

Combined Supplementation of Probiotics and Enzymes Improves Performance and Regulates Rumen Microbiota in Fattening Goats

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Research

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Abstract

Background: The aim of this study was to explore the effects of growth performance, slaughter performance, serum biochemical, immune and antioxidant indexes and rumen microflora fed with a basal diet (CON group), added *B. subtilis* and *B. licheniformis* (PRO group), supplemented with *B. subtilis*, *B. licheniformis* and enzyme preparations (COM group) on fattening goats.

Methods: 39 male goats were randomly divided into 4 groups with 13 individuals in each group for feed period of 80 d. Goats were fed as follows: CON diet, PRO diet with *B. subtilis* and *B. licheniformis*, and COM diet with *B. subtilis*, *B. licheniformis* and compound enzymes.

Results: ADFI of COM group significantly increased compared with CON group and PRO group ($P < 0.01$), as well as COM group and PRO group dramatically promoted ADG versus with CON group ($P < 0.05$). As a consequence, the body weight of fattening goats in the COM group was predominantly higher than that in the CON group ($P < 0.01$). In addition, the PRO group and COM group enhanced the TNF- α ($P < 0.05$) and IL-10 content ($P < 0.01$) in the serum. No differences were observed in serum biochemical and antioxidant indexes of three groups ($P > 0.05$). Likewise, the GR values of PRO group and COM group were noteworthy improved in comparison with CON group ($P < 0.01$). The VFA contents in rumen fluid were insignificantly different ($P > 0.05$). COM group also enriched the relative abundance of *Proteobacteria* compared with CON group and PRO group ($P < 0.05$). Nevertheless, the relative abundance of *Actinobacteria* decreased of PRO group and COM group in rumen fluid microorganisms ($P < 0.05$). Apparently, COM group significantly enriched nitrogen metabolism, glycolysis and TCA cycle ($P < 0.05$), whereas nucleotides biosynthesis was notably reduced ($P < 0.05$).

Conclusion: The combined feed of probiotics and enzymes had more profound effects than probiotics feed. Consequently, supplementation with *B. subtilis* and *B. licheniformis* and enzymes in the basal diet of fattening goats, which could improve growth performance, slaughter performance, immunity and accommodate rumen microbiota.

Background

Bacillus have been widely added to animal feed, particularly *Bacillus subtilis* and *Bacillus licheniformis* were used extensively, which can fight cancer, resist oxidation and emerge vitamins [1]. Moreover, *B. subtilis* can produce antibiotics and as dominant microorganisms isolated in animal gastrointestinal tracts [2, 3]. *B. licheniformis* can produce a variety of digestive enzymes and antimicrobial peptides, which can inhibit the activities of harmful microflora in animal gastrointestinal tracts [4–6]. Enzyme preparations include amounts of enzymes, xylanase, β -mannanase and β -glucanase can eliminate antinutrients in feedstuff, whereby increasing the utilization rate of feedstuff [7–9]. Protease plays a pivotal role in digestion and absorption of nutrients [10]. Cellulase can degrade cellulose into glucose in feedstuff, which is conducive to the nutrient metabolism [11, 12]. Supplementing with amylase in basal diet can compensate for the lack of endogenous enzymes in organism [13].

The compound biological preparations of *Bacillus licheniformis*, *Saccharomyces cerevisiae* and protease fed fattening lambs, which improved the growth performance, antioxidant competence and immunocompetence, also increased the rumen microbial diversity [14]. Supplementation with probiotics and cellulose was confirmed of having the impact on improving growth performance and promoting the expression of functional genes in rumen. The combined feed of probiotics and cellulose has an immense importance for the health of sheep [15]. Nevertheless, the functions of probiotics (*B. subtilis*, *B. licheniformis*) and enzymes (xylanase, protease, amylase, β -mannanase, cellulase, β -glucanase) on fattening goats are still unknown. Herein, this research investigated the effects of *B. subtilis*, *B. licheniformis* and enzymes on growth performance, slaughter performance, antioxidant capacity, immune ability and rumen microorganisms of fattening goats.

Materials And Methods

Probiotics and enzymes

Bacillus subtilis (1×10^{11} CFU/g) and *Bacillus licheniformis* (1×10^{11} CFU/g) were provided by Kangyuan Biotechnology Company. Xylanase (4000 U/g), protease (1000 U/g), amylase (300 U/g), β -mannanase (150 U/g), cellulase (200 U/g), and β -glucanase (150 U/g) were obtained from Guangdong VTR Bio-Tech Co., Ltd.

Experimental design

The procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University and the experiments were followed by the National Research Council Guide. 39 male goats (Yantse River Delta White) with near weights and healthy state were randomly divided into 3 groups and 13 individuals in each group: CON group, fed a basal diet; PRO group, fed a basal diet with *B. subtilis* (600 mg/kg) and *B. licheniformis* (600 mg/kg); COM group, fed a basal diet with *B. subtilis* (600 mg/kg), *B. licheniformis* (600 mg/kg) and compound enzymes (200 g/t). For PRO group and COM group, *B. subtilis*, *B. licheniformis* and compound enzymes and other ingredients were made to granule then fed to goats as experimental diets. The basal diets were purchased from Jiangsu Agriportal Co., Ltd. The composition and nutritional levels are shown in Table 1. The goats were raised in house and fed with basal diets twice a day at 08:00 and 16:00, goats had fed and water *ad libitum*.

Table 1
Dietary composition and nutrient levels of basal diet (air-dry basis).

Items	CON ^a	PRO ^b	COM ^c
Ingredients, %			
Corn	45.00	45.00	45.00
Corn germ meal	12.00	12.00	12.00
Malt root	6.00	6.00	6.00
Pleurotus eryngii residue	5.00	5.00	5.00
Soybean meal	12.00	12.00	12.00
Rice husk	5.00	5.00	5.00
Soybean hull	10.00	10.00	10.00
Premix ^d	5.00	5.00	5.00
Total	100.00	100.00	100.00
Nutrient levels ^e			
DM, %	89.73	89.73	89.73
CP, %	14.87	14.87	14.87
EE, %	2.62	2.62	2.62
Ash, %	7.23	7.23	7.23
NDF, %	27.10	27.10	27.10
ADF, %	13.94	13.94	13.94
Ca, %	1.17	1.17	1.17
P, %	0.44	0.44	0.44
Living BS count, CFU/g	-	1.0 × 10 ¹¹	1.0 × 10 ¹¹
Living BL count, CFU/g	-	1.0 × 10 ¹¹	1.0 × 10 ¹¹
Xylanase, U/g	-	-	4.0 × 10 ³
Protease, U/g	-	-	1.0 × 10 ³
Amylase, U/g	-	-	300.00
β-mannanase, U/g	-	-	150.00
Cellulase, U/g	-	-	200.00

Items	CON ^a	PRO ^b	COM ^c
β-glucanase, U/g	-	-	150.00

CON = control feedstuff; PRO = probiotics feedstuff; COM = combined feedstuff.

a. (basal diet).

b. (basal diet + 0.06% *Bacillus subtilis* + 0.06% *Bacillus licheniformis*).

c. (basal diet + 0.06% *Bacillus subtilis* + 0.06% *Bacillus licheniformis* + 0.02% enzyme preparations).

d. The premix provided the following per kg of the diet: VA 3 000 IU, VD₃ 750 IU, VE 6 mg, nicotinamide 11 mg, Cu (as copper sulfate) 11 mg, Fe (as ferrous sulfate) 40 mg, Mn (as manganese sulfate) 50 mg, Zn (as zinc sulfate) 50 mg, Se (as sodium selenite) 0.25 mg, Co (as cobalt chloride) 0.5 mg, I (as calcium iodate) 0.4 mg.

e. Nutrient levels were measured values.

Sample collection

The ruminal contents were collected by a gastric catheter on day 20, day 40, day 60 and day 80 of the experimental process, after the filtration of four layers of gauze, the rumen fluid was transferred to 50 mL tube, then quickly measured the pH of rumen fluid using a pH meter (Wuhan, China) and frozen in liquid nitrogen and stored at -80 °C.

Blood samples of four goats in each group were collected on day 80 and centrifugated at 3000 rpm for 10 min to obtain the serum samples then stored at -20 °C before detection. Serum biochemical, immune and antioxidant indexes were analyzed using kits (Nanjing Jiancheng, Biotechnology, Jiangsu, China).

The content of NH₃-N was detected according to the means [16]. The VFA contents were measured according to the following method: 1 mL rumen fluid samples were blended with 0.2 mL mixed solution of metaphosphoric acid and crotonic acid (metaphosphoric acid 25 g/100 mL, crotonic acid 0.6464 g/100 mL), and placed at -20 °C for 12 h. After thawing, subsequently, quickly injecting 0.6 mL samples to gas chromatography (Agilent 7890A GC, Japan).

Growth performance

The goats were weighted without being fed on the 1st and 80th days of the experiment to determine the initial weight and final weight, then calculated ADG of each goat. $ADG = (\text{final weight} - \text{initial weight}) / \text{experimental days}$. The feed intake of goats was recorded each day during the experimental period, $ADFI = \text{total feed intake} / \text{experimental days}$, thus F/G was determined: $F/G = ADFI/ADG$.

Slaughter performance

Five goats in each group were randomly selected to slaughter and fasted 12 h before slaughter. Live weight before slaughter and carcass weight were recorded. Carcass weight = live weight before slaughter – head weight – skin weight – hoof weight – tail weight – reproductive organs weight – internal weight (remain kidney and perirenal lipid). Then dressing percentage was determined: Dressing percentage = carcass weight / live weight before slaughter. Loin eye area was calculated using a planimeter (KP-90 N) followed a previous study [17]. GR value was recorded by using a vernier caliper (Shanghai, China) in accordance with the method [18].

16S sequencing

DNA of samples was extracted using the E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., San Antonio, TX, USA) according to the procedures of manufacturer. DNA was eluted in 50 mL Elution buffer and stored at -20 °C before determination in the PCR by LC-Bio Technology (Hangzhou, China).

The hypervariable V3-V4 region of the prokaryotic (bacterial and archaeal) small-subunit (16S) rRNA was amplified with primers (F: 5'-CCTACGGGNGGCWGCAG-3' and R: 5'-GACTACHVGGGTATCTAATCC-3'). In accordance with the following procedure to amplify PCR: denaturation at 98 °C for 30 s; 32 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s; then a final extension at 72 °C for 10 min. The amplification was conducted on a volume of 25 µL reaction mixture. PCR products were verified by 2% agarose gel electrophoresis and purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA). The quantity of the amplicon library was evaluated on the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). The NovaSeq PE250 platform was prepared to sequence the libraries.

Bioinformatic analysis

Samples were sequenced on an Illumina NovaSeq platform provided by LC-Bio. Sequences with a similarity of $\geq 97\%$ were assigned to the same operational taxonomic units (OTUs) by Vsearch (V 2.3.4). OTUs were chosen by the RDP (Ribosomal Database Project) classifier (<http://sourceforge.net/projects/rdp-classifier/>) (Wang *et al.*, 2007). Alpha diversity was analyzed to evaluate the bacterial community and calculated with QIIME (Version 1.8.0), including Chao1, Observed_species, Goods_coverage, Shannon, Simpson. The R package (V 3.4.4) was used to make other diagrams. Moreover, the functional predictions of bacterial metabolism were performed by PICRUSt (<https://huttenhower.sph.harvard.edu/galaxy/>).

Statistics and analysis

SPSS 23.0 was performed to analyze data through one-way ANOVA and Duncan's test was conducted to compare the differences. The results were shown as the Mean \pm Standard Error (M \pm SE). The differences among the three groups were described as significant differences at $P < 0.05$ and trends at $P < 0.10$. GraphPad Prism 8.0 was used to make graphs.

Results

As Table 2 shows, the final weight and ADG of goats significantly increased in the PRO and COM group compared with that in the CON group ($P < 0.05$). COM treatment dramatically enriched ADFI in comparison with that in CON and PRO. There were no significant differences in F/G among the three groups ($P > 0.05$).

Table 2
Effects of probiotics combined with enzymes on growth performance of fattening goats.

Items	CON	PRO	COM	P-value
Initial weight, kg	13.87±2.55	13.87±0.58	15.33±3.32	0.711
Final weight, kg	26.27±0.61 ^b	31.40±0.35 ^{ab}	33.73±4.54 ^a	0.034
ADG, g/d	157.96±31.11 ^b	221.94±2.92 ^a	232.91±32.91 ^a	0.024
ADFI, g/d	826.02±100.28 ^b	841.76±123.98 ^b	904.54±125.29 ^a	0.001
F/G	5.46±1.21	3.82±0.05	3.96±0.52	0.069

^{a,b} Means a row with different small letter superscripts significantly differ ($P < 0.05$), while with no letter or the same letter superscripts mean no significant difference ($P > 0.05$). The same as below. The values were expressed as "mean ± standard deviation".

Slaughter performance

As shown in Table 3, probiotics feed and combined feed had no notable effects on live weight before slaughter, carcass weight, dressing percentage and loin eye area of fattening goats compared with those in CON ($P > 0.05$). Nevertheless, supplementing with probiotics or combined with enzymes pronouncedly enhanced the GR value than that in CON ($P < 0.05$).

Table 3
Effects of probiotics combined with enzymes on slaughter performance of fattening goats.

Items	CON	PRO	COM	P-value
Live weight before slaughter, kg	27.84±1.53	30.12±0.70	28.72±1.71	0.066
Carcass weight, kg	13.62±1.05	14.44±0.57	14.38±1.41	0.426
Dressing percentage, %	48.89±1.56	47.96±2.03	50.00±2.28	0.301
Loin eye area, cm ²	19.67±3.14	16.97±1.53	14.40±2.96	0.126
GR value, mm	6.65±1.05 ^b	11.34±0.56 ^a	11.32±0.41 ^a	0.001
^{a,b} Means a row with different small letter superscripts significantly differ ($P < 0.05$), while with no letter or the same letter superscripts mean no significant difference ($P > 0.05$).				

Serum biochemical indexes

Figure 1 shows the effects of biochemical indexes in fattening goats fed probiotics and combined with enzymes. There were no differences in ALT, AST, ALP, TP, ALB, GLB, TG, TC, UN, Cr and HDL contents of three groups ($P > 0.05$). The results illustrated that supplementation of probiotics and combined with enzymes had no dramatical function of changing the physiological state of goats ($P < 0.05$).

Serum immune and antioxidant indexes

No differences were observed in SOD, MDA, GSH-Px and T-AOC contents among CON, PRO and COM groups ($P > 0.05$, Fig. 2). Furthermore, administrating probiotics and combined with enzymes predominantly increased the TNF- α and IL-10 concentrations of goat serum ($P < 0.05$), while the content of IL-6 had no remarkable changes in the PRO and COM group in comparison with that in the CON group ($P > 0.05$).

Rumen fermentation parameters

Figure 3 shows the rumen fermentation parameters of fattening goats administration of probiotics or combined with enzymes. Feeding PRO or COM unaffected the pH, NH₃-N and VFA contents compared with the CON, while the isobutyrate content significantly increased in the PRO group ($P = 0.040$) and tended to enrich in the COM group ($P = 0.064$) than that in the CON group.

Rumen microbiota and interactions

As shown in Table 4, no dramatical differences were detected in the Chao 1, Goods_coverage, Observed_species, Shannon and Simpson indexes in CON, PRO and COM group, which indicated that supplementing probiotics and combined with enzymes had no central effects on alpha diversity of rumen fluid microorganisms in fattening goats. Principal Coordinate Analysis (PCoA) demonstrated 30.47% variation of total rumen microbiota based on unweighted_unifrac. Besides, the stacked bar chart

exhibited that *Bacteroides* and *Firmicutes* were the dominant phylum of rumen microbiota. Compared with CON group, the relative abundance of *Proteobacteria* pronouncedly enriched in COM group, while the relative abundance of *Actinobacteria* significantly reduced in PRO and COM (Fig. 4B). Additionally, heatmap manifested remarkable bacteria at the genus level of three groups (Fig. 4C). Circos showed *Prevotella_1*, *Prevotella_7* and *Prevotellaceae_unclassified* accounted for a large proportion in three groups (Fig. 4D). Fig. 3E shows sixteen predominant in three groups. Specifically, adding probiotics significantly advanced the relative abundance of *Butyrivibrio* and *Clostridiales_unclassified* ($P < 0.05$), the relative abundance of *Bilophila* and *Succiniclasicum* dramatically enhanced in COM group ($P < 0.05$).

Figure 4F reflected the evolutionary branching cladogram of rumen microflora in three groups. The PRO group had no biomarker of rumen microflora. The dominant biomarkers were *Butyrivibrio*, *Shuttleworthia* and *Syntrophococcus* in the COM group. LEFSe analysis found that *Aeromonas_sp_18III_A01_071* was a biomarker in PRO group (LDA SCORE > 3), and there were seventeen biomarkers in COM, in which the number of biomarkers was the largest among three groups. Specifically, the relative abundance of *Firmicutes*, *Lachnospiraceae*, *Ruminococcus_1_unclassified* and *Ruminococcus_1*(LDA SCORE >4) were significantly improved in COM group. Sparcc analyzed the most abundant microbial species in the top 30 to get the correlation between two bacteria (Fig. 4H). The results indicated that *Bacteroidaceae_unclassified* and *Prevotellaceae_unclassified*, *Bacteroidetes_unclassified* and *F082_unclassified* had the strongest positive correlations, a negative correlation existed in *Prevotella_1* and *Lachnospiraceae_NK3A20_group* (Fig. 4I).

Table 4

Effects of probiotics combined with enzymes on alpha diversity of rumen fluid microorganisms in fattening goats.

Items	CON	PRO	COM	P-value
Chao1 index	982.40±186.19	788.82±300.58	1077.62±273.23	0.319
Goods_coverage index	0.9998±0.0002	0.9998±0.0003	0.9993±0.0006	0.157
Observed_species index	981.50±185.68	787.75±299.43	1072.00±267.95	0.321
Shannon index	7.87±0.38	7.27±0.56	7.87±0.52	0.192
Simpson index	0.99±0.01	0.98±0.01	0.98±0.01	0.537

The values included four duplicates.

PICRUSt functional predictions

PICRUSt (Phylogenetic investigation of communities by reconstruction of unobserved states) were applied to predict the functional abundance of microflora. The COM group significantly upregulated nitrogen metabolism, ATPase and GMP reductase compared with the CON group (Fig. 5A). The results found that the contents of DNA synthesis, some proteins and transcriptional regulators were significantly enriched in COM based on the KO database (Fig. 5B). Furthermore, glyoxylate cycle, glycolysis and

glyoxylate were dramatically upregulated in the COM group compared with that in the CON group, COM group significantly upregulated nucleotide synthesis and TCA cycle in functional proteins in the COM group compared with that in the CON group (Fig. 5D).

Correlation analysis goat performance, serum indexes, rumen fermentation parameters and rumen microbiota

Pearson's correlation was emphasized to analyze the relevance of goat performance, basic parameters and rumen microbiota (Fig. 6). At the phylum level, *Actinobacteria* notably diminished in the COM group and had positive correlations with F/G and MDA content of serum ($P < 0.05$), and a negative correlation with ADG ($P < 0.05$). Besides, ADG significantly increased ($P < 0.05$), F/G had a tendency to mitigate in COM ($P < 0.10$), the changes were consistent with these correlations. Moreover, *Proteobacteria* pronouncedly enriched in COM and positively correlated with carcass weight ($P < 0.05$), negatively correlated with SOD and T-AOC contents of serum ($P < 0.05$). Accordingly, COM had increased carcass weight and reduced contents of SOD and T-AOC. Additionally, *Patescibacteria* ameliorated in COM and had a positive relationship with $\text{NH}_3\text{-N}$ and a negative relationship with butyrate ($P < 0.05$), the increased *Patescibacteria* led to the enrichment of $\text{NH}_3\text{-N}$ and the decrease of butyrate in COM.

Furthermore, the relative abundance of *Spirochaetes* enlarged in PRO and positively related with the contents of IL-10 and $\text{NH}_3\text{-N}$ ($P < 0.05$), which were in accordance with the improved contents of IL-10 and $\text{NH}_3\text{-N}$ in PRO. *Firmicutes* remarkably reduced in PRO and positively related to AST content ($P < 0.05$), which contributed to the attenuation of AST content in PRO.

At the genus level, *Saccharofermentans* reduced in PRO and COM and were positively related with F/G and MDA ($P < 0.01$), loin eye area, Cr, TVFA and valerate ($P < 0.05$), and negatively with ADG ($P < 0.05$), pH ($P < 0.01$). Probiotics feed and combined feed decreased the abundance of *Olsenella* and had positive relationships with UN, TG, TC, Cr, butyrate and valerate ($P < 0.05$), negative relationships with TNF- α , $\text{NH}_3\text{-N}$, isovalerate and A/P ($P < 0.05$). Besides, COM feed improved *Bacteroidaceae_unclassified* and positively related to carcass weight and dressing percentage ($P < 0.05$). *Prevotellaceae_unclassified* were negatively correlated with SOD ($P < 0.01$) and T-AOC ($P < 0.05$) in the COM group. Additionally, *Lachnospiraceae_NK3A20_group* reduced in PRO and had positive relationships with GSH-Px ($P < 0.01$) and Cr ($P < 0.05$), a negative relationship with ALB ($P < 0.01$). *Prevotellaceae_UCG-001* were positively correlated with IL-10 and $\text{NH}_3\text{-N}$ in PRO ($P < 0.05$).

Discussion

These results observed that feeding probiotics combined with enzymes enhanced the growth performance and slaughter performance of fattening goats. Besides, COM had more advanced effects than PRO. In addition, the immunity was enhanced and the rumen bacterial structure was changed in the PRO group and COM group, which might be the reasons to improve the performance of fattening goats.

A number of studies have shown the profound effects on pigs supplemented with *B. subtilis* and *B. licheniformis* [20–24]. Nevertheless, there were little researches into which the influence of *B. subtilis* and *B. licheniformis* were conducted on goats. Moreover, various researches have studied the effects of compound enzymes on weaned pigs [25, 26]. In addition, combined feed probiotics and enzymes had predominant effects on growth performance and rumen bacteria of sheep [15]. Based on these reports, we investigated the functions of *B. subtilis*, *B. licheniformis* and multiple enzymes on fattening goats, individually and in combination. Importantly, the effects of combined feed were better than single feed which was explored in this study.

The results observed that ADG significantly increased of fattening goats in the PRO group. A previous study reported that the basal diet administration of *B. subtilis* and *B. licheniformis* significantly improved the growth performance of piglets [20]. The growth performance of finisher pigs was noteworthy improved supplemented with *B. subtilis* and *B. licheniformis* [23]. Adding *B. subtilis*, *B. licheniformis*, *B. coagulans* and *C. butyricum* ameliorated the weaning pig performance [27]. Some findings above indicated that feeding *B. subtilis* and *B. licheniformis* played a central role in improving the growth performance of pigs. In our study, supplementing *B. subtilis* and *B. licheniformis* also accelerated the improvement of goat performance. Furthermore, COM group raised the final weight, ADG and ADFI in goats in comparison with CON group. There was a report that supplementing cellulose increased the growth performance of Hu sheep [28]. Previous research found that treatment of compound probiotics and cellulase enabled the increase of the total weight gain and ADG in sheep [15]. It may be explained that supplementation with enzymes could promote the absorption of nutrients, enhance the feed efficiency, keep the healthy condition [29–31]. Additionally, more combinations of probiotics and enzymes should be investigated to put to good use in the feed industry.

Slaughter performance reflects the applicability in animals, which decides the economic profit in the practical application [32]. Adding cellulase and xylanase to steers failed to improve the carcass and slaughter percentage, which were similar to our research results [33]. Pigs fed probiotics included *Bacillus subtilis* and *Clostridium butyricum* had no effect on loin eye area [34]. A previous study reported that supplementation with cellulose did not significantly change the dressing percentage and loin eye area of Hu sheep [28]. Consistently, PRO and COM feed unaffected the loin eye area of fattening goats. Compared with the CON group, the GR value dramatically in PRO group and COM group, while there was no significant difference between PRO group and COM group, which indicated that probiotics administration helped to deposit the muscle fat, besides, combined feed cannot enhance the deposition effect. The results showed that probiotics alone or combined with enzymes could improve the slaughter performance of fattening goats.

We elucidated that PRO and COM feed enriched the contents of IL-10 and TNF- α compared with CON feed. IL-10 and TNF- α play vital roles in inflammation [35]. TNF- α affects the carbohydrate and immune response of animals, which is a kind of proinflammatory cytokines [36, 37], and the content of TNF- α would enrich if the tissues were inflammatory [38]. IL-10 performs an important effect on inflammatory reaction as an anti-inflammatory factor, importantly, inducing the function of immunity [39, 40].

Supplementing probiotics could influence the immune function, inducing the improvement of immune cytokines contents [41–43]. Studies reported that administration of *B. subtilis* lead to the benefit of the host immune system [44, 45]. CD4+ cells are part of the T lymphocytes, whose subpopulations are Th cells that include Th1 and Th2 cells [46]. Th2 secretes different cytokines, such as IL-2, IL-4, IL-6 and IL-10, which play a major role in immune regulation and inflammatory responses [46, 47]. Importantly, Th2 accelerates the multiplication and differentiation of B lymphocytes, resulting in the production of antibody and intense responses of immune systems [46]. Apparently, *B. subtilis* secretes the anti-inflammatory cytokines IL-10 to regulate the immune response [48]. Consequently, supplementation with *B. subtilis*, *B. licheniformis* and in combination with compound enzymes had an intense importance for enhancing the immunity of fattening goats.

Rumen pH, NH₃-N and VFA contents are vital parameters in ruminants which illustrate the function and steady state of the rumen [14], and VFA are principal products of rumen fermentation, which relate the balance of energy in ruminants [49]. Probiotics could improve the function of rumen via maintaining the pH and enriching the VFA concentrations [14]. A previous study found that *Lactobacillus plantarum* and *Bacillus subtilis* feed unaffected the pH, NH₃-N and VFA concentrations, which is consistent with our results [50]. Administration of *B. licheniformis* enriched the total VFA contents and reduced the NH₃-N content [51]. There were no remarkable changes in the rumen fermentation parameters of cows fed with *B. subtilis* [51]. Nevertheless, the influence of rumen fermentation of fattening goats fed with probiotics or combined with enzymes still remains unclear. In our study, we found that adding probiotics (*B. subtilis* and *B. licheniformis*) or blended enzymes insignificantly influenced the rumen pH, NH₃-N and VFA concentrations of fattening goats in basal diet. Moreover, PRO and COM had potential tendencies to increase the isobutyrate contents. Isobutyrate predominantly produced from the oxidative-deamination and decarboxylation of ruminal isoleucine, which illustrated that PRO feed and COM feed had the potential to enhance the metabolism of isoleucine [52].

The microbiota has profound effects on the health and performance of animals. Hence, it is necessary to regulate diversity based on the composition and structure of bacteria in the host [53]. Interestingly, the alpha diversity of rumen microorganisms (Chao 1 index, Goods_coverage index, Observed_species index, Shannon index and Simpson index) showed no significant differences ($P > 0.05$). Administration of probiotics combined with enzymes had no impact on the bacterial diversity of goat rumen. At the phylum level of rumen microorganisms, COM group remarkably upregulated the relative abundance of *Proteobacteria* in contrast with CON group and PRO group ($P < 0.05$). Besides, the relative abundance of *Actinobacteria* in both PRO and COM significantly decreased compared with that in CON ($P < 0.05$). The combined feed showed notable upregulation of *Ruminococcaceae*, *Lachnospiraceae* and *Succinaceae* in ruminal microorganisms ($P < 0.05$). *Prevobacteriaceae*, *Clostridium* and *Succinaceae* are strict anaerobes, and *Ruminococcus* can produce a large amount of VFA in the hindgut of cows [54–55], which may lead to the enrichment of isobutyrate in goat rumen. A previous study found that supplementation with compound enzymes (xylanase, α -amylase, β -glucanase and protease) to pigs resulted in the increase of the abundance of *Firmicutes* in treatment group, which is in coherence with our findings [56]. The

colonization of anaerobic microbes contributes to the creation of an anaerobic environment in rumen, which is conducive to the maintenance of rumen health and effective digestion in rumen, therefore, promoting the health of fattening goats. *Butyrivibrio* was one of the distinct bacteria in the COM group ($P < 0.05$), which was in agreement with that previously reported [57]. *Prevotella* is a kind of potential pathogenic bacterium [58]. The relative abundance of *Prevotella_1* significantly reduced in COM in comparison with that in PRO, indicating that combined feed of probiotics and enzymes could decrease the number of pathogenic bacteria to keep the health of fattening goats. *Clostridiales* beneficially maintained the balance of animal intestines and optimized the structure of intestinal microenvironments, thus inhibiting the reproduction of harmful bacteria in the intestine [59]. The relative abundance of *Clostridiales_unclassified* dramatically enriched in COM compared with that in PRO, which plays a central role in keeping the balance of goat rumen.

This study first revealed the metabolic functions added to probiotics or blended enzymes of fattening goats. COM group significantly upregulated nitrogen metabolism, DNA synthesis, glyoxylate cycle, nucleotide synthesis and TCA cycle. COM greatly regulated the rumen microbes possibly be a reason to the differences of functional metabolism [60].

The correlation analysis confirmed there were relevance among goat performance, serum biochemical, immune and antioxidant indexes, rumen fermentation parameters and rumen microorganisms, which indicated that feeding probiotics or combined with enzymes regulated the rumen microbiota to ameliorate the performance and immunity of fattening goats. Particularly, *Saccharofermentans* and *Olsenella* positively related to VFA and improved the performance and serum indexes of goats. Consequently, these results above emphasized that PRO feed and COM feed had beneficial effects on the rumen microflora to facilitate the performance of goats. The performance and healthy state of lactating sows were promoted supplementation with *B. subtilis* and *B. licheniformis* [24], which was aligned to our research results. Accordingly, it elucidated an original method to enhance the performance and immunity of fattening goats by attenuation of the relative abundance of *Saccharofermentans* and *Olsenella* in rumen.

Conclusion

In conclusion, this research reveals the profound effects on the growth performance, slaughter performance, serum biochemical, immune and antioxidant indexes, rumen fermentation parameters and rumen microbiota of fattening goats administration of *B. subtilis*, *B. licheniformis* and combined with enzymes, which explains the potential mechanism to improve the performance and immunity of PRO feed and COM feed. Nonetheless, further research is still worthy of being investigated in regulating the performance and immunity of fattening goats added PRO and COM feed.

Abbreviations

DM: dry matter; CP: crude protein; EE: ether extract; Ash: crude ash; NDF: neutral detergent fiber; ADF: acid detergent fiber; Ca: calcium; P: phosphorus; BS: *Bacillus subtilis*; BL: *Bacillus licheniformis*; ADG: Average daily gain; ADFI: Average daily feed intake; F/G: Feed to gain ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TP: Total protein; ALB: Albumin; GLB: Globulin; TG: Triglyceride; TC: Total cholesterol; UN: Urea nitrogen; Cr: Creatinine; HDL: High density lipoprotein; TNF- α : Tumor necrosis factor α ; IL-6: Interleukin 6; IL-10: Interleukin 10; SOD: Superoxide dismutase; MDA: Malondialdehyde; GSH-Px: Glutathione peroxidase; T-AOC: Total antioxidant capacity; TVFA: Total volatile fatty acid; PCoA: Principal coordinates analysis; LefSe: Linear discriminant analysis effect size; LDA: Linear discriminant analysis; PICRUST: Phylogenetic investigation of communities by reconstruction of unobserved states; TCA: Tricarboxylic acid cycle.

Declarations

Authors' contributions

Jiawei Lu: conceptualization and writing – original draft. Zili Chen: methodology. Qin gao: investigation. Peizhen Li: visualization. Jingang Wang: investigation. Yu Cai: writing – review and editing. Zhibo Wang: resources. Dongxu Li: writing – review and editing. Huixia Li: writing – review and editing. Feng Wang: supervision. Yanli Zhang: project administration.

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Availability of data and materials

The datasets analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University and the experiments were followed by the National Research Council Guide.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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Figures

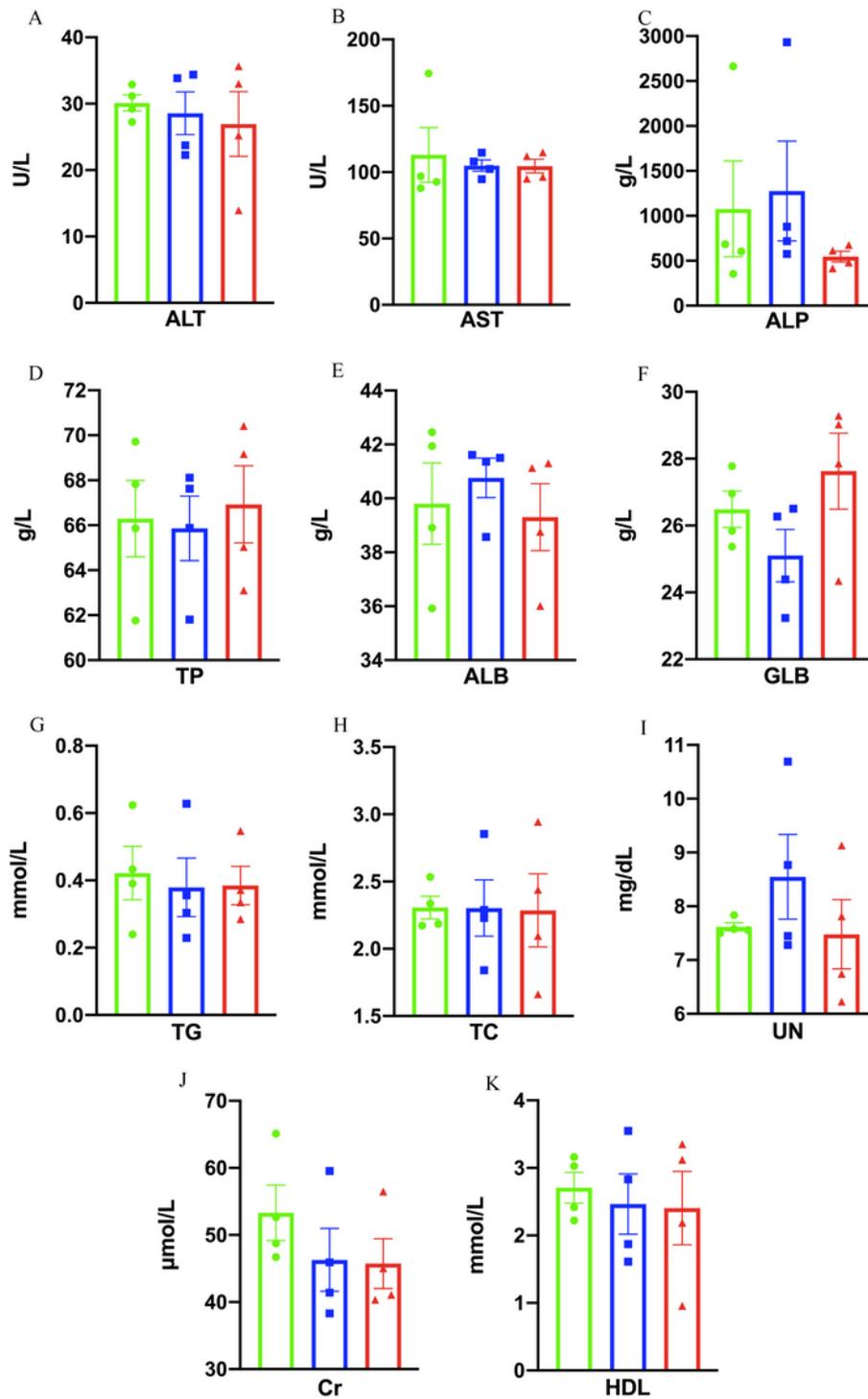


Figure 1

Serum biochemical indexes of fattening goats. A. ALT; B. AST; C. ALP; D. TP; E. ALB; F. GLB; G. TG; H. TC; I. UN; J. Cr; K. HDL. Values included four duplicates.

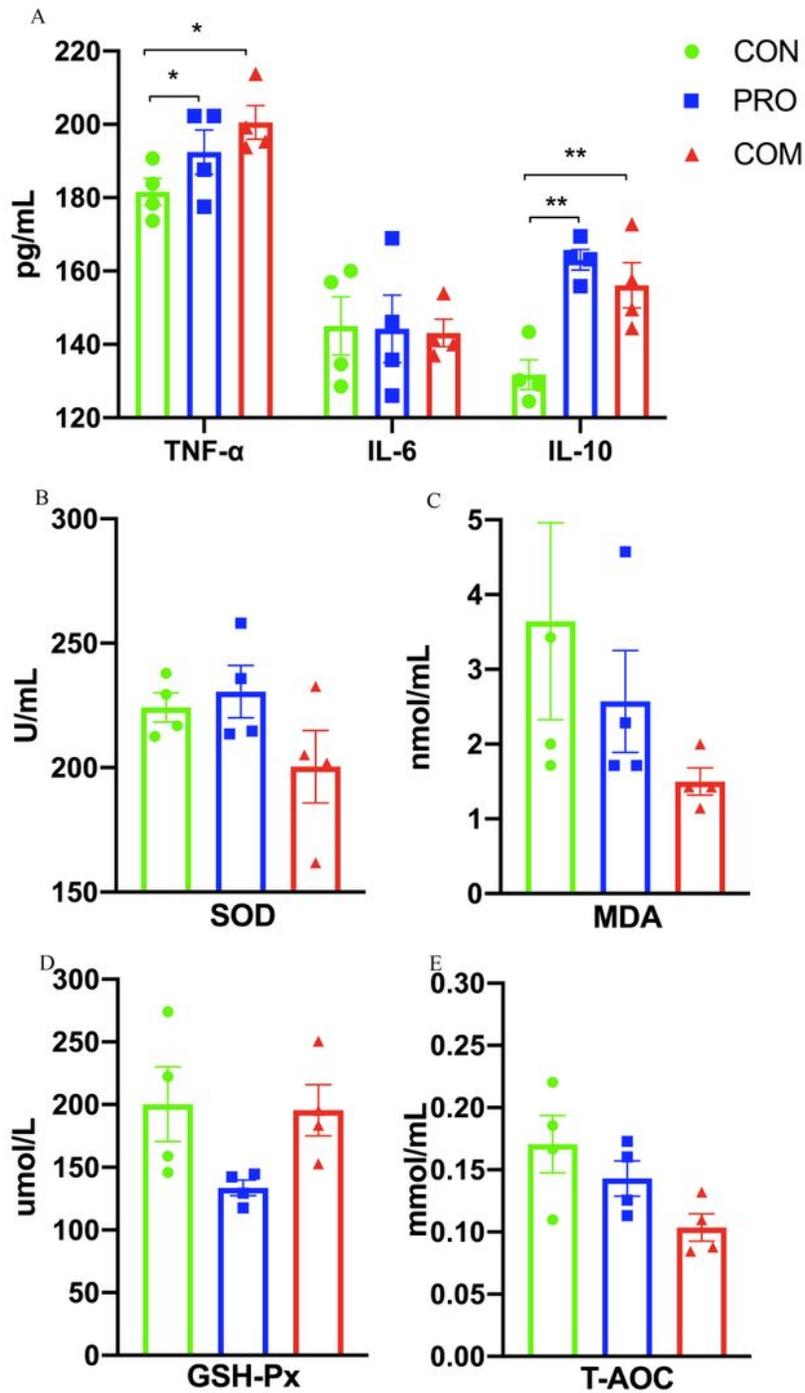


Figure 2

Serum immune and antioxidant indexes of fattening goats. A. Serum immune indexes. B. SOD. C. MDA. D. GSH-Px. E. T-AOC. * Represented $P < 0.05$ and ** represented $P < 0.01$ in comparison with CON. Values included four duplicates.

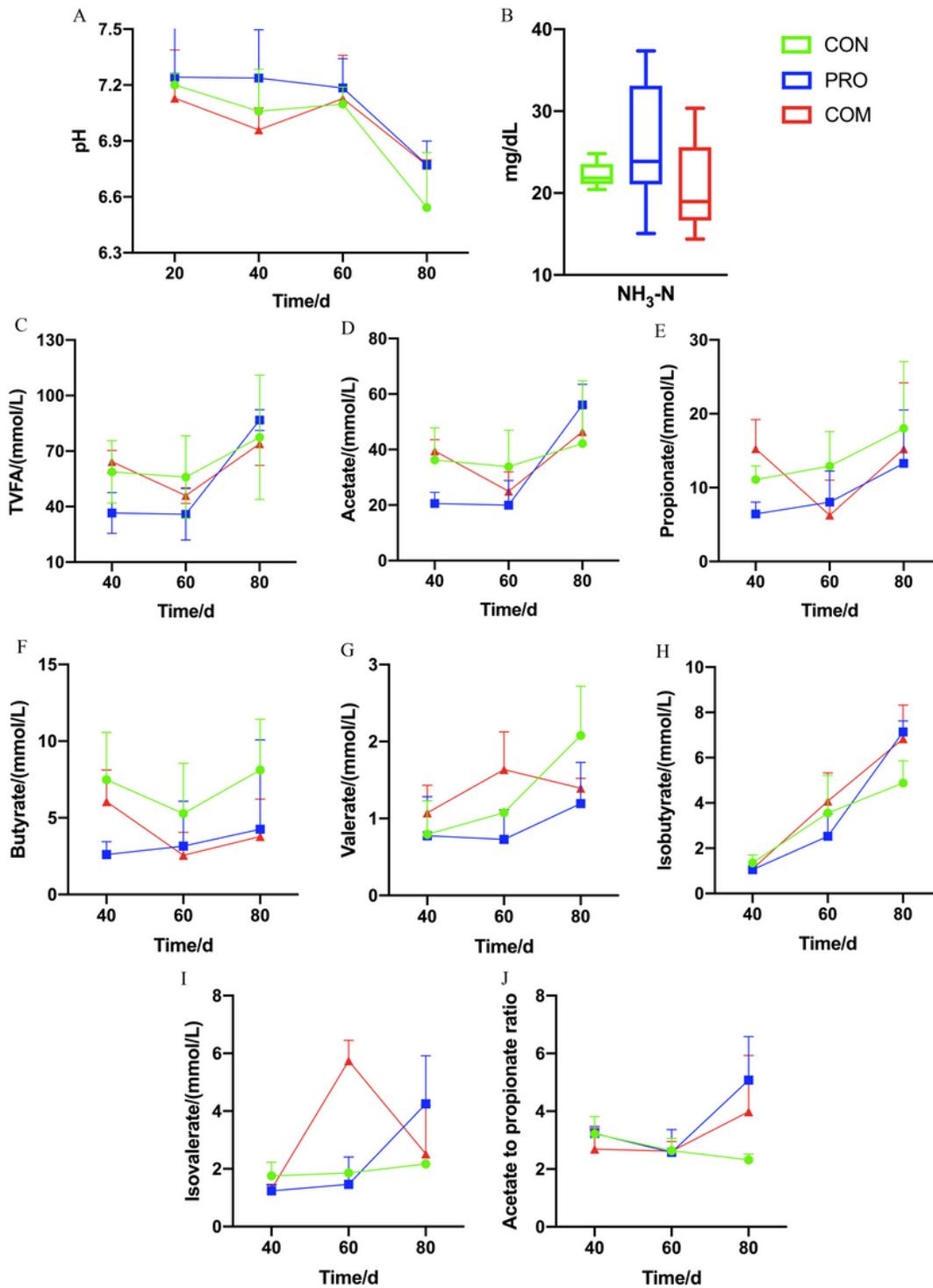


Figure 3

Rumen fermentation parameters of fattening goats. A. pH; B. NH₃-N; C. TVFA; D. Acetate; E. Propionate; F. Butyrate; G. Valerate; H. Isobutyrate; I. Isovalerate; J. Acetate to propionate ratio. Values included three duplicates.

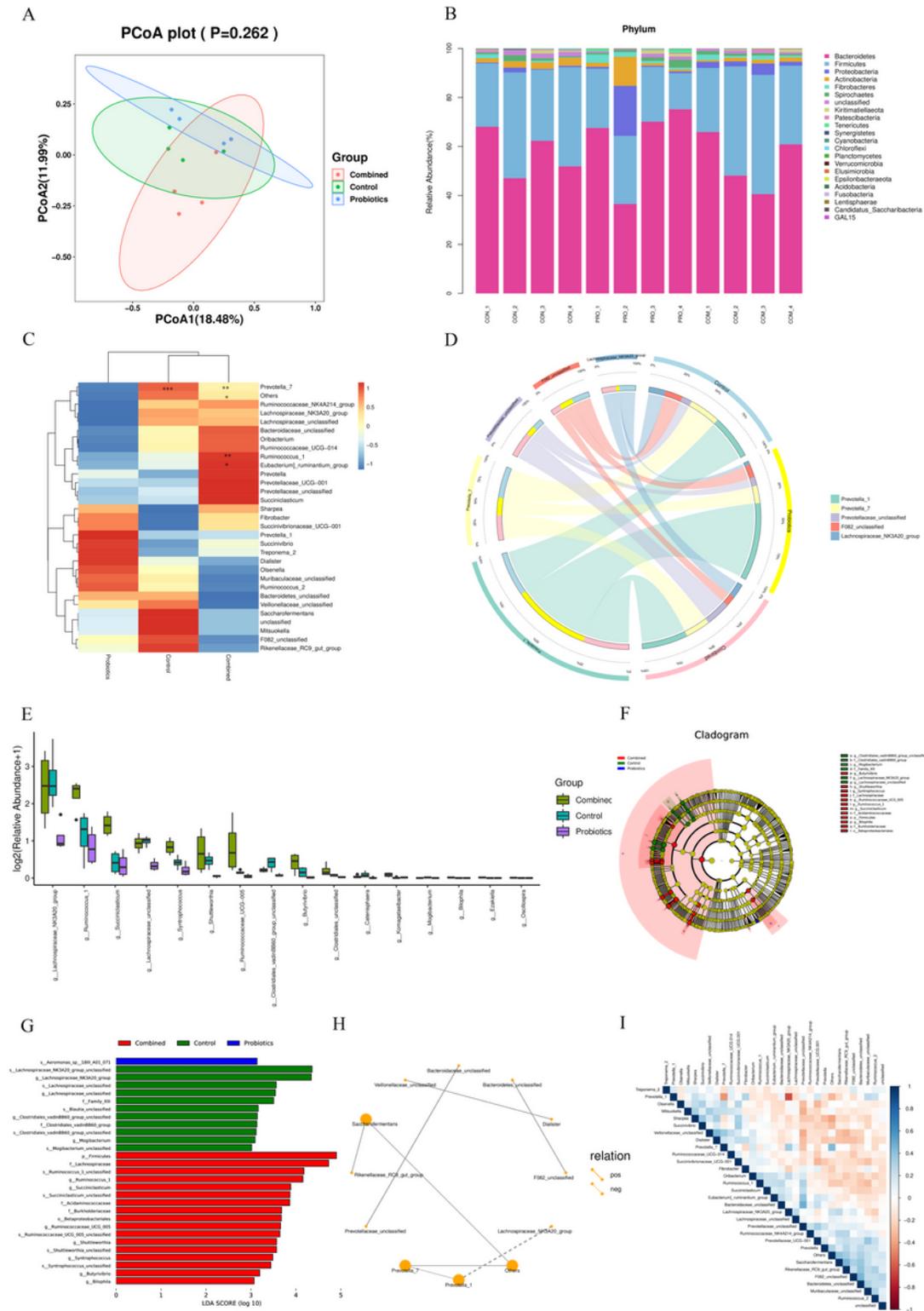


Figure 4

Exhibition of rumen microflora and relationships. A. Principal coordinates analysis based on unweighted unifrac. B. Relative abundance histogram of rumen microorganisms at phylum level. C. Relative abundance heatmap of bacterial genera. The significant differences are shown as *** $P < 0.001$, ** $0.001 < P < 0.01$, and * $0.01 < P < 0.05$. Values included four duplicates. D. Circos of distinguished phylum and the relative abundance of bacteria in the top 5. E. Difference analysis of barplot at significant genera

among three groups. F. LEFSe evolutionary diagram of rumen microflora. G. LDA histogram of remarkable biomarkers. H. Sparcc network of dominant genus. The relationships were shown by correlation coefficients $-0.4 < \rho < 0.4$. Full line illustrates positive correlations, and the dotted line illustrates negative correlations. The node size means the number of other microorganisms associated with bacteria. I. Corrheatmap of dominant genera. Values included four duplicates.

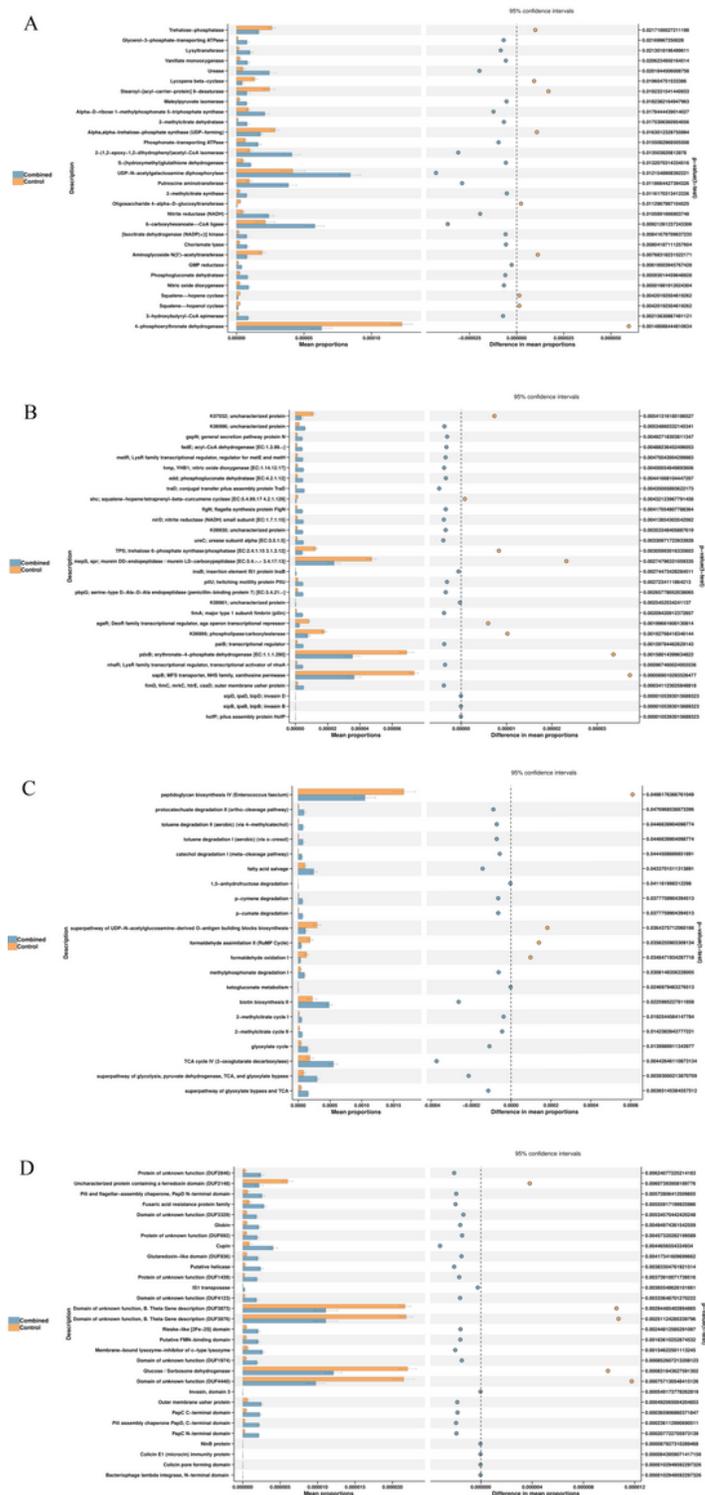


Figure 5

PICRUSt functional predictions. A. EC. B. KO. C. Pathways. D. PFAM. Values included four duplicates.

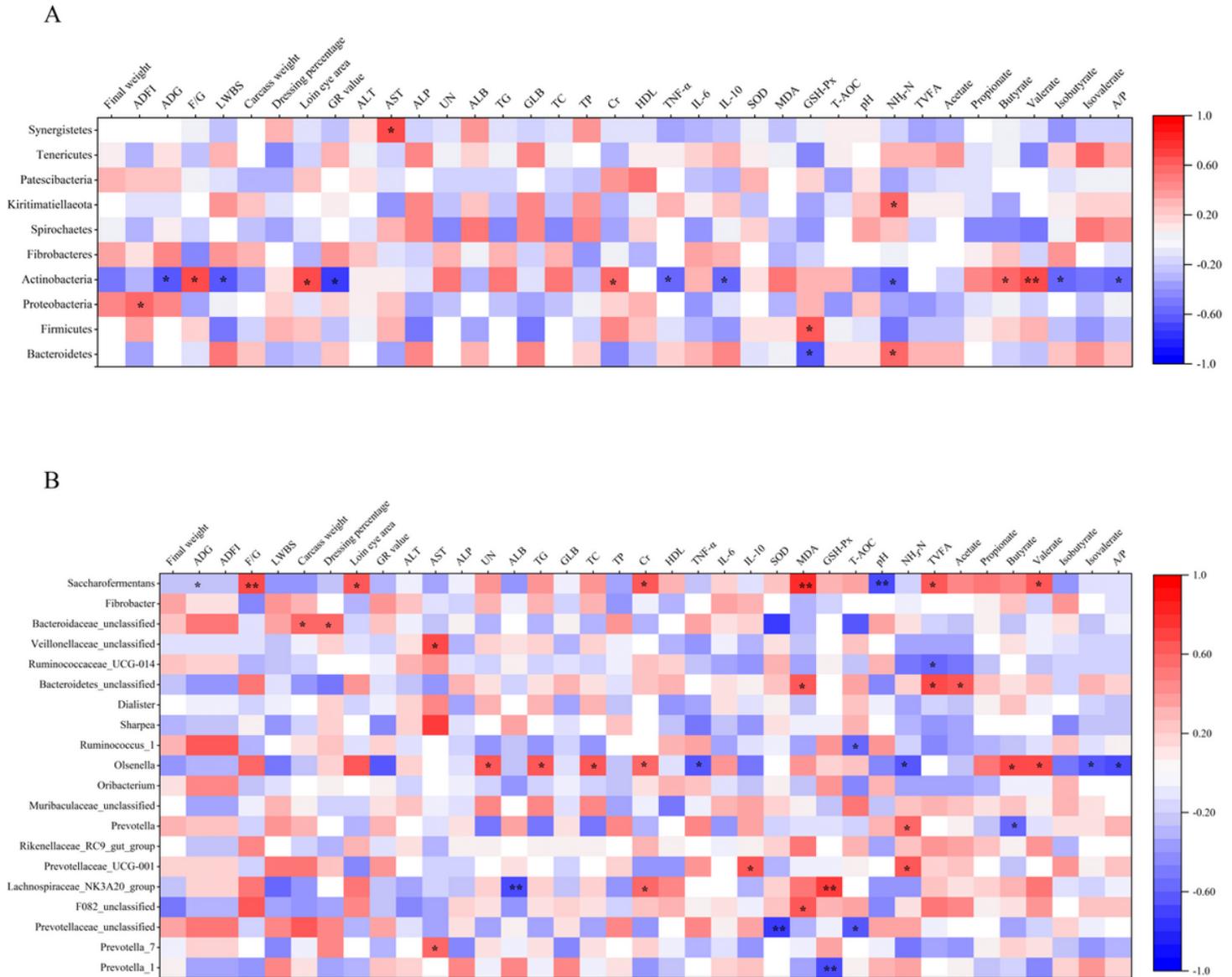


Figure 6

Relationships of goat performance, serum indexes, rumen fermentation parameters and rumen microorganisms. A. At the phylum level. B. At the genus level. Red represents a positive correlation and blue represents a negative correlation. The darker the color, the stronger the correlation. The significance is shown as ** $0.001 < P < 0.01$, and * $0.01 < P < 0.05$. Values included nine duplicates.