The footprints of gut microbial–mammalian co-metabolism. *J Proteome Res.* 2011;10(12):5512–22. 10.1021/pr2007945.
44. Meister A. Glutathione metabolism and its selective modification. *J Biol Chem.* 1988;263(33):17205–8. 10.1016/S0021-9258(19)77815-6.

Figures

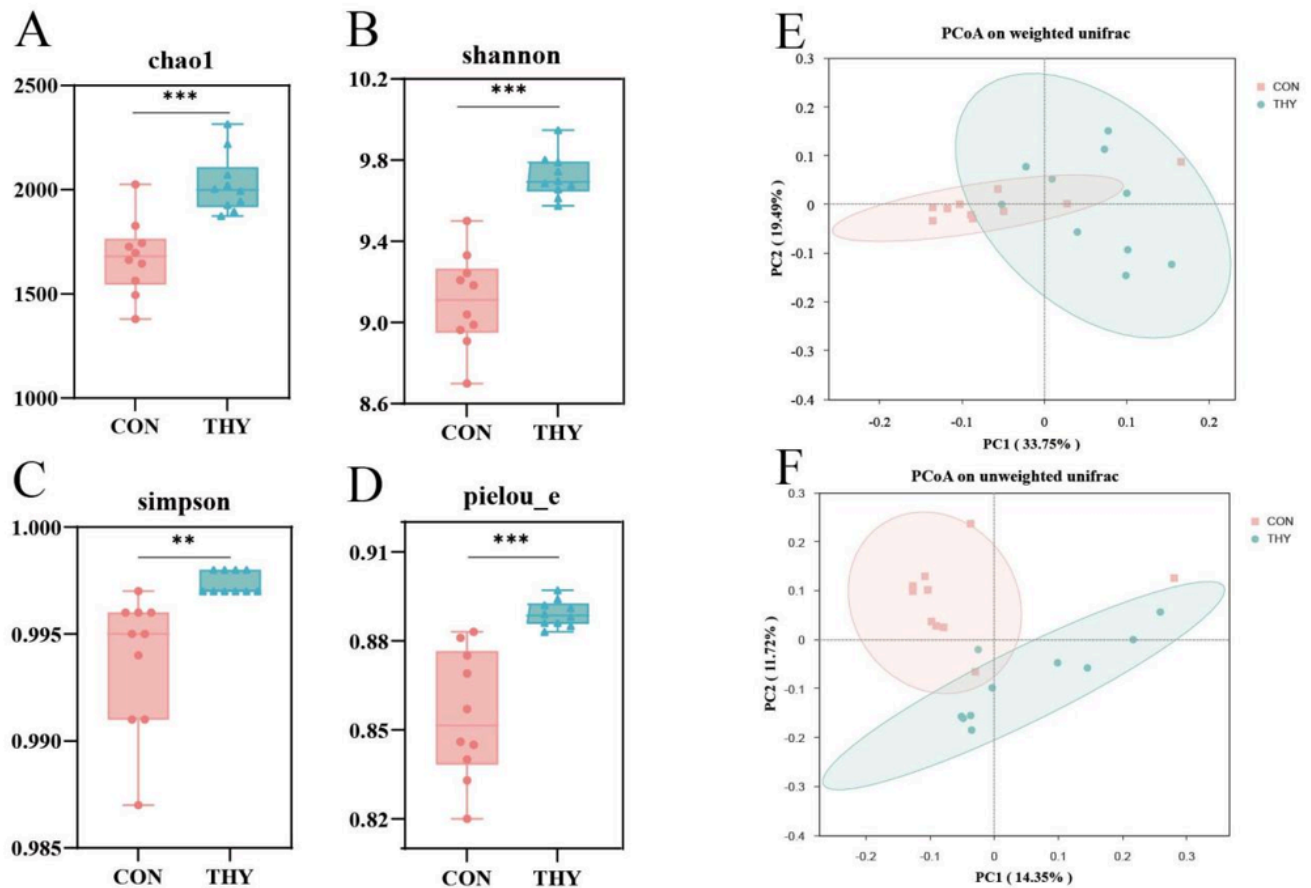


Figure 1

Effects of dietary thymol supplementation on rumen bacterial diversity and community structure of Texel × Kazakh crossbred growing rams. (A) Chao1 index. (B) Shannon index. (C) Simpson index. (D) Pielou's evenness index. Box plots display the median and interquartile range, with individual points representing individual samples (n = 10). (E) PCoA based on weighted UniFrac distances. (F) PCoA based on unweighted UniFrac distances. Ellipses represent 95% confidence intervals. P values were obtained by PERMANOVA (999 permutations). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CON = control group; THY = thymol group.

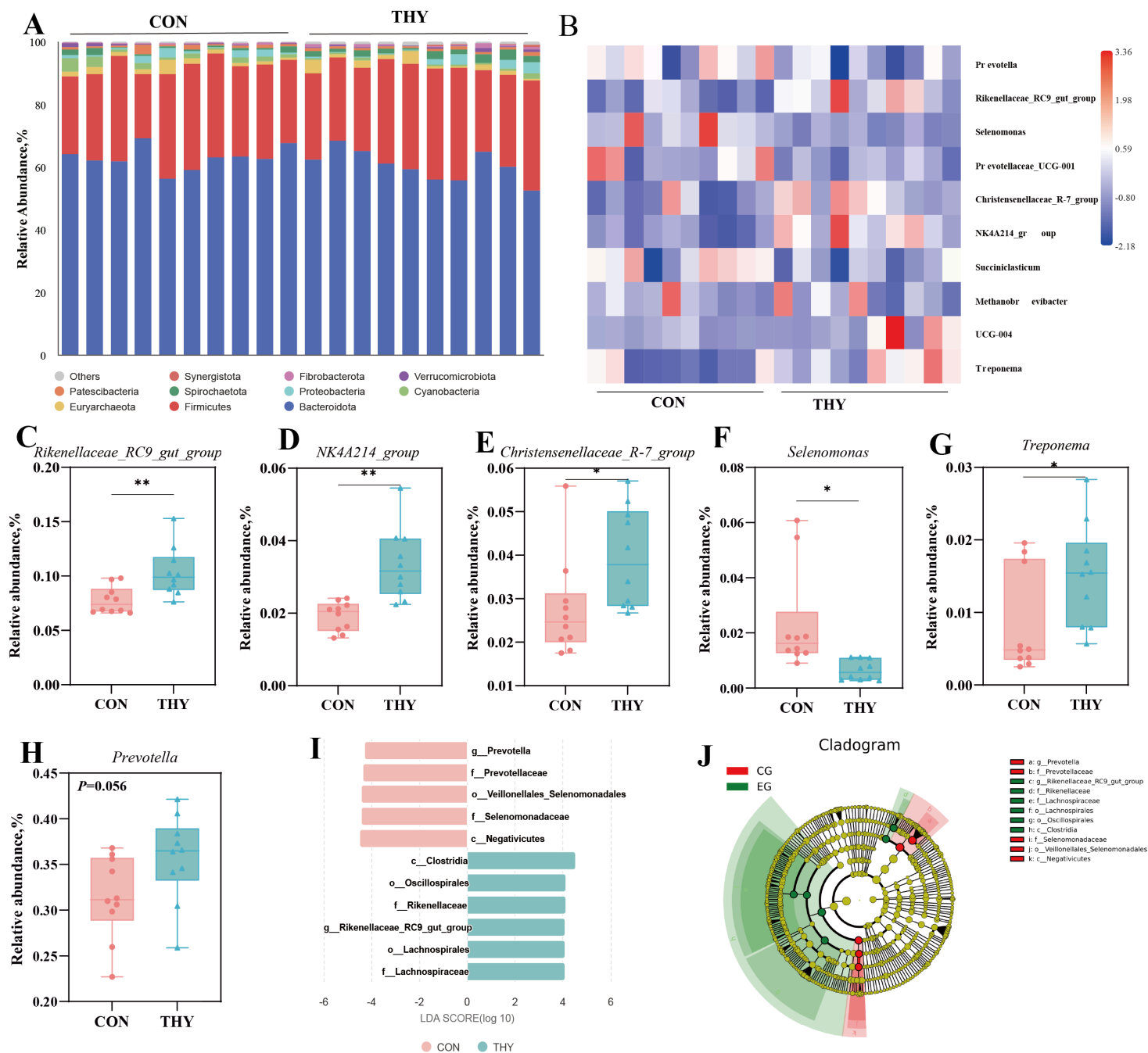


Figure 2

Effects of dietary thymol supplementation on rumen bacterial community composition and differentially abundant taxa of Texel × Kazakh crossbred growing rams. (A) Relative abundance at the phylum level (top 10), presented as group means. (B) Clustered heatmap at the genus level (top 30), with color intensity reflecting relative abundance; clustering based on Euclidean distance. (C–H) Relative abundances of significantly differentially abundant genera: (C) *Rikenellaceae_RC9_gut_group*, (D) *NK4A214_group*, (E) *Christensenellaceae_R-7_group*, (F) *Treponema*, (G) *Selenomonas*, (H) *Prevotella*. Box plots display the median and interquartile range, with individual points representing individual samples (n = 10). (I–J) LEfSe analysis (LDA score > 2.0): (I) LDA score histogram, (J) Cladogram (phylum to genus).

Red nodes indicate taxa enriched in the THY group; green nodes indicate taxa enriched in the CON group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CON = control group; THY = thymol group. PCoA = principal coordinates analysis; LfSe = linear discriminant analysis effect size; LDA = linear discriminant analysis.

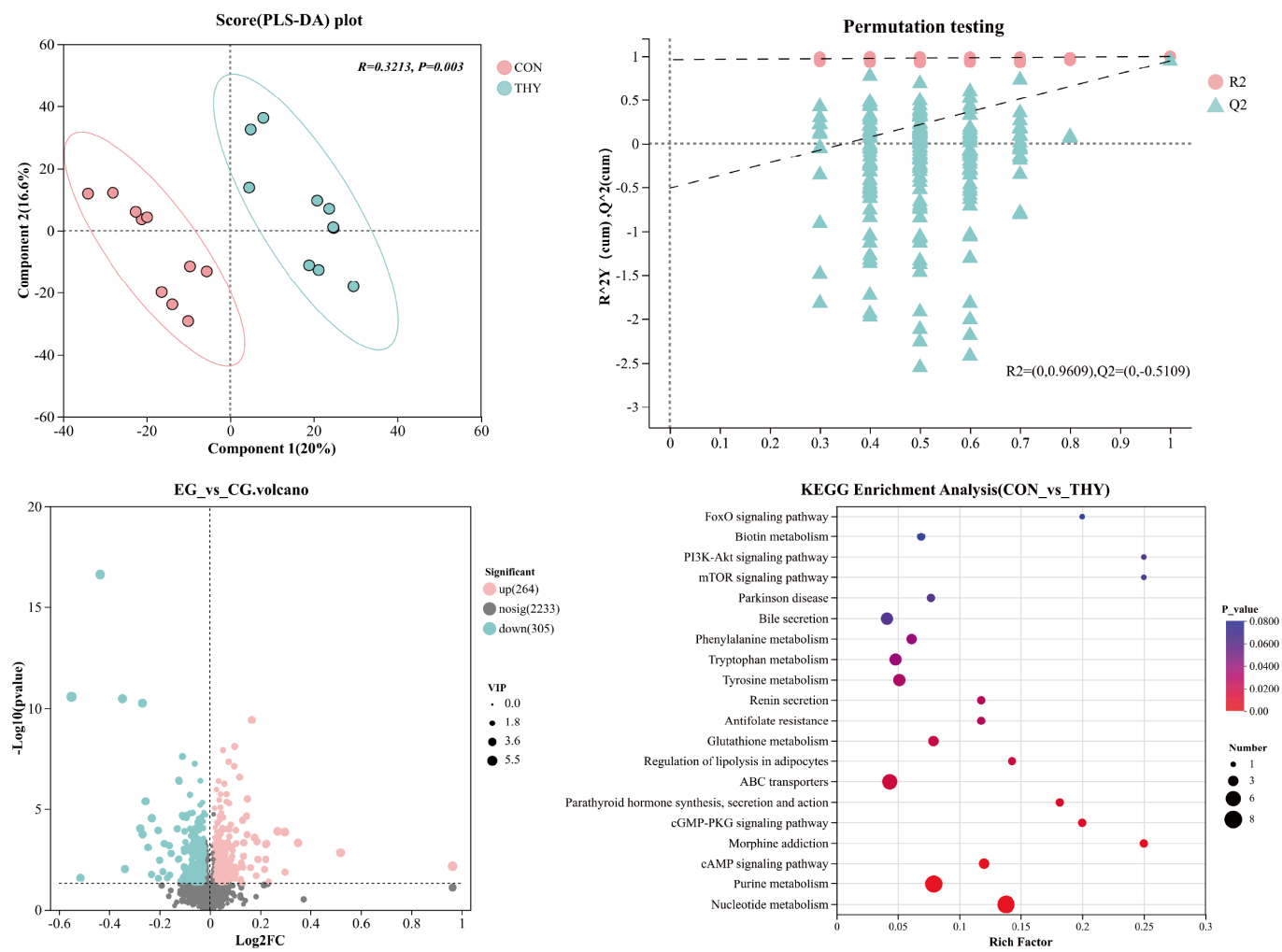


Figure 3

Effects of dietary thymol supplementation on the rumen fluid metabolome of Texel × Kazakh crossbred growing rams.(A) PLS-DA score plot. (B) Permutation test of the PLS-DA model. (C) Volcano plot of differential metabolites (VIP > 1.0, $P < 0.05$). Pink: up-regulated (n = 264); cyan: down-regulated (n = 305). (D) KEGG pathway enrichment analysis of differential metabolites. Dot size represents the number of metabolites, and color (red to blue) indicates the P-value. CON = control group; THY = thymol group. PLS-DA = partial least squares discriminant analysis; VIP = variable importance in projection; KEGG = Kyoto Encyclopedia of Genes and Genomes.

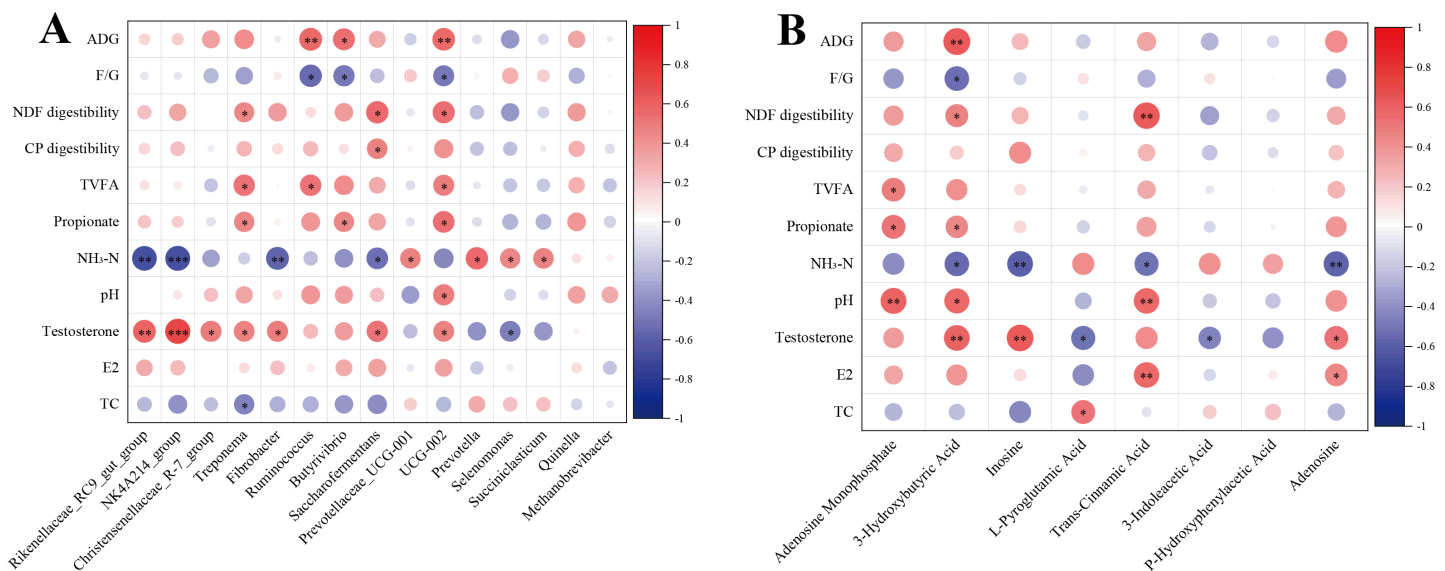


Figure 4

Integrated Spearman correlation heatmaps of rumen microbiota, key metabolites, and host phenotypes in Texel × Kazakh crossbred rams. (A) Correlation heatmap between 15 differentially abundant genera at the genus level (rows) and 11 host phenotypic indicators (columns). The color bar on the right indicates the direction of change in the THY group relative to the CON group. (B) Correlation heatmap between 8 representative metabolites from the significantly enriched KEGG pathways (rows) and the same 11 host phenotypic indicators (columns). $P < 0.05$, $*P < 0.01$.

ADG = average daily gain; F/G = feed-to-gain ratio; NDF = neutral detergent fiber; CP = crude protein; TVFA = total volatile fatty acids; NH₃-N = ammonia nitrogen; E2 = estradiol; TC = total cholesterol; AMP = adenosine monophosphate; CON = control group; THY = thymol group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterialThymol20250521.pdf](#)
- [TableS1DifferentialMetabolites.xlsx.csv](#)