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RESEARCH ARTICLE

Microbial Metabolic Shifts and Inflammation-Related Gene Expression Underlie Protective Effects of Slow Digestible Starch on Rumen Epithelium of Finishing Lambs

Ke Tian[1,§], Hua Yuan[1,§], Shixiu Qiu[3,§], Zhenying Xu[3], Aldian Dicky[4], Wenming Huang[1], Gan Luo[2,*] and Yongju Zhao[1,*]

[1]College of Animal Science and Technology, Southwest University, Chongqing, 400715/402460, China; [2]College of Animal and Veterinary Science, Southwest Minzu University, Chengdu, 610000, China; [3]Chengdu Academy of Agriculture and Forestry Sciences, Chengdu 611130, China; [4]Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan. [§]Equal contributions
*Corresponding author: luogan19941115@163.com (GL); zyongju@163.com (YZ)

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ABSTRACT

This study aimed to investigate the effect of dietary slow-digestible starch (RS) on rumen microbial metabolic alterations and barrier function, and to reveal their interrelationships in lambs using native starch (NS) as the control. Twenty Hu lambs (25.1±1.1 kg) were recruited in a 56-day finishing trial and allocated into two groups offered either a corn NS (35% of dietary dry matter) or a corn RS (35% of dietary dry matter) diet. Results showed that dietary RS improved lamb weight gain and feed efficiency ($P<0.05$). Meanwhile, RS increased the rumen minimum pH ($P<0.05$) and decreased lipopolysaccharide and lactate concentrations ($P<0.05$). Dietary RS enhanced bacterial diversity and the abundances of Ruminococcus, while reducing the abundance of Prevotella ($P<0.05$). Metabolomic analysis showed that RS increased the relative concentrations of bile acids and decreased those of purines ($P<0.05$), leading to enriched primary bile acid biosynthesis and purine metabolism ($P<0.05$). Regarding rumen morphology, dietary RS increased rumen papilla length and surface area, and increased the thickness of the stratum corneum ($P<0.05$). Regarding gene expressions, dietary RS upregulated *Claudin-1* and *ZO-1*, but downregulated *TLR4*, *IL-6* and *TNF-α* expressions ($P<0.05$). Overall, dietary RS promoted rumen health by modulating microbial compositions and shifting metabolic alterations toward bile acid and purine metabolisms. Our study provides new insights into the molecular mechanisms of rumen ecosystem–host interactions in finishing lambs and highlights the potential role of RS in improving rumen health.

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INTRODUCTION

Mutton serves as an essential dietary component in most global regions. As the world enters the post-pandemic era, the mutton market is recovering and exhibiting substantial growth (FAO, 2025). However, subacute ruminal acidosis (SARA) induced by finishing diets remains a major limitation to achieving optimal growth performance in lambs. Though research targeting either enhancement of rumen ecosystem balance or improvement of rumen barrier function through dietary supplements has yielded satisfactory results (Dai *et al*., 2017; Wang *et al*., 2021), the resilience of the rumen ecosystem requires the continuous inclusion of supplements to maintain the desired regulatory effects, and those effective but unconventional supplements certainly impose extra financial burdens on producers. Given that cereals are typically included at high proportions in lamb finishing diets (Humer and Zebeli, 2017) and that the rapid fermentation of cereal starch is a major factor contributing to rumen SARA, addressing cereal starch and its rumen utilization pattern represents a promising approach to alleviating rumen SARA and enhancing rumen health during finishing period.

Cereals used in animal agriculture, such as corn and barley, contain a substantial proportion of native starch (NS) that consists of regularly polymerized α-1,4- and α-1,6-glycosidic bonds that are susceptible to hydrolysis by salivary and pancreatic α-amylases. In contrast, slow digestible starch (RS) is the irregular supramolecular form of NS characterized by a dense crystalline structure and resistance to enzymatic hydrolysis (Dobranowski and

Stintzi, 2021). Owing to its restorative effects on the gastrointestinal ecosystem, dietary inclusion of RS has been extensively studied in recent years (Ni *et al*., 2023). In ruminants, dietary RS confers benefits including alleviation of systemic inflammation (Yang *et al*., 2018) and improvement of weight gain (Liu *et al*., 2020) compared to NS by moderating rumen ecosystem fluctuations. As demonstrated by Jin *et al*. (2023), such regulatory effect of dietary RS involves coordinated interactions among microbes, metabolites, and tissue genes expressions. Supporting this notion, studies utilizing dietary RS inclusion have observed increased abundance of Ruminococcus spp. (Metzler-Zebeli *et al*., 2015) and elevated primary bile acid (BA) concentrations (Tian *et al*., 2024b). However, the specific microbial alterations and metabolic pathways through which RS exerts beneficial effects remain unclear. Concurrently, previous work has shown that dietary RS alleviates goat systemic inflammation via suppressing rumen lipopolysaccharides (LPS) concentration (Wang *et al*., 2024a). Given that rumen LPS primarily originates from microbial lysis and that excessive rumen LPS impairs rumen epithelium (Plaizier *et al*., 2018), dietary RS-improved sheep growth was thereby largely attributed to the improved rumen barrier function (Liu *et al*., 2020). The aforementioned observations collectively emphasize the microbe-metabolite-host tissue interplay underlying RS-mediated effects and underscore the need to elucidate the involved mechanisms.

In the present study, we hypothesized that dietary RS would improve rumen health in finishing lambs, with the benefits driven by microbial-metabolic and inflammatory and tight junction gene expression alterations.

MATERIALS AND METHODS

Experimental design and animal management: Twenty healthy Hu lambs of similar age (6 months old) and live weight (25.1±1.1 kg), raised at the Southwest University experimental farm were allocated and balanced for 2 weeks by feeding the same cereal-free diet (Standard of Meat-Type-Sheep Nutritional Requirements of China (NY/T816–2021) prior to the trial. After the balancing period, lambs were randomized into either an NS group (n=10; received a diet containing 35% corn NS on a dry matter (DM) basis as the sole cereal starch, or a RS group (n=10; received a diet containing 35% corn RSS on a DM basis as the solo cereal starch). The experimental lambs were the same cohort of animals as used in our published study (Tian *et al*., 2026), in which the basis for starch inclusion level was also described. The trial lasted 8 weeks, during which all lambs were fed daily at 0700 and 1700, with free access to water and mineral blocks. Dietary formulation was shown in Supplementary Table 1. The NS and RS (No. X&Y-1945) were provided by Quanyin Xiangyu Biotechnology Co., Ltd. (Beijing, China) in powder forms, the NS was straightly obtained from corn, and the RS was produced by repeatedly subjecting NS to a heat-moisture treatment process. To prevent feed selection, dietary concentrates including wheat bran, soybean meal, and starches were mixed and extruded into 1.0×0.5cM pellets and synchronously offered with forage parts at feeding, daily orts were controlled below 5% of dietary DM. During the finishing trial, no obvious abnormal behavior or health compromise was observed in lambs.

Table 1: Dietary effect on growth performance of finishing lambs

Item	NS	RS	SEM	P-value
Initial weight, kg	25.10	25.27	0.249	0.736
Final weight, kg	33.48	35.56	0.397	0.005
DMI, kg	0.99	0.96	0.011	0.182
ADG, kg	0.14	0.17	0.007	0.021
Feed gain ratio	7.35	5.74	0.359	0.021

ADG, average daily gain; DMI, dry matter intake; NS, native starch group; RS, slow digestible starch group; SEM, standard error of the mean.

Sample collection: Body weight was measured on two consecutive days at both the start and the end of the experiment to calculate average daily gain (ADG) and feed: gain ratio. Two days before slaughter, rumen fluid was collected at 0, 3, 6 and 9 h before morning feed for measuring pH, the initial 60mL was discarded to avoid saliva contamination. Three portions of rumen fluid collected at 3 h were snap-frozen using liquid nitrogen, stored under -80°C, for fermentation, microbial and metabolic analyses. Slaughter was conducted on day 56. After 12 hours of fasting, all lambs were slaughtered by professionals using the Islam exsanguination practice at the campus slaughterhouse. The rumen epithelium was immediately collected, scissored, rinsed three times in PBS and cut into portions. One portion was cut into 0.5×0.5×1cM pieces and fixed in 4% paraformaldehyde for the hematoxylin and eosin (H&E) staining. A 1gm portion was immediately frozen at -120°C for subsequent RNA extraction.

Rumen fermentation analysis: The short-chain fatty acid (SCFA) in rumen fluid was measured using same GC with SH-RTX-WAX capillary columns (30 m×0.25μM×0.25mM, Shimadzu, Japan). Aliquot of 1μL for each sample was injected into GC (split ratio=50:1) for analysis. The temperature gradient was set as: initial temperature of 100°C, held for 1 min, a 5°C rise/min to 190°C and held for 15 min. The carrier gases were O_2 (0.4 Mpa), N_2 (0.7 Mpa) and H_2 (0.3 Mpa) and flow rate was 1mL/min. Temperatures of injector and detector were 230°C and 240°C, respectively. Ammonia nitrogen (NH_3-N) was determined by the phenol-hypochlorite method. Lactate was determined using commercial kit (A019-2-1) purchased from Jiancheng Bioengineering Institute Co., Ltd, China. Rumen LPS concentration was determined using assay kit purchased from Xiamen Bioendo Technology Co., Ltd (Xiamen, China), with the reference endotoxin of *E. coli* O111:B4.

Morphological analysis: For H&E staining, the epithelium tissue was sectioned thickness, followed by dewaxing, dehydrating, and rinsing. Subsequently, the tissue was stained with hematoxylin, differentiated, and returned to blue. Next, the tissue was decolorized and stained with eosin. Finally, the tissue was again dehydrated and rinsed with xylene. The pretreated tissue was observed using a microscope (B80i, Nikon, Tokyo, Japan). Measurement of papilla and stratum morphology were performed using Image-Pro Plus software (Ver. 6.0, Rockville, MD, USA).

Real-time quantitative PCR analysis: Epithelium RNA was extracted using a commercial kit (EZB-RN4; EZBioscience, China). The extracted RNA concentration

was measured using a NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific, USA). After reverse transcription with 4× ABScript miRNA First-Strand Synthesis Kit (ABclonal Technology, China), qRT-PCR was performed on a QuantStudio 6 RealTime PCR System (Thermo Fisher, USA) using 2× universal SYBR Green (Abclonal Technology, China), the program was 95°C for 10 min followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). The mRNA expression of the target genes relative to the reference gene (β-actin) were computed by $2^{-\Delta\Delta Ct}$ method.

Microbial analysis: Microbial DNA was extracted from the rumen fluid using a DNA isolation kit (Omega Bio-tek, GA, USA). The extracted DNA was purified and quantified on the QuantiFluor™ ST blue fluorescence quantitation system (Promega, USA) and 1% agarose gel electrophoresis, respectively. The universal primers (F: 5'-CCTAYGGGGRBGCASCAG-3'; R: 5'-GGACTACNNGGGGTATCTAAT-3') were used to amplify the V3-V4 regions of the bacterial 16S rRNA gene. After the amplification, the Illumina MiSeq platform was applied to construct paired-end sequencing libraries (2 × 300 bp).

Metabolic analysis: Metabolic analysis of rumen fluid was performed using a Vanquish ultrahigh-performance liquid chromatography-tandem system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (MS; Thermo Fisher, Germany) using our previously published protocol (Aldian *et al*., 2023). A 1-mL aliquot of each sample, quality control (QC; for data normalization), and blank (ultrapure water) was mixed with 20μL of myricetin (No. M6760, Merck KgaA, Shanghai, China; 1 mg dissolved in 5mL of methanol) as the internal standard, vortexed for 3 min. Subsequently, 1980μL of liquid chromatography tandem mass spectrometer (LC-MS) grade methanol was added, vortexed again for 15 min and centrifuged at 16,000×g at 4°C for 10 min. Finally, 1mL of the supernatant was collected and transferred into an LC-MS sampling vial. The MS was set as: capillary voltage of 2.5 kV, dissociation voltage of 30 V, desolvation temperature of 500°C and source temperature of 150°C. The QC sample was detected after every four rumen fluid samples for monitoring purposes.

Statistical analysis: Each lamb was considered the experimental unit for all analyses. Data on growth performance, rumen pH and LPS, and epithelial gene expressions were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. SPSS software (v20.0, IBM, Armonk, NY, United States) was used for all statistical analysis. Results were presented as means ± standard errors of the mean (SEM). Statistical significance was set at $P<0.05$.

Microbial raw data were quality filtered with fastp and merged with FLASH (Ver. 1.2.11). Then the high-quality sequences were de-noised using DADA2 plugin in the Qiime2 (Ver. 2020.2) pipeline with recommended parameters, which obtains single nucleotide resolution based on error profiles within samples. DADA2 denoised sequences are usually called amplicon sequence variants (ASVs). Then ASVs assigned to spike-in sequences were filtered out and reads were counted. A standard curve (based on read counts versus spike-in DNA copy number) for each sample was generated, the quantitative absolute abundance of each ASV in a sample was determined. Taxonomic assignment of ASVs was performed using the Naïve bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S rRNA database (Ver. 138).

Metabolic raw data were converted via the Reifycs Analysis Base File Converter (Reifycs Inc. Tokyo, Japan) and processed with MS-DIAL (ver. 4.9; systemsomicslab.github.io/compms/msdial/main.html). The MS alignment result from the MS-DIAL was submitted to MetaboAnalyst 5.0 (www.metaboanalyst.ca) and then normalized using internal standard and log transformation. Normalized data were calculated for supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) and performed permutation test of OPLS-DA results. Subsequently, student's *t* test and log_2-fold change (log_2FC) were calculated, metabolites with first principal component of variable importance in the projection values > 1 and P values < 0.05 were recognized as differential metabolites (DMs) between groups. Volcano plot program in the MetaboAnalyst was generated for overview of DMs between the NS and RS groups. Finally, DMs were analyzed using Pathway Analysis embedded in the MetaboAnalyst, and validation of the affected pathways was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For microbial and metabolomic analyses, P values derived from multiple comparisons were adjusted using the Benjamini-Hochberg false discovery rate (FDR) method. In addition, given the sufficient biological replication (n=10 per group) and the performance of QC sample throughout the analyses, technical replicates were not included for the 16S rRNA gene sequencing or metabolomics analyses. To investigate the relationships between rumen morphology with microbes and DMs, spearman's correlation analysis was performed using R software.

RESULTS

Lamb growth performance and rumen fermentation: Changes in lamb growth performance are displayed in Table 1. Dietary RS inclusion enhanced the final weight and ADG, decreased feed gain ratio ($P<0.05$). Dietary effects on rumen fermentation indices are shown in Table 2. Lambs in RS group showed a higher minimum rumen pH than those in NS group ($P<0.01$). For SCFAs, dietary RS increased propionate concentrations and reduced acetate concentrations ($P<0.01$) but exerted no effect on total SCFA or butyrate ($P>0.05$). For rumen inflammatory stimuli, dietary RS decreased LPS and lactate concentrations ($P<0.05$).

Rumen morphology and barrier function: For papilla morphology, dietary RS enhanced papilla length and surface area ($P<0.05$; Fig. 1). Regarding epithelium morphology, dietary RS enhanced stratum corneum thickness ($P<0.05$; Fig. 2). However, dietary RS exerted limited impact on the stratum granulosum, spinosum, or epithelium thickness ($P>0.05$). For inflammatory responses, dietary RS decreased *TLR4*, *IL-6*, and *TNF-α* expressions ($P<0.05$) but showed no effect on *IL-1β*

(P>0.05). With respect to tight junctions, dietary RS increased *Claudin-1* and *ZO-1* (P<0.05) but exerted no effect on *Claudin-4* and *Occludin* (P>0.05).

Table 2: Dietary effect on rumen fermentation indices of finishing lambs

Item	NS	RS	SEM	*P*-value
pH variations				
Mean pH	6.31	6.35	0.043	0.616
Minimum pH	5.65	5.97	0.073	0.023
Maximum pH	6.77	6.86	0.026	0.078
SCFAs				
Total SCFA, mmol/L	92.11	89.19	1.142	0.195
Acetate, mmol/L	62.24	55.28	1.233	0.002
Propionate, mmol/L	18.16	22.03	0.561	< 0.001
Butyrate, mmol/L	11.71	11.77	0.148	0.811
Inflammatory stimuli				
LPS, $\times 10^3$ EU/mL	15.28	10.52	1.115	0.029
NH_3-N, mg/dL	17.24	18.00	0.311	0.227
Lactate, mmol/L	1.68	1.54	0.034	0.025

LPS, lipopolysaccharides. NH_3-N, ammonia nitrogen. SCFA, short-chain fatty acid. NS, native starch group; RS, slow digestible starch group; SEM, standard error of the mean.

Changes in rumen microbial compositions: The α diversity analysis showed that RS group had higher Chao and Simpson than NS group (P<0.05; Fig. 3A) and the β diversity analysis showed that clear separation between NS and RS groups was observed in the PCA plot, of which PC1 explains 26.18% of variance and PC2 explains 9.93% of variance (Fig. 3B). For community compositions, at the phylum level, ten bacterial phyla with relative abundances higher than 0.5% were identified in at least one group (Fig. 4A), in which the Bacillota and Bacteroidota were the most abundant phyla, accounted for 50.22 and 24.75% of total bacteria phylum, respectively. At the genus level, the relative abundance of top 20 bacterial genera is displayed in Fig. 4B, which accounted for 70.06% of the total bacteria abundance. Among them, *Xylanibacter* and *Olsenella* were the most abundant genera that occupy 9.00 and 9.05 %, respectively of total bacteria genus. The relative abundance of Ruminococcus, unclassified_Clostridia, Rikenellaceae_ *RC9*_group, Christensenellaceae_*R7*_ group, and *F082* were higher, while Syntrophococcus was lower in RS group (P<0.05). Linear discriminant analysis (LDA; Fig. 4C) score showed that Ruminococcus and Prevotella respectively represent the most important variants for microbial improvement and reduction in RS group.

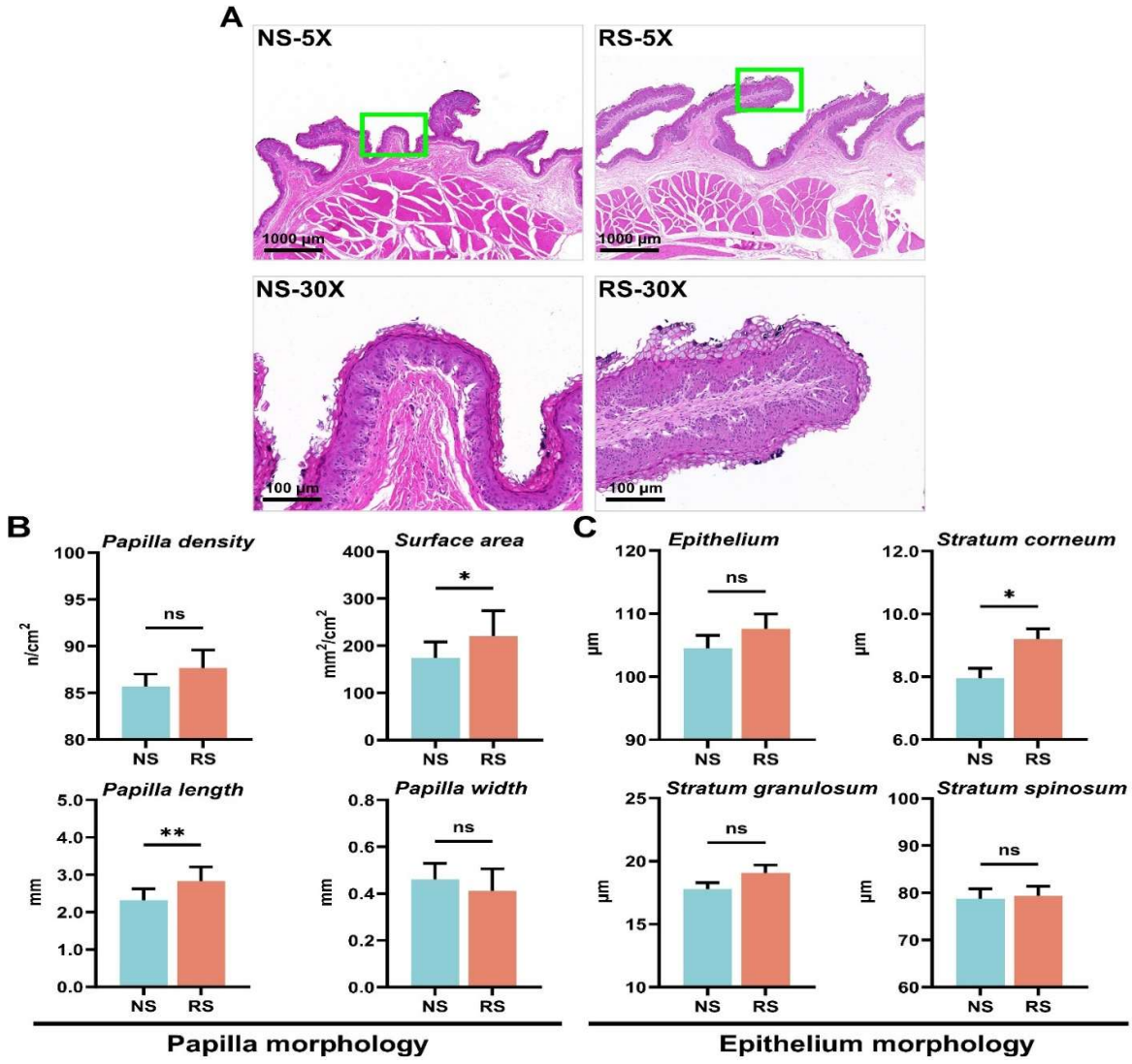

Fig. 1: Dietary effect on rumen epithelium morphology. (A) hematoxylin and eosin staining. (B) papilla morphology. (C) epithelium morphology. NS, native starch group; RS, slow digestible starch group.

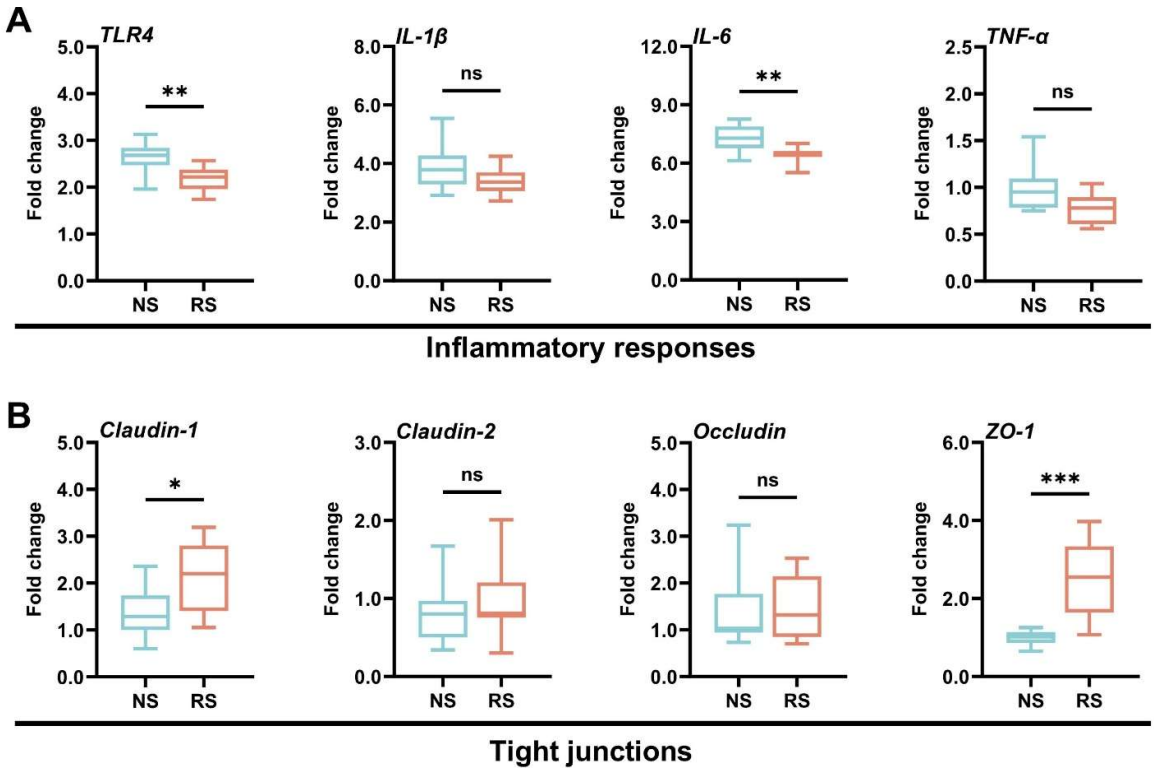

Fig. 2: Rumen epithelial gene expression in lambs fed different diets. (A) Gene expressions related to inflammation. (B) Gene expressions related to barrier function; *IL*, interleukin; *TLR*, toll-like receptor; *TNF*, tumor necrosis factor; *ZO-1*, zonula occludens-1.

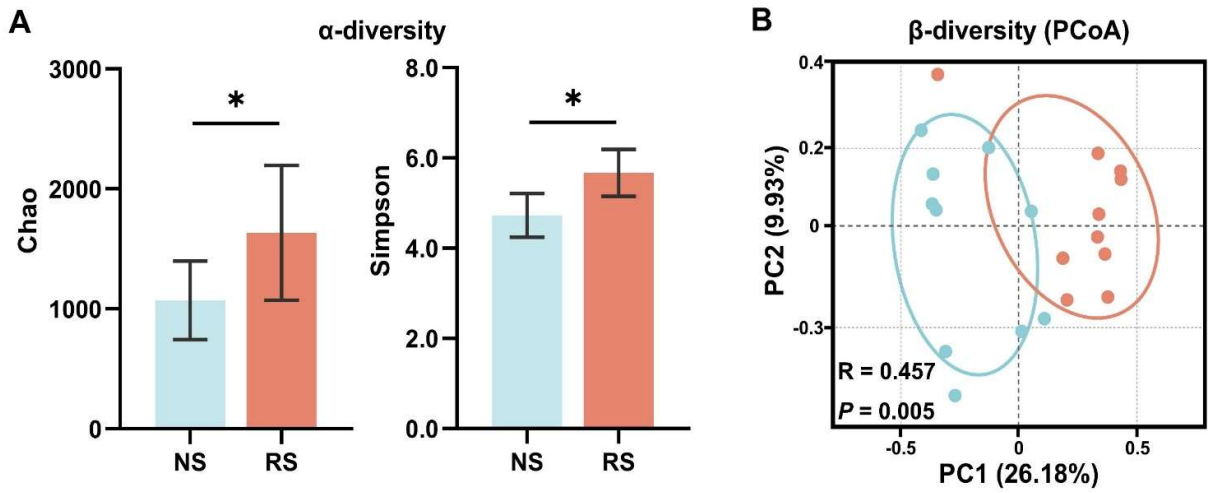

Fig. 3: Dietary effect on rumen bacterial diversities. (A) α diversities expressing as Chao (left) and Simpson (right) indices. (B) β diversities expressing as principal coordinates analysis score plots. NS, native starch group; RS, slow digestible starch group.

Variations in rumen metabolites: A total of 2498 MS peaks were identified in the ruminal fluid. After quality filtering against the database embedded in the MS-DIAL, 180 metabolites were identified, and 50 DMs were distinct between the two groups. As shown in the volcano plot (Fig. 5A). The OPLS-DA score plots of all samples were inside the 95% Hotelling T-squared ellipse (Fig. 5B), and there was distinct separation between NS and RS groups in the OPLS-DA plots, the R^2Y intercepts of permutation tests were 0.986 and Q^2 was 0.84, representing acceptable interpretability and predictability of the analytical model. The log2FC of DMs were depicted in Fig. 5C, the DMs based on RS group vs. NS group contained various components including purine derivatives, such as xanthosine (log_2FC=3.64) and cyclic GMP (log_2FC=-1.44); bile acid (BA), such as isolithocholic acid (log_2FC=7.95) and ursodeoxycholate (log_2FC=3.47); and amino acids such as tyrosine (log_2FC=1.28) and methionine (log_2FC=1.15). KEGG database mapping and pathway enrichment analysis showed that several metabolic pathways were significantly enriched (Fig. 5D, E), and the primary terms within the enriched pathway include purine metabolism (impact=0.09; -log10; P=4.15), primary bile acid biosynthesis (impact=0.16; -log10; P=2.97) and TCA cycle (impact=0.43; -log10; P=1.32).

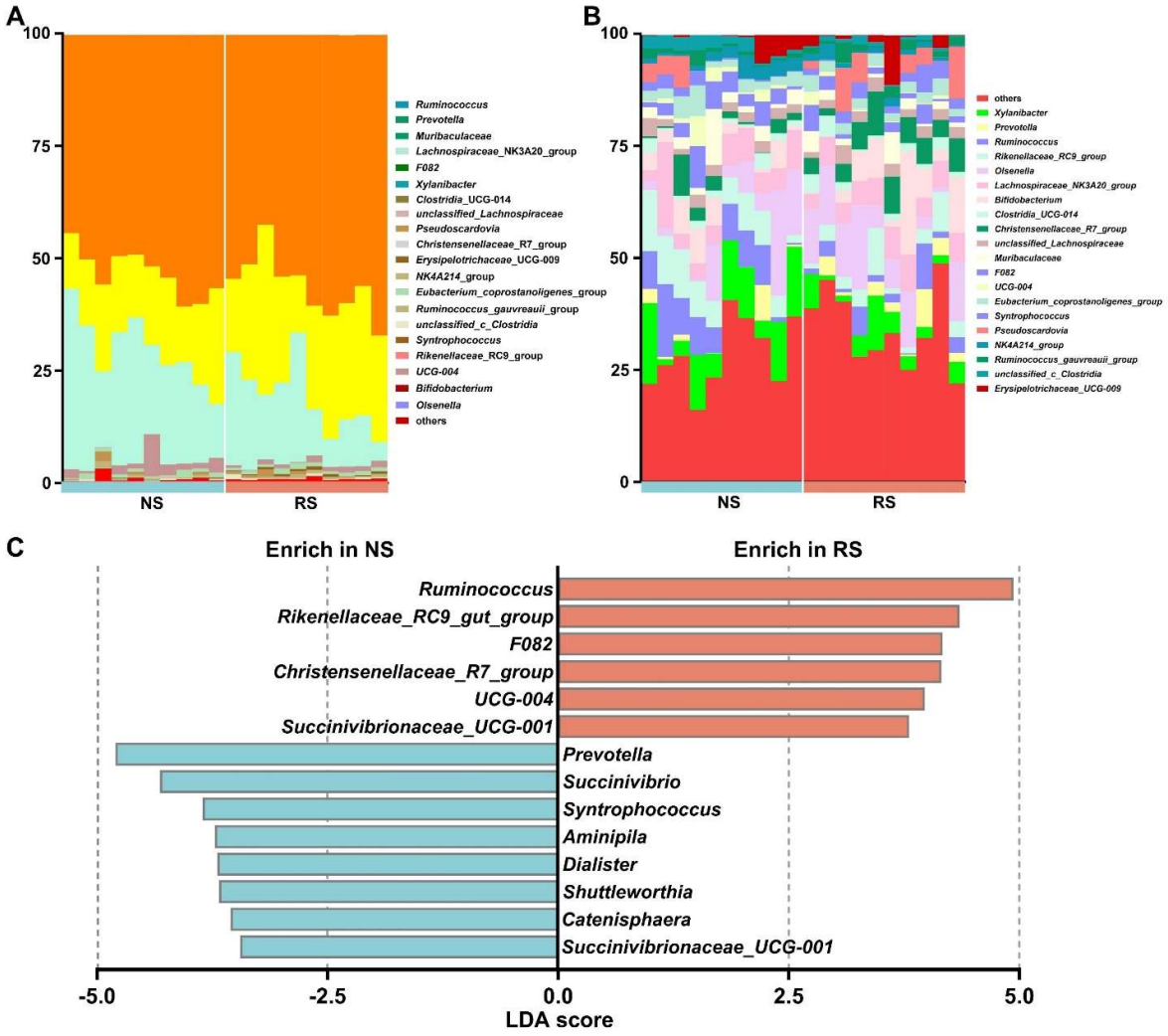

Fig. 4: Rumen bacterial variations in response to dietary effects. (A) Relative abundance of bacteria at phylum level. (B) Relative abundance of bacteria at genus level. (C) Linear discriminant analysis (LDA) of differential microbes. NS, native starch group; RS, slow digestible starch group.

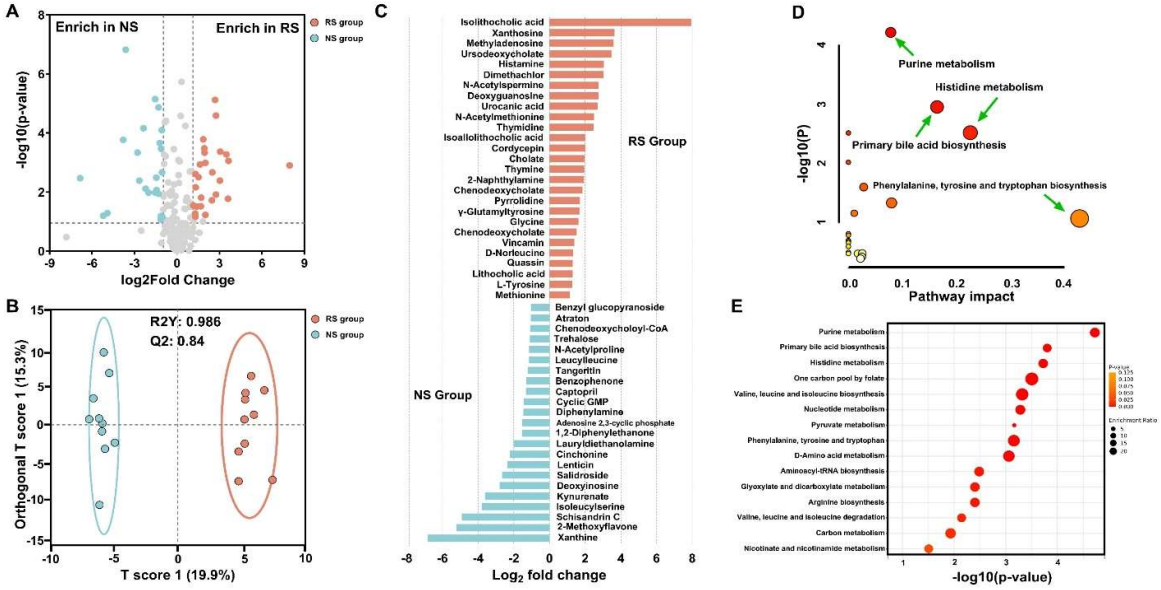

Fig. 5: Rumen metabolic alterations of lamb fed different diets. (A) Volcano plot of identified metabolites. (B) Orthogonal partial least-squares discriminant analysis (OPLS-DA) of differential metabolites. (C) Log2 fold changes of differential metabolites. (D) Pathway enrichment analysis based on differential metabolites. (E) Pathways analysis against KEGG database. NS, native starch group; RS, slow digestible starch group.

Correlations between rumen microbes, metabolites, and morphology: Strong associations were observed among microbes, metabolites, and morphological indices (Fig. 6). Negative correlations were detected between rumen morphology and microbes (P<0.05; Right). Although a strong correlation was noticed between Ruminococcus and Prevotellaceae_UCG-003 (Spearman's r coefficient>0.05), the association of Ruminococcus with rumen morphology was limited (P>0.05). Concerning DMs-morphology interactions, rumen papilla length and stratum corneum were correlated with DMs (P<0.05; Left), but not for papilla surface area (P>0.05). Lithocholic acid was positively correlated with the papilla length (P<0.05). Among the purines, xanthosine and xanthine exhibited negative correlation, while methyl adenosine showed a positive correlation with morphological indices (P<0.05).

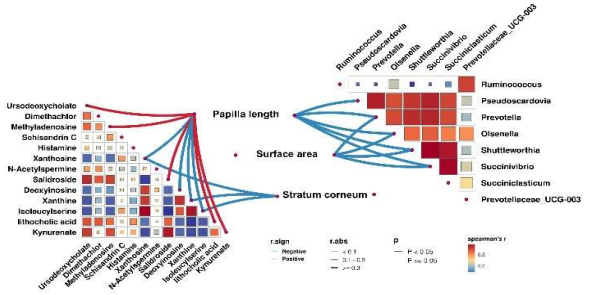

Fig. 6: Spearman correlation between microbes and metabolites with rumen morphological indices. Significant correlations were linked using solid lines, colored lines/squares represent positive and negative correlation, respectively.

DISCUSSION

As reported by Tian *et al*. (2024a), the health-promoting effects of dietary RS in ruminants are multifactorial, that is, on the one hand, the slower rumen hydrolysis rate of RS facilitates a sustained propionate production, which was confirmed by both the RS-induced increase in propionate level observed by Metzler-Zebeli *et al*. (2015) and the elevated propionate level in our study. On the other hand, by preventing drastic rumen pH depression to reduce the risk of metabolic disorders like SARA, thereby redirecting nutrients toward growth rather than disease compensation (Plaizier *et al*., 2018). The present study observed reduced acetate and elevated propionate levels in RS group. The increased propionate level can be construed as sustained rumen fermentation of RS (Tian *et al*., 2026). Due to the lower *pKa* value, lactate (*pKa* 3.9) contributes more to rumen pH depression than SCFA (*pKa* 4.8). If rumen pH drops below 5.6, glucose in the rumen is largely transformed into lactate via lactate dehydrogenase and pyruvate hydrogenase (Russell and Hino, 1985). Fortunately, dietary RS prevented the drastic pH depression through enhancing rumen minimum pH in RS group, thereby helping to avoid rumen acidity and injury of epithelium.

The genus Prevotella, which specializes in carbohydrate degradation and typically dominates under high-starch finishing periods (Liu *et al*., 2020), are often negatively correlated with epithelium integrity (Plaizier *et al*., 2018). Although lower abundance of Prevotella collaborated with the enhanced rumen barrier function in the RS group, it also implied a reduced availability of non-structural carbohydrates or constrained hydrolytic capacity in the rumen (Liu *et al*., 2020). Interestingly, the increased Ruminococcus abundance observed in RS group may compensate for such deficiencies. Multiple studies, including our prior work, reported RS-mediated increases in gastrointestinal Ruminococcus (Ze *et al*., 2012; Jin *et al*., 2023; Tian *et al*., 2024a) and the concurrent rise of phylum Bacillota is consistent with a potential involvement of Ruminococcus in RS degradation. Generally, Ruminococcus spp. functions as primary cellulose and hemicellulose degraders and produces acetate as a major fermentation product in ruminants consuming high forage diet (Plaizier *et al*., 2018). Therefore, the elevated propionate level is unlikely to straightly originate from Ruminococcus, as this genus cannot efficiently hydrolyze the α-1,6 linkages in RS (Tian *et al*., 2024a), which may also explain the limited correlation observed between Ruminococcus and rumen morphology in our results. Instead, we assume that Ruminococcus participates as primary degraders (Cerqueira *et al*., 2020), breaking down the complex and irregular structure of RS to facilitate subsequent propionate synthesis by other microbes and such process also reduced the abundance or replaced the role of Prevotella in starch degradation.

Previous studies had observed a simultaneous rise in gastrointestinal Ruminococcus with BA following RS ingestion (Wang *et al*., 2024b). Bile acids are important mediators in body inflammatory responses (Zhao *et al*., 2023) and are intensively synthesized in the rumen (Liang *et al*., 2023). The increases in lithocholic acid and isolithocholic acid in the RS group align with previous *in-vitro* and *in-vivo* experiments (Tian *et al*., 2024b; Wang *et al*., 2024b) who used dietary RS inclusion. The increased lithocholic acid concentration was positively correlated with the abundance of Ruminococcus, which is consistent with previous reports suggesting that members of this genus possess bile acid-transforming capabilities (Jia *et al*., 2018), although the relationship between these variables requires further validation. More importantly, the elevated BAs may contribute to improved rumen barrier function via BA receptor in animal tissues capable of downregulating *TLR4* expression and triggering anti-inflammatory signaling cascade (Zhao *et al*., 2023). On the other hand, although associated with protein utilization, purines were negatively correlated with rumen barrier function. Previous studies indicated that purines participate in inflammatory responses via exerting cytotoxic effect on cell cycle (Linden *et al*., 2019), and research in yak observed that SARA-induced mitochondrial dysfunction in epithelium cells was closely correlated with purine metabolism (Wang *et al*., 2024b). This suggests that the reduction in purines abundance of the RS group may also contribute to the enhanced rumen barrier function. Therefore, the elevated BA concentrations and reduced purine were associated with improved rumen barrier function.

The rapid fermentation of dietary NS and its companioned microbial-metabolic alterations are known to injure rumen epithelium integrity and compromise barrier function, thereby provoking systemic inflammation, and in turn attenuate epithelium thickness and decrease tight junction expressions (Świerk *et al*., 2023; Liu *et al*., 2025). The increased papilla length and surface area, as well as greater stratum corneum thickness in RS group not only

indicate improved rumen barrier function but align with the enhanced lamb growth performance, since greater papilla surface area can promote nutrient assimilation (Wang *et al*., 2024a). As tight junctions have been documented to be negatively correlated with systemic inflammation (Lai *et al*., 2022), improvements in rumen barrier function can thus be attributed to the suppressed expression of rumen *TLR4* and *IL-6* observed in the present study, which was partly consistent with the result that dietary RS increased rumen LPS concentration. Surprisingly, correlations between metabolites and microbes with papilla surface area were limited. As suggested by Plaizier *et al*. (2018), a key reason may be that the observed rumen minimum pH in both NS and RS groups did not persist long enough to induce severe epithelial injury. Other possible explanations could be attributed to the relatively short trial duration (8 weeks) and the similar dietary compositions between experimental groups (Jiao *et al*., 2015). Nevertheless, the altered bile acid profile may also reflect shifts in rumen fermentation patterns rather than direct microbial regulation alone.

Conclusions: Dietary RS inclusion shifted the rumen microbes toward a more balanced ecosystem and was associated with alterations in bile acid and purine metabolism. These changes simultaneously occurred with improved tight junction and reduced inflammatory gene expressions, which may collectively contribute to enhanced rumen barrier function in finishing lambs. The present findings provide supportive evidence for the potential application of RS in intensive ruminant production systems. However, given the relatively small sample size and short experimental duration, further long-term studies with larger cohorts and multi-omics validation are required to confirm the underlying mechanisms.

Ethics approval and consent to participate: Protocols of the present study were approved by the Southwest University Committee for Animal Care and Use (approval ID: LAC2025-2-0109). The study was carried out in line with the Guidelines for Ethical Review of Animal Welfare (Ministry of Science and Technology of China, 2018) and the Guidelines for Animal Research and Welfare of Southwest University (2024-209).

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