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Article

Effects of Yeast Cell Walls on Rumen Fermentation, Microbial Composition, and Metabolite Profiles in Early-Weaned Simmental Calves

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Simple Summary

Early weaning can improve cow reproductive efficiency, but it may challenge calves because the rumen is still developing and must adapt quickly from milk to solid feed. We tested whether adding a yeast cell wall product to a calf starter diet could support rumen function during this transition. Twenty-four newborn Simmental heifer calves were fed the same starter diet, with half receiving 5 grams of yeast cell wall per calf each day. At weaning age, we collected rumen fluid to measure fermentation products, identify key rumen microbes, and profile small metabolites. Calves given the yeast cell wall produced more butyric acid, a fermentation product that helps rumen tissue grow and mature. They also showed a higher level of a fiber-digesting bacterium called *Ruminococcus* and clear shifts in metabolites, especially compounds involved in nucleotide metabolism. Links between *Ruminococcus*, butyric acid, and several increased metabolites suggest that the supplement promoted a coordinated change in rumen microbes and metabolism. Overall, yeast cell wall supplementation may be a practical nutritional strategy to support rumen development and health in early-weaned beef calves.

Abstract

This study aimed to evaluate how dietary yeast cell wall (YCW) supplementation in the starter feed affects ruminal fermentation parameters, microbial community composition, and metabolite profiles in early-weaned Simmental calves. Twenty-four newborn Simmental heifer calves (initial body weight: 37.53 ± 2.50 kg) were randomly assigned based on birth date sequence into the experimental group and the control group (12 calves per group). Calves in the experimental group (YCW) received a daily supplement of 5 g/head/day of yeast cell wall in the starter diet, whereas those in the control group (CON) received no supplementation. The experimental period lasted for 100 days, with weaning conducted at 70 days of age. On day 70, rumen fluid samples were randomly collected from six calves per group for analysis of rumen fermentation parameters, microbial community composition, and metabolomic profiles. (1) YCW supplementation significantly increased ruminal butyrate concentration and the relative abundance of the genus *Ruminococcus* ($p < 0.05$); (2) Metabolomic analysis identified 43 differential metabolites (20 upregulated and 23 downregulated), with nucleotide metabolism-related compounds such as guanylic acid and deoxycytidine monophosphate being prominently enriched ($p < 0.05$); (3) Spearman correlation analysis further revealed positive associations between *Ruminococcus* and both butyrate levels and selected upregulated metabolites, including guanylic acid ($p < 0.05$). Dietary yeast cell wall supplementation enhanced ruminal fermentation in early-weaned Simmental calves by increasing butyrate concentration and altering the ruminal microbiota and metabolome. Enrichment of *Ruminococcus* and nucleotide-associated metabolites, with positive correlations to butyrate, indicates a coordinated shift

in the microbiota–metabolite axis. These findings support YCW as an effective nutritional strategy to promote rumen development and health during the early weaning period.

Keywords: yeast cell wall; simmental calves; rumen fermentation parameters; rumen microorganisms; rumen metabolites

1. Introduction

In China, the beef cattle industry predominantly employs the cow-calf production system. Calves are typically weaned between 5 and 6 months of age, resulting in an extended lactation period for dams, which in turn delays breeding and conception timelines. This delay contributes to reduced reproductive efficiency[1]. To balance maternal production performance with calf growth requirements, strategically advancing weaning age and shortening lactation duration within the cow-calf management framework has emerged as a key intervention to improve cow productivity and reproductive performance while maintaining calf health and developmental quality.

Early weaning represents a critical management strategy in modern intensive beef cattle production, serving to alleviate the lactation burden on dams, reduce feed expenses, and facilitate the transition of calves from liquid to solid feed [2]. The transition period from birth to weaning for calves is a critical stage for the rumen to shift from a “single stomach” (relying on milk for digestion) to a “ruminant digestion” system: the rumen volume of newborn calves is only about 1.1 L, and the nipple height is less than 1 mm [3]. Through the stimulation of solid feed, the epithelium proliferates, the nipples elongate, and the microbial community establishes, ultimately forming an efficient fermentation system [4]. However, the weaning process involves a drastic change in the dietary structure, shifting from easily digestible milk to a solid diet mainly consisting of fibrous carbohydrates and plant proteins. This transition often leads to imbalance in the rumen microbial community, disrupted fermentation patterns, and specific manifestations such as a decrease in fiber-decomposing bacteria, an increase in lactate-producing bacteria, intensified rumen pH fluctuations, and a reduction in volatile fatty acid (VFA) production, especially a decrease in butyric acid, which is crucial for rumen development [5]. This, in turn, triggers mucosal oxidative stress, inflammatory responses, diarrhea, and growth retardation, causing various health problems. Therefore, there is a need to identify a feed additive capable of mitigating the challenges associated with early weaning, including microbial dysbiosis and reduced fermentation efficiency.

The yeast cell wall (YCW), a natural feed additive derived from *Saccharomyces cerevisiae*, is primarily composed of bioactive components such as β -glucan, mannan oligosaccharides (MOS), and chitin. These constituents not only exhibit immunomodulatory effects and support intestinal barrier integrity [6], but also directly modulate rumen fermentation by optimizing volatile fatty acid (VFA) profiles, thereby improving energy utilization efficiency [7]. Research has demonstrated that dietary supplementation with 0.4% YCW in weaned calves significantly increases total ruminal VFA concentration and the proportion of butyric acid, while stabilizing ruminal pH [8]. As the primary energy substrate for ruminal epithelial cells, elevated butyrate levels promote cellular proliferation and differentiation and upregulate the expression of tight junction proteins, thus enhancing gut barrier function [9]. Additionally, supplementation at a dose of 5 g/head/day has been shown to reduce ammonia nitrogen ($\text{NH}_3\text{-N}$) concentration, increase microbial protein synthesis, and improve the ruminal fermentation microenvironment [10]. Furthermore, β -glucan and MOS serve as fermentable substrates that provide supplementary energy for fibrolytic bacteria, potentially enhancing host energy metabolism pathways [11]. Although the effects of YCW on rumen function in adult ruminants have been relatively well documented, systematic investigations into its specific impacts on rumen microbial community structure and metabolic profiles in early-weaned calves remain limited.

Therefore, this study integrated 16S rRNA gene sequencing with LC–MS/MS-based metabolomics to systematically evaluate the impact of dietary YCW supplementation on ruminal

fermentation, microbial community structure, and metabolic profiles in early-weaned Simmental calves. We hypothesized that YCW would enhance butyrate production, enrich fiber-degrading bacteria, and promote a beneficial microbiota–metabolite axis during the early weaning period.

2. Materials and Methods

2.1. Animal Subjects and Experimental Design

All experimental procedures were conducted in accordance with the 4th Edition of the Guide for the Care and Use of Agricultural Animals in Research and Teaching. The protocol was reviewed and approved by the Animal Ethics Committee of Gansu Agricultural University (Approval No.: GSAU-ETH-AST-2023-036). The experiment was conducted from January to May 2024 at Shengze Beef Cattle Farm in Hezheng County, Linxia City, Gansu Province, China. A total of 24 healthy newborn Simmental heifer calves (initial body weight: 37.53 ± 2.50 kg), all offspring of the farm’s first-parity cows, were selected and randomly assigned to either the experimental group or the control group (n = 12 per group) based on birth date using an alternating allocation method. Calves were housed in four pens, with three animals per pen (one pen per treatment group).

The experimental group (YCW) received a daily supplement of 5 g/head of yeast cell wall (YCW) in the starter diet [12], whereas the control group (CON) received no supplementation (Table 1). The feeding protocol is outlined as follows: from days 0 to 28, calves were reared under a mother-calf free-suckling system, with starter feed offered daily at fixed times and locations to encourage intake (Table 2). Starting at one month of age, calves were transitioned to restricted suckling via physical separation from their dams. A stepwise weaning protocol was implemented throughout the trial, progressively reducing both the frequency and duration of nursing bouts, culminating in complete weaning by day 70. Throughout the 100-day experimental period, all calves had ad libitum access to fresh water. The YCW product (Lesaffre, France) was derived from *Saccharomyces cerevisiae* and contained ≥20.0% mannan oligosaccharides, ≥20.0% β-glucan, ≤25.0% crude protein, and ≤6.0% moisture. On day 70, six calves were randomly selected from each group, and rumen fluid was collected via ruminal fistula for the analysis of rumen fermentation parameters, rumen microbial composition, and metabolomic profiles.

On day 1 postpartum, each calf received an intramuscular injection of vitamin ADE (3 mL/head; Zhongmu Veterinary Pharmaceutical Co., Ltd.). Vaccination against foot-and-mouth disease (serotypes O and A) was performed using a bivalent inactivated vaccine on days 7, 21, and 35 (2 mL/head; Lanzhou Institute of Veterinary Research). Deworming was carried out via oral administration of ivermectin on days 14, 28, and 42 (0.2 mg/kg body weight; Puleke Animal Health, Ivermectin Microemulsion Oral Solution). Daily monitoring included observation of mental status, fecal consistency, and feed intake. Calves exhibiting diarrhea were immediately isolated and treated according to established protocols with oral rehydration solution (composition: 40 g glucose + 3.5 g NaCl + 1.5 g KCl + 2.5 g NaHCO₃ dissolved in 2 L warm water) and enrofloxacin oral solution.

Table 1. Calf feeding program.

Day	Lactation Arrangement	Starter Feed Offered (g)	Starter Feeding Schedule
0-7		10~20	---
8-14	Unrestricted suckling	80~100	1-2/d
15-21		80~100	(for feeding stimulation)
22-28		80~100	2 × 2-h sessions daily
29-36		80~100	3 × 2-h sessions daily
37-43	4 × 1-h sessions daily	150~200	
44-50	3 × 1-h sessions daily	200~500	Ad libitum access
	3 × 0.5-h sessions daily	500~800	

51-70		
(weaning phase)	1 × 0.5-h sessions daily	1000~1400

Table 2. Composition and nutrient levels of concentrates (Air-Dried Basis)%.

Ingredients	Content	Nutrient level	Content
Corn	40.54	DM	87.95
Soybean meal	32.00	CP	22.17
Wheat bran	5.80	EE	5.79
Cottonseed meal	5.30	Ash	5.91
Puffed soybeans	5.00	NDF	12.23
Whey powder	4.00	ADF	6.18
Molasses	4.00	Ca	0.91
CaCO3	1.60	P	0.59
Soybean oil	0.80		
NaCl	0.60		
CaHPO4	0.10		
MgO	0.10		
Selenium yeast	0.02		
1Premix	0.14		
Total	100.00		

The premix provides the following per kilogram: Per kg premix provides: Cu:1000 mg, Fe:5600 mg, Mn: 4200 mg, Zn:9000 mg,I:100 mg, Se:40 mg, Co:35 mg,Vitamin A:900 IU,Vitamin D3:400 IU,Vitamin E:6500 IU.

2.2. Rumen Fluid Sample Collection

On day 70 of the experimental period, rumen fluid samples were collected from six randomly selected calves at 2 hours post morning feeding (09:00–10:00). Each calf was sampled using a sterilized oral catheter (inner diameter: 5 mm; length: 100 cm) to obtain 30 mL of rumen fluid, with strict adherence to aseptic procedures. Immediately after collection, samples were filtered through four layers of sterile gauze (pore size: 150 μm), and the filtrate was transferred into pre-chilled 15 mL centrifuge tubes and temporarily stored on ice (< 30 min). Within 15 minutes of sampling, pH values were measured in triplicate using a portable pH meter (STARTER 300, Ohaus, Shanghai) calibrated with standard buffer solutions at pH 4.0, 7.0, and 10.0. The mean of the three readings was recorded as the final pH value. The filtered rumen fluid was aliquoted into four sterile cryovials (5 mL per tube): two tubes were supplemented with 0.5 mL of 25% metaphosphoric acid for fixation and stored at –80 °C for volatile fatty acid (VFA) analysis; one tube was supplemented with 0.1 mL of 50% sulfuric acid and stored at –20 °C for ammonia nitrogen (NH₃-N) determination; and one tube was left untreated, rapidly frozen in liquid nitrogen, and subsequently transferred to a –80 °C ultra-low temperature freezer for microbial 16S rRNA gene sequencing and LC-MS-based metabolomics analysis.

2.2.1. Determination of Volatile Fatty Acids and Ammonia Nitrogen (NH₃-N)

The concentration of volatile fatty acids (VFA) was determined by gas chromatography (Agilent 6890N, Agilent, USA) equipped with a flame ionization detector (FID), using 2-ethylbutyric acid (2EB) as the internal standard. Separation was achieved on a DB-FFAP capillary column (30.0 m × 0.25 mm × 0.25 μm) under the following temperature program: initial temperature of 85 °C held for 1 min, then increased at 10 °C/min to 180 °C and maintained for 3 min. The FID detector and injection port temperatures were set at 250 °C and 280 °C, respectively. The NH₃-N concentration was quantified using the phenol-chloramine T colorimetric method with a 721 spectrophotometer, following the procedure described by Eschenlauer et al. [13].

2.2.2. 16S rRNA Gene Sequencing and Bioinformatics Analysis

All raw sequencing data generated from the microbial omics analysis were processed using the MajorBio Cloud platform (<https://cloud.majorbio.com>). Total genomic DNA was extracted from rumen microbial samples using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). Negative controls (no-template) and positive controls (standard strain DNA) were included in all extraction and amplification steps to monitor contamination and validate assay performance. High-quality DNA was used as the template for PCR amplification targeting the V3–V4 hypervariable region of the 16S rRNA gene using universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The 25 µL PCR reaction system contained 12.5 µL of 2× KAPA HiFi HotStart ReadyMix, 0.5 µM of each primer, and 10 ng of template DNA. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. Amplified products were purified using Vazyme VAHTSTM DNA Clean Beads, and sequencing libraries were constructed by ligating dual-indexadapters compatible with the Illumina platform. High-throughput paired-end sequencing (2 × 250 bp) was performed on an Illumina NovaSeq 6000 platform. Raw reads were assembled into contiguous sequences using FLASH v1.2.11, and chimeric sequences were identified and removed using UCHIME v8.1 (denovo mode), yielding high-quality clean reads. Operational taxonomic units (OTUs) were clustered at a 97% sequence similarity threshold using USEARCH (v11.0), and taxonomic annotation was conducted against the SILVA reference database (release 138). Alpha diversity indices were calculated using mothur (v1.30.2), and rarefaction curves and rank-abundance curves were generated to assess sequencing depth and community richness. Principal coordinate analysis (PCoA) was performed based on Bray–Curtis dissimilarity to assess microbial community structure. The LefSe method (LDA>2.5, P<0.05) was applied to identify taxonomic groups that significantly differed between groups. Microbial functional profiles were then predicted using PICRUSt2 and mapped to the KEGG and COG databases to analyze differences in metabolic pathways.

2.2.3. Rumen Metabolomics Analysis

Rumen fluid samples were thawed on ice, and 200 µL of each sample was transferred into a microcentrifuge tube. To this, 600 µL of cold acetonitrile-methanol (1:1, v/v) containing 0.02 mg/mL L-2-chlorophenylalanine (used as internal standard) was added. The mixture was vortexed for 30 s and subjected to ultrasonic extraction in an ice-water bath (40 kHz, 4 °C) for 30 min. Following extraction, the samples were centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was collected and concentrated to dryness under a stream of nitrogen at 35 °C. The residue was reconstituted in 100 µL of 80% (v/v) aqueous methanol, filtered through a 0.22 µm nylon membrane, and transferred to autosampler vials for injection. Chromatographic separation was performed on a HSS T3 column (100 mm × 2.1 mm, 1.8 µm) maintained at 40 °C with a flow rate of 0.40 mL/min and an injection volume of 3 µL. The mobile phases consisted of: solvent A, water-acetonitrile (95:5, v/v) containing 0.1% formic acid; and solvent B, acetonitrile-isopropanol-water (47.5:47.5:5, v/v/v) containing 0.1% formic acid. Mass spectrometric detection was carried out in both positive and negative electrospray ionization (ESI) modes with a mass scanning range of m/z 70–1050. Ion source parameters were set as follows: sheath gas flow rate 50 psi, auxiliary gas flow rate 13 psi, auxiliary gas temperature 425 °C, capillary temperature 325 °C, spray voltage ±3500 V, and collision energy ramped at 20–40–60 eV.

The raw data were processed using Progenesis QI (Waters) for peak detection, alignment, quantification, and normalization. Metabolites were identified by matching the MS/MS spectra to reference spectra in databases including HMDB and METLIN, as well as a custom-built database (Meiji BioCloud library). The data matrix was uploaded to the Meiji BioCloud Platform (<https://cloud.majorbio.com>) for preprocessing: missing values were removed according to the 80% rule, and the remaining missing values were imputed using the minimum value. Following total sum normalization and filtering of QC samples with RSD>30%, the data were log₁₀-transformed.

Differential metabolites were screened using the following criteria: variable importance in projection (VIP)>2 from the OPLS-DA model, $p < 0.01$ from univariate t-tests, and fold change (FC)>1.5 or <0.67. The identified differential metabolites were then subjected to KEGG pathway enrichment analysis using MetaboAnalyst 5.0 to interpret their biological significance.(相关性分析没有)

2.3. Statistical Analysis of Data

All raw data were processed using Excel 2016. Experimental results are expressed as mean $\pm$ SEM. Statistical analyses were conducted using SPSS 25.0. For fermentation parameters (volatile fatty acids, VFA; ammonia nitrogen, NH₃-N), after confirming normality (Shapiro-Wilk test) and homogeneity of variance (Levene’s test), differences between groups were assessed using the independent-samples t-test. Microbial diversity indices were compared using the Wilcoxon rank-sum test (non-parametric testfor two-group comparisons). Correlations between variables wereevaluated using Spearman’s rank correlation coefficient. Statistical significance was declared at $p < 0.05$, and a $0.05 \leq p < 0.10$ was considered a trend toward significance.

3. Results

3.1. Rumen Fermentation Characteristics

YCW significantly increased ruminal butyrate concentration ($p < 0.05$), but did not affect ruminal pH, total volatile fatty acid (VFA) production, or the concentrations of other predominant VFAs ($p > 0.05$) (Table 3).

Table 3. Effect of yeast cell wall supplementation on rumen fermentation parameters in early-weaned calves (mmol/L).

Items	Group		P-value
	CON	YCW	
pH	6.33 $\pm$ 0.17	6.40 $\pm$ 0.27	0.660
Ammonia Nitrogen (mg/dL)	11.53 $\pm$ 2.62	10.92 $\pm$ 2.28	0.737
Acetic acid	44.90 $\pm$ 6.74	45.52 $\pm$ 5.76	0.893
Propionic acid	17.33 $\pm$ 1.41	16.54 $\pm$ 3.97	0.722
Butyric acid	4.85 $\pm$ 0.76 ^a	6.79 $\pm$ 0.25 ^b	0.001
Isobutyric acid	1.99 $\pm$ 0.35	2.55 $\pm$ 0.52	0.133
Valeric acid	3.39 $\pm$ 1.11	3.51 $\pm$ 1.02	0.877
Isovaleric acid	4.04 $\pm$ 0.60	3.83 $\pm$ 0.78	0.683
Total Acid	76.53 $\pm$ 9.88	78.77 $\pm$ 3.09	0.681

Note: Comparisons between the Con and Trt groups based on independent-samples t-test,mean $\pm$ SEM;*. Con, control; Ycw, treatment; a,bMeanswithinthesamerow withunlikesuperscripts differ,p<0.05.

3.2. Rumen Microbial Community

Analysis of the rumen microbial community based on 16S rRNA gene sequencing (Figure 1A) identified a total of 1,862 operational taxonomic units (OTUs) across all samples. Specifically, 1,087 OTUs were detected in the CON group and 1,421 in the YCW group, with 646 OTUs shared by both groups. The rarefaction curves (Figure 1B) reached saturation plateaus, indicating that the current sequencing depth adequately captured the majority of microbial diversity within the samples. Principal coordinate analysis (PCoA) was performed based on the Bray-Curtis distance matrix, revealing a discernible separation trend in community structure between the two groups ($p = 0.08$, indicating a trend, Figure 1C). Furthermore, α -diversity indices were used to assess intra-community microbial richness and diversity. As shown in Figure 2A–D, no significant differences were observed in the richness indices (Chao1 and ACE) or diversity indices (Shannon and Simpson) between groups ($p > 0.05$).

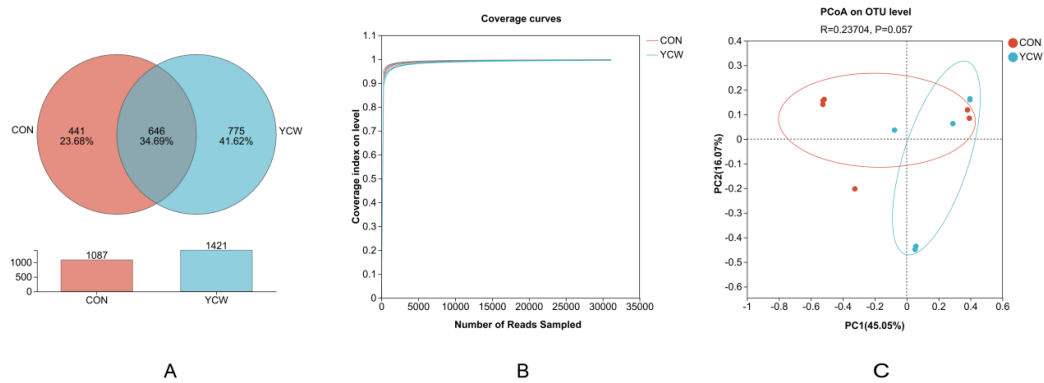


Figure 1. Rumen microbial diversity analysis (A) Venn diagram (B) Dilution curve (C) PCoA analysi.

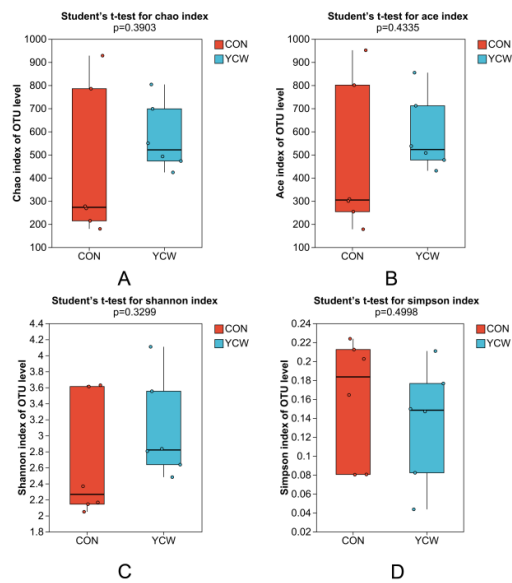


Figure 2. Alpha diversity index.

3.3. Rumen Microbial Species Composition

At the phylum level (Figure 3A), the five most abundant bacterial phyla in the rumen microbiota of calves were Firmicutes, Bacteroidota, Actinobacteriota, Patescibacteria, and Proteobacteria. The YCW group exhibited a higher relative abundance of Firmicutes compared to the CON group, with a trend toward increase ($0.05 \leq p < 0.10$)(Table 4). At the genus level(Figure 3B), the ten most prevalent genera included *Lachnospiraceae_NK3A2O_group*, *norank_f_Eubacterium_coprostanoligenes_group*, *Pseudoscardovia*, *Ruminococcus*, *Acetitomaculum*, *Olsenella*, *Coprococcus*, *Christensenellaceae_R-7_group*, *Ruminococcus_gauvreauii_group*, and *Prevotella*. Statistical analysis indicated a significant increase in *Ruminococcus* abundance in the YCW group compared to the control group ($p < 0.05$)(Table 4).

Table 4. Species composition of calf rumen microorganisms at phylum level and genus level(Top 5).

Items	Group		P-value
	CON	YCW	
Phylum level			
p__Firmicutes	64.24 ± 15.59	80.47 ± 15.29	0.092
p__Actinobacteriota	1.53 ± 0.71	11.46 ± 1.36	0.229
p__Bacteroidota	30.81 ± 2.66	5.63 ± 3.83	0.128

p__Patescibacteria	2.66 ± 0.02	1.95 ± 0.11	0.748
p__Proteobacteria	0.46 ± 0.16	0.25 ± 0.19	0.261
Genus level			
g__Lachnospiraceae_NK3A20_group	11.94 ± 1.81	19.32 ± 1.03	0.378
g__norank_f__Eubacterium_coprostanoligenes_group	11.12 ± 5.05	11.30 ± 7.67	0.810
g__Pseudoscandia	19.82 ± 1.76	0.058 ± 0.02	0.494
g__Ruminococcus	0.79 ± 0.22 ^b	14.83 ± 1.86 ^a	0.030
g__Prevotella	0.36 ± 0.64	6.12 ± 1.73	0.128

Note: Comparisons between the Con and Trt groups based on independent-samples t-test, mean±SEM; *, Con, control; Ycw, treatment; a,b Means within the same row with unlike superscripts differ, p<0.05.

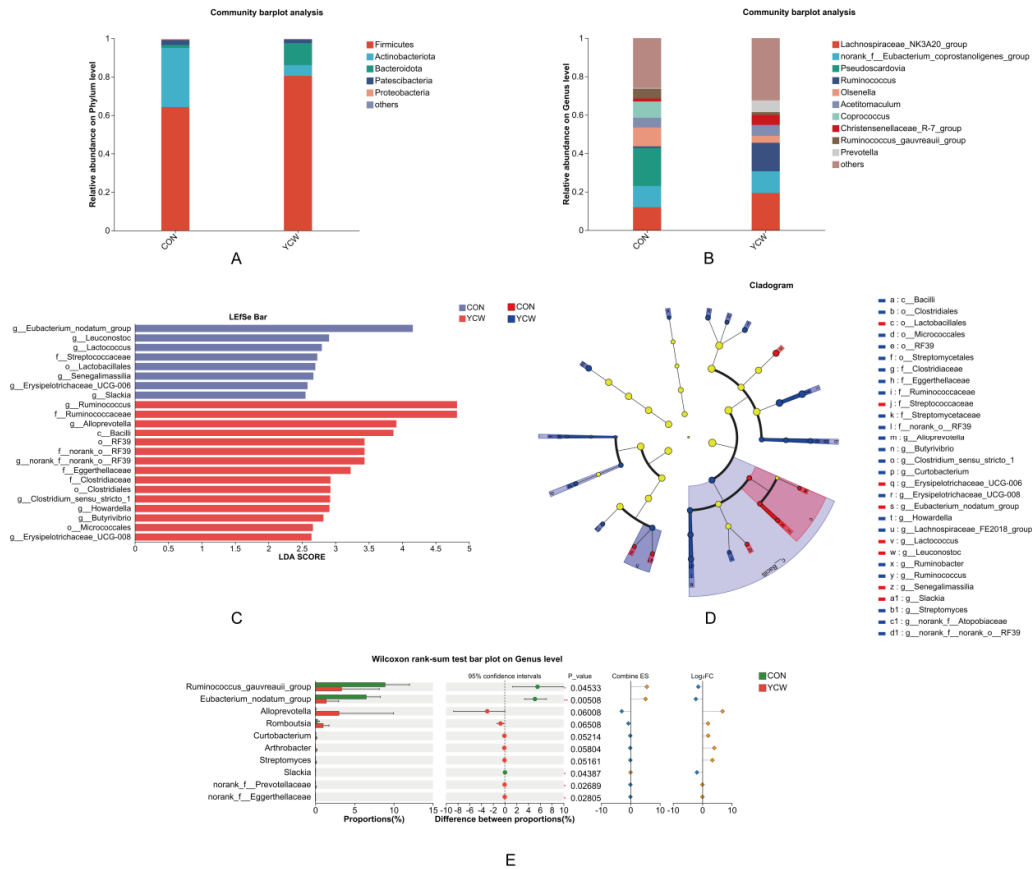


Figure 3. Species composition analysis (A) Species composition at the phylum level (B) Species composition at the genus level (C) LEfSe Analysis Note: Phylogenetic Tree and LDA Bar Chart (LDA Score ≥ 2.5) (D) Comparison of differences at the genus level (%) between different groups of ruminal flora.

3.4. LEfSe Analysis for Multi-Level Differential Species Identification

To identify microbial taxa that differ significantly between groups, LEfSe analysis was performed with an LDA threshold > 2.5 and $p < 0.05$. The phylogenetic distribution and LDA bar plot (Figure 3C-D) revealed distinct microbial biomarkers enriched in each group. Specifically, the CON group was characterized by eight discriminative taxa, including *g_Eubacterium_notatum_group*, *g_Leuconostoc*, and *g_Lactococcus*, whereas the YCW group exhibited significant enrichment of 15 taxa, such as *g_Ruminococcus*, *f_Ruminococcaceae*, and *g_Alloprevotella* ($p < 0.05$).

Figure 3D further shows that the *Ruminococcus_gauvreauii_group* and *Eubacterium_notatum_group* were significantly less abundant in YCW than in CON ($p < 0.05$), while *norank_f_Prevotellaceae* and *norank_f_Eggerthellaceae* were significantly elevated in the YCW group ($p < 0.05$).

3.5. Correlation Analysis and Functional Prediction

To investigate the associations between rumen microbial composition and fermentation profiles, a correlation analysis was performed (Figure 4A). Butyric acid concentration was found to be significantly positively correlated with the relative abundance of *Ruminococcus*, *Acetitomaculum*, and *Christensenellaceae_R-7_group* ($p < 0.05$), and significantly negatively correlated with *Coprococcus* and *Ruminococcus_gauvreauii_group* ($p < 0.05$). Furthermore, isobutyric acid was negatively correlated with *Ruminococcus* and *Prevotella*, acetic acid was negatively correlated with *Pseudoscariovia*; and isovaleric acid was positively correlated with *Olsenella* ($p < 0.05$).

Functional potential of the microbial community was predicted using PICRUSt2 (Figure 4B). Among KEGG level-2 pathways, the five most abundant metabolic functions were Carbohydrate metabolism, Amino acid metabolism, Energy metabolism, Translation, and Replication and repair. The YCW group exhibited a higher abundance in carbohydrate metabolism compared to the CON group, although this difference did not reach statistical significance ($0.05 \leq p \leq 0.10$).

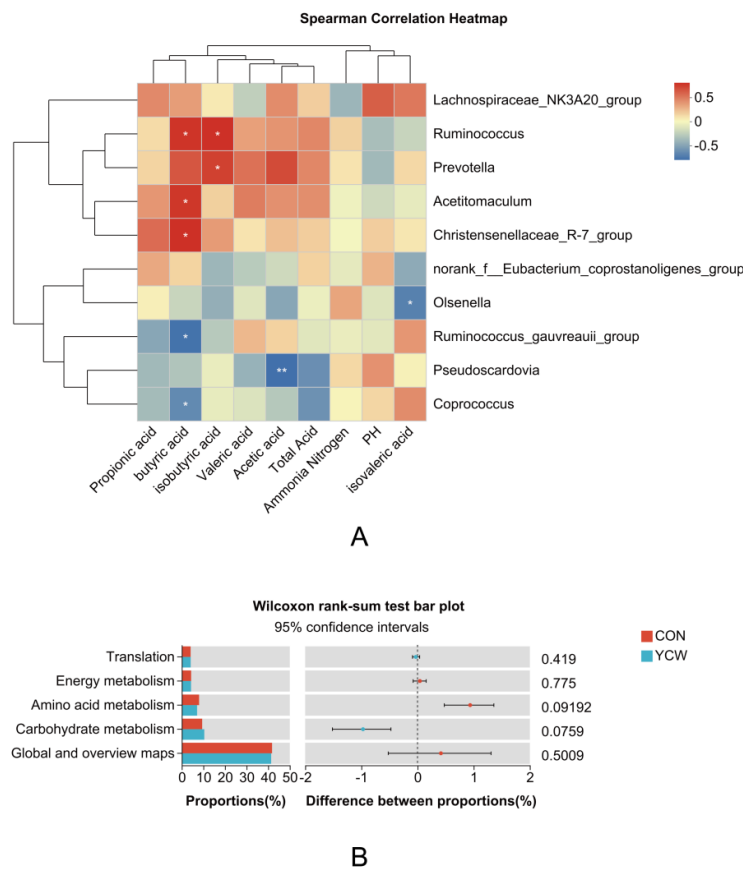


Figure 4. Functional prediction analysis of bacterial microbiota and Correlation heatmap (A) Correlation analysis between rumen microbial composition and fermentation characteristics (B) Prediction of rumen microbial PICRUSt2 function in calves.

3.6. Rumen Metabolites

To ensure the reliability of the orthogonal partial least squares-discriminant analysis (OPLS-DA) model, 200 permutation tests were performed. The results (Figure 5A–B) revealed a negative intercept of the Q^2 regression line on the Y-axis, and both the R^2 and Q^2 values from the permuted models were substantially lower than those of the original model, indicating that the model is robust, free from overfitting, and possesses strong predictive capability. Therefore, the model was suitable for subsequent identification of differential metabolites. Venn diagram analysis (Figure 5C) showed that

the CON and YCW groups harbored 2,985 and 3,018 unique metabolites, respectively, with 2,712 metabolites commonly present in both groups. Principal component analysis (PLS-DA)(Figure 5B) and partial least squares discriminant analysis (PCA) (Figure 5D), performed on the complete set of metabolites, revealed a distinct separation between the YCW group and the CON group, indicating a marked alteration in the rumen metabolic profile following yeast cell wall supplementation.

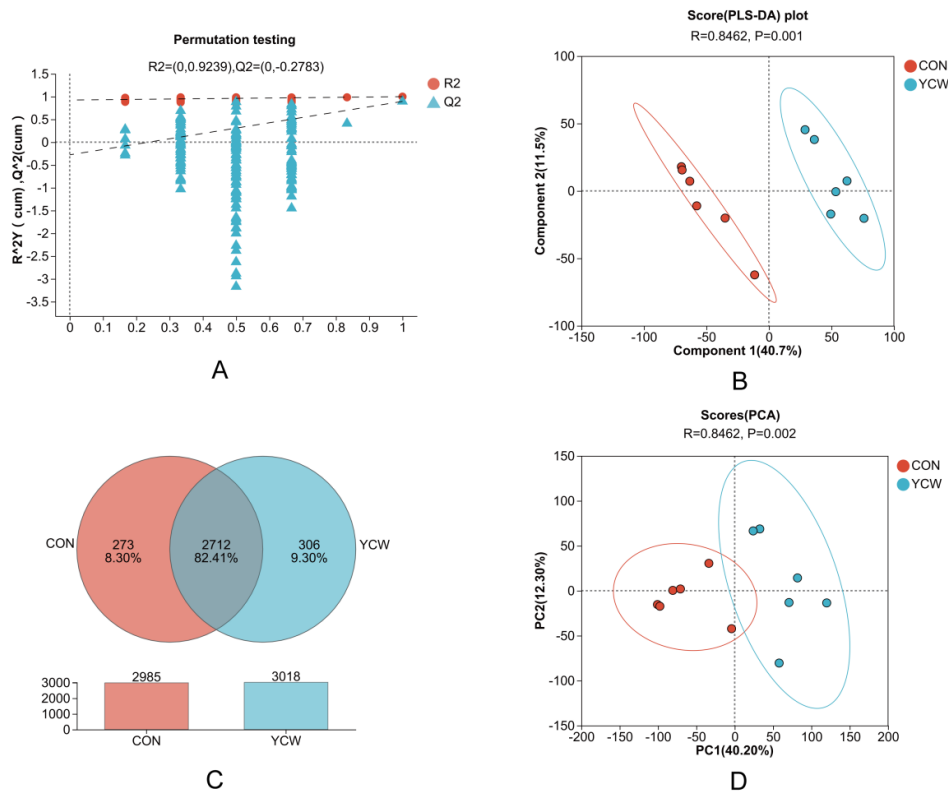


Figure 5. Rumen metabolites of different groups (A–B) OPLS-DA analysis plot (C) Venn plot (D) PCA plot.

3.7. Analysis of Differential Metabolites and Associated Metabolic Pathways

A total of 43 differential metabolites were identified by integrating the OPLS-DA model (VIP>2) with univariate t-tests ($p < 0.01$) in both positive (POS) and negative (NEG) ion modes. As shown in Figure 6A, compared with the CON group, 20 metabolites were significantly up-regulated and 23 were down-regulated in the YCW group. The top 20 key differential metabolites ranked by $|\log_2\text{FC}|$ are listed in Table 4 (9 up-regulated, 11 down-regulated). KEGG pathway enrichment analysis (Figure 6B) revealed that these metabolites were enriched in nine metabolic pathways. Notably, nucleotide metabolism was significantly enriched ($p < 0.05$), while lysine biosynthesis and sphingolipid metabolism exhibited pronounced enrichment trends, suggesting potential biological relevance.

3.8. Correlation Analysis Between Rumen Microorganisms and Differential Metabolites

To elucidate the potential interactions between rumen microbiota and metabolites, Spearman correlation analysis was performed (Figure 6C). The results revealed a significant correlation network between specific differential microbial genera and sets of differential metabolites. Specifically, *Eubacterium_nodatum_group* and *Lactococcus*—genera enriched in the CON group—were positively correlated with most down-regulated metabolites ($p < 0.05$), including 7-Methylinosine, (12E)-10-Hydroxytetradec-12-enoylcarnitine, and LysoSM (d18:1). In contrast, genera enriched in the YCW group exhibited strong positive correlations with multiple up-regulated metabolites ($p < 0.05$). Notably, several genera enriched in YCW (*Alloprevotella*, *Lachnoclostridium*, *Ruminococcus*,

Ruminobacter, and *UCG-004*) showed strong positive correlations with metabolites that were elevated by YCW. For example, these genera were closely associated with the higher concentrations of guanylic acid, LysoPE (18:4/0:0), baohuoside I, and 4-chloropentanone in the YCW group. These findings indicate a potential link between yeast cell wall-induced shifts in rumen microbial composition and concomitant remodeling of the metabolic profile.

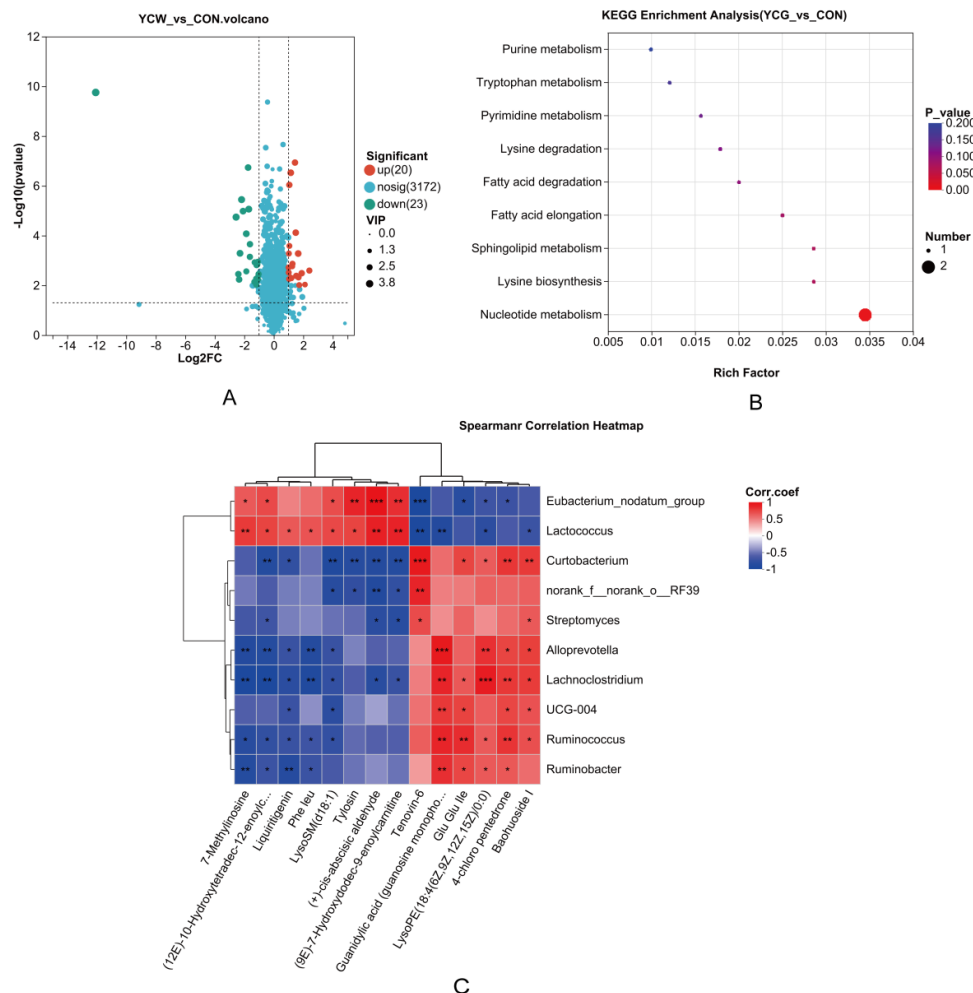


Figure 6. Number of Differentially Metabolized Compounds Among Groups and KEGG Pathway Enrichment Analysis (A)Volcano plot (B) Top 20 most enriched pathwaysin (C)Association study of differentially abundant microbial taxa and metabolomic profiles.

Table 5. Differential metabolites of rumen fluid in MIX mode.

Items	Log2FC	P-value	VIP
Upregulated metabolites			
guanylic acid (guanosine monophosphate)	1.65	0.001	2.84
LysoPE (18:4(6Z,9Z,12Z,15Z)/0:0)	1.15	0.000	2.90
'7,8-dihydroxy-5,6-dimethoxy-2-phenylchromen-4-one'	1.49	0.000	2.73
Baohuoside I	1.89	0.003	2.69
Kaempferol 3,7,4'-Trimethyl Ether	1.44	0.000	2.58
Haloperidol glucuronide	1.05	0.000	2.52
Deoxycytidine monophosphate	1.52	0.004	2.54
Convallatoxin	1.67	0.005	2.40

Albendazole-2-aminosulfone	0.98	0.000	2.41
Downregulated metabolites			
2-[(5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoxy] propane-1,3-diol	-12.07	0.000	3.63
Auraptenol	-2.56	0.000	3.18
LysoSM(d18:1)	-2.18	0.000	3.17
Tylosin	-1.70	0.000	3.09
(+)-cis-abscisic aldehyde	-2.29	0.001	2.87
Formyl-5-hydroxykynurenamine	-1.87	0.000	2.81
(12E)-10-Hydroxytetradec-12-enoylcarnitine	-2.09	0.000	2.77
(9E)-7-Hydroxydodec-9-enoylcarnitine	-1.74	0.000	2.74
N6-(L-1,3-Dicarboxypropyl)-L-lysine	-2.35	0.006	2.64
N-(furan-2-ylmethyl)-2-(6-oxopyridazin-1-yl) acetamide	-2.40	0.004	2.67
7-Methylinosine	-1.17	0.002	2.38

Note: Log2FC = log2 fold change (logarithm base 2); VIP = Variable Importance in Projection.

4. Discussion

Yeast supplementation has the potential to modulate the rumen microbial community by creating a more favorable environment for cellulolytic bacteria, thereby altering the profile and proportions of individual volatile fatty acids (VFAs) in the rumen [14]. Rumen pH serves as a key integrative indicator of fermentation dynamics, influencing both the structural composition of the microbial population and the metabolic pathways associated with VFA production. In this study, rumen pH values in both groups of early-weaned calves remained within the established physiological range for healthy calves [15], consistent with findings from prior studies [16]. β -glucan may contribute to ruminal buffering through the promotion of salivary and epithelial bicarbonate secretion and by stabilizing ruminal fermentation patterns, thus helping to maintain pH homeostasis. Butyrate plays a pivotal role in rumen development, serving not only as the primary energy substrate for ruminal epithelium but also as a stimulant for epithelial cell proliferation and differentiation [17,18]. Lascano [19] demonstrated that yeast-derived additives can increase the relative abundance of *Ruminococcus* and enhance butyrate production. Similarly, Amin [14] reported elevated ruminal butyrate concentrations in calves following yeast supplementation. The present findings align with these results, potentially due to yeast-induced stimulation of *Megasphaera elsdenii* growth [20], a bacterium known to metabolize lactate into butyrate, thereby contributing to the observed increase in butyrate concentration in the YCW group. Evidence from previous research indicates that enhanced butyrate supply can accelerate rumen papillae development, expand the absorptive surface area, and consequently improve solid feed intake in early-weaned calves, thereby supporting their subsequent growth and development [21].

The primary mechanism underlying roughage utilization in ruminants is centered on the rumen, a specialized digestive organ. Functioning as a self-regulated, continuous fermentation system, the rumen serves as a metabolic “hub” in ruminant physiology [22]. The yeast cell wall (YCW) not only acts as a prebiotic by selectively modulating microbial balance but also functions as an immune stimulant and toxin adsorbent, exerting multi-pathway synergistic effects that collectively foster a more stable, healthy, and efficient rumen microenvironment [23]. In this study, YCW supplementation did not significantly alter alpha or beta diversity indices of the ruminal microbiota in calves, but it led to the enrichment of taxa including the phylum Firmicutes, the genus *Ruminococcus*, and the *Christensenellaceae_R-7_group*. These findings are consistent with previous studies: Zhu et al. [24] reported that selenium-enriched yeast influences ruminal microbial composition, particularly increasing the abundance of Firmicutes, while Song et al. [25] demonstrated that *Candida* (NJ-5) yeast remodels the ruminal microbiota in goats by elevating the relative abundance of *Ruminococcus* and *Christensenellaceae_R-7_group*, a finding that aligns with the results of the present study. YCW may induce a moderate shift in microbial composition toward Firmicutes,

thereby promoting a butyrate-dominated fermentation pattern, enhancing fiber degradation and utilization efficiency, and concurrently maintaining overall stability of the gut microbial ecosystem. Notably, *Ruminococcus* and *Christensenellaceae_R-7_group* are well-recognized cellulolytic bacterial groups [26] that facilitate plant cell wall breakdown, increase the release of fermentable intermediates such as acetyl-CoA, and thereby indirectly stimulate butyrate biosynthesis pathways—ultimately contributing to the optimization of the ruminal fermentation environment [27]. LDA analysis identified *g_Ruminococcus*, *f_Ruminococcaceae*, and *g_Butyrvibrio* as bacterial taxa associated with fiber degradation and butyrate production [28]. Chen et al. [29] demonstrated that YCW supplementation enhances the formation of fermentable oligosaccharides and fiber-derived intermediate metabolites in the rumen. The genus *g_Butyrvibrio* possesses metabolic capabilities to utilize plant cell wall components such as xylan and pectin, contributes to butyrate synthesis, and is also recognized as a key participant in the biohydrogenation of unsaturated fatty acids within the rumen. These findings align with the results of the present study. The increased abundance of *g_Alloprevotella* suggests that YCW promotes the establishment of a microbial community with enhanced capacity for plant polysaccharide degradation and utilization of soluble carbohydrates. This observation is consistent with previous reports [30], which indicate that yeast-derived additives can improve the rumen's synergistic degradation of starch and structural carbohydrates, thereby enhancing overall fermentation efficiency. Within the rumen, the class *c_Bacilli* primarily encompasses lactic acid-producing bacteria and *Bacillus*-related taxa, whose members rapidly ferment soluble carbohydrates and generate intermediates such as lactate. Under conditions of high-concentrate feeding or excessive fermentable substrate availability, unchecked lactate production may lead to its accumulation and increase the risk of subacute ruminal acidosis [31]. In this study, the observed increase in butyrate concentration without a concomitant decrease in ruminal pH indicates that lactate metabolism in the rumen is likely governed by a balanced “production-utilization” dynamic rather than pathological lactate accumulation.

LEfSe analysis further confirmed that YCW supplementation enriched microbial taxa associated with fiber degradation and carbohydrate metabolism, including the unclassified family Prevotellaceae (*norank_f_Prevotellaceae*) and the unclassified family Eggerthellaceae (*norank_f_Eggerthellaceae*) [32,33]. Correlation analysis revealed a significant positive association between *Ruminococcus* abundance and butyric acid concentration, which is consistent with findings reported by Pan et al. [34] from correlation-based analyses in the rumen of Qinchuan cattle. *Ruminococcus* is a well-characterized genus involved in fiber and cellulose degradation; its increased abundance typically reflects enhanced breakdown and fermentation flux of plant cell wall polysaccharides, thereby providing greater availability of fermentable substrates for butyrate-producing microbes and facilitating elevated butyrate production.

This study utilized LC-MS technology to investigate the impact of YCW supplementation on rumen metabolites in early-weaned calves. The results showed that upregulated metabolites were predominantly enriched in pathways associated with nucleotide metabolism. Wang et al. [35] reported that supplementation with yeast culture alters ruminal amino acid, lipid, and vitamin metabolism in ruminants, with these metabolic shifts occurring concurrently with changes in microbial composition. Notably, the concentrations of guanosine monophosphate (GMP) and deoxycytidine monophosphate (dCMP) were significantly elevated, suggesting enhanced capacity for nucleic acid synthesis and repair within the ruminal microbiota. GMP serves as a direct precursor to guanosine triphosphate (GTP), a molecule essential for DNA replication, transcription, translation, and other cellular growth and repair processes. Thus, the increased GMP levels may be interpreted as a metabolic indicator of accelerated microbial growth and nucleic acid turnover [36]. This study revealed a significant positive correlation between the relative abundance of *Ruminococcus* and GMP levels, suggesting that the shift in rumen microecology toward a more favorable environment for fiber-degrading bacteria following YCW supplementation is accompanied by enhanced microbial nucleic acid synthesis and turnover, particularly within purine and nucleotide metabolic pathways. This interpretation aligns with previous studies recognizing nucleic acids and purine-related

compounds as reliable endogenous markers of ruminal microbial biomass and biosynthetic activity [37]. Beyond their role as biosynthetic precursors, these nucleotide metabolites may also function as intermicrobial signaling molecules, modulating population dynamics and stress response mechanisms. Notably, research on a yeast cell wall-containing complex additive has shown that dietary supplementation with exogenous nucleotides can further improve intestinal morphology, redox homeostasis, and immune function, implying a potential synergistic interaction between the observed enhancement in nucleotide metabolism and the health-promoting effects of YCW [38]. Modified nucleosides are primarily derived from RNA turnover and degradation, and their formation pathways vary across microbial taxa. Therefore, the observed reduction in 7-Methylinosine levels in this study is likely attributable to YCW-induced remodeling of the ruminal microbiota and a more stable fermentation environment—characterized by a stable pH and elevated butyrate concentration. This ecological stabilization may reduce overall RNA degradation rates or alter the metabolic flux of modified nucleoside production and utilization, ultimately leading to decreased accumulation of 7-Methylinosine [29]. Notably, the concentration of the phospholipid-related metabolite LysoSM (d18:1) was significantly lower in the YCW group. LysoSM (d18:1) is known to mediate pro-inflammatory and pro-oxidative stress signaling pathways; its reduction suggests a shift toward a quiescent state of local ruminal inflammation and a decrease in oxidative stress levels [39]. Thus, the downregulation of LysoSM (d18:1) further supports an improvement in inflammatory homeostasis within the rumen microenvironment. This finding aligns with the observed enhancements in fermentation performance and ruminal pH stability, indicating that YCW not only improves fermentative function but also exerts beneficial effects on host inflammatory regulation by modulating key metabolic signaling pathways [40,41]. LysoPE (18:4(6Z,9Z,12Z,15Z)/0:0) plays a critical role in maintaining cell membrane integrity and modulating inflammatory responses. Its increased abundance reflects improved epithelial barrier function and enhanced local tissue health. The positive correlation between *Ruminococcus* abundance and LysoPE (18:4(6Z,9Z,12Z,15Z)/0:0) is consistent with findings from Wang et al. [35] in sheep, where yeast culture supplementation increased the abundance of *Ruminococcus* and other fiber-degrading taxa, while differentially regulated metabolic pathways were enriched in lipid metabolism. Collectively, these results indicate that YCW reshapes ruminal metabolite profiles through the enrichment of specific microbial populations. The coordinated shifts in microbial composition and metabolic activity provide valuable insights into the mechanistic basis of YCW's physiological effects.

5. Conclusions

In conclusion, supplementing the starter diet of early-weaned Simmental calves with 5 g/head/d of YCW effectively modulated ruminal microbial composition and metabolic profiles. YCW increased ruminal butyrate concentration, enriched the fiber-degrading genus *Ruminococcus*, and primarily affected nucleotide metabolism pathways. These findings provide a mechanistic basis for using YCW as a functional dietary additive to support rumen development and health during early weaning.

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