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Rumen Microbiome Dynamics and Their Influence on Digestibility, Fermentation, and Metabolism Across Parities in Sanhe and Holstein Cows Under Uniform Diets

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ABSTRACT

The link between serum metabolism and milk production in Sanhe and Holstein cows has been explored previously, showing that metabolic responses vary with breed and parity. However, the role of the rumen microbiome in these differences remains unclear, despite its central function in nutrient absorption and metabolic processing. In this study, we investigated rumen fermentation characteristics and nutrient digestibility in Sanhe (S1–S4) and Holstein (H1–H4) cows across four parities, alongside a high-resolution analysis of their rumen microbial communities. Among Sanhe cows, dry matter digestibility and ammonia nitrogen levels showed significant variation, with S1 tending to have higher volatile fatty acid concentrations. Holstein cows exhibited notable differences in crude protein digestibility, higher isovaleric acid in H1, and a lower acetate-to-propionate ratio in H3. Microbiome profiling revealed patterns consistent with these metabolic changes: S1 diverged from S2–S4, while H1 and H2 were distinct from H3 and H4. While the overall microbial composition was broadly similar between the two breeds, relative abundances differed. For example, Rhizophagus (Glomeromycetes), Neocallimastix, and Piromyces were more prevalent in early-parity cows, and pathways such as autophagy, plant-pathogen interactions, and endocytosis were enriched. In older Sanhe cows, ATP-binding cassette transporter pathways were more prominent. Furthermore, specific carbohydrate-active enzymes (GH84, GH37) were closely associated with physiological traits and milk production indicators. Overall, these results highlight the intricate interactions between the rumen microbiome and metabolism, suggesting that microbial composition shifts may underlie parity-dependent differences in lactation performance.

Keywords: Rumen microbiome, Dairy cows, Breed differences, Parity, Lactation

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Introduction

The rising global demand for high-quality dairy and beef products has brought renewed focus to cattle breeding and management practices. Among the many breeds, Holstein cows are widely recognized and extensively studied due to their global prevalence and dairy productivity [1]. At the same time, indigenous breeds, such as China's Sanhe cattle, represent valuable genetic resources with both dairy and meat potential [2, 3]. Selective breeding has enhanced the dual-purpose traits of Sanhe cattle, yet compared with well-characterized breeds like Holstein, knowledge about their production performance and physiological features remains limited.

Lactation is the primary driver of productivity in dairy cows, and parity exerts a significant influence on lactation efficiency due to variations in basal metabolism and energy demands across successive lactations [4]. In prior work, we systematically characterized the metabolic and physiological profiles of Sanhe and Holstein cows from

parities 1 through 4 [5]. The results demonstrated pronounced differences between primiparous and multiparous Sanhe cows, suggesting substantial shifts in nutrient metabolism and lactation-related pathways with increasing parity. Conversely, Holstein cows displayed a different pattern: metabolic profiles in the first and second parities were largely similar, while the third and fourth parities showed distinct trends [6].

The rumen microbiome is a central determinant of feed utilization, nutrient absorption, and overall production performance in dairy cows [7]. Despite its importance, most studies have focused either on microbial development in weaned calves [8] or on differences in microbial composition between cows with varying production levels [9, 10]. Little is known about how the rumen microbiome changes across different parities in lactating cows. For instance, Xue *et al.* [11] reported that the abundance of Fibrobacteres and SR1 varied among mid-lactation cows from parities 2 to 7, whereas the primary functional bacterial populations remained stable. Moreover, lactation traits are closely intertwined with core rumen microbial communities [12–14], highlighting the rumen microbiome as a critical target for understanding milk quality regulation.

We propose that, even under identical diets, differences in serum metabolism and lactation performance between Sanhe and Holstein cows of various parities are partially driven by alterations in the rumen microbial community. This study aims to elucidate how variations in rumen microbiome composition affect fermentation processes and metabolic activity in Sanhe and Holstein cows across parities 1 to 4. Integrating these findings with previous research will provide a comprehensive view of the interplay between host physiology, rumen microbiota, and metabolic adaptations, ultimately improving our understanding of the Sanhe breed and informing sustainable breeding and management strategies for dairy cattle.

Materials and Methods

Animal management and experimental setup

All experimental procedures were conducted in accordance with the guidelines approved by the Animal Experiment Ethics Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Lactating Sanhe and Holstein cows representing parities 1 through 4 were selected from a single farm, ensuring consistent feeding and management practices across all groups. Sanhe cows were classified as S1 (n=10), S2 (n=9), S3 (n=10), and S4 (n=10), while Holstein cows were categorized as H1 (n=10), H2 (n=7), H3 (n=9), and H4 (n=9). Detailed protocols regarding animal selection and feeding strategies are described in our previous studies on Sanhe [5] and Holstein [6] cows.

Fecal sample collection and nutrient analysis

Fecal samples were collected rectally twice daily over a five-day period, at 2 hours before feeding and 2 hours after feeding. Samples were preserved immediately in 10% sulfuric acid and stored at −20°C for subsequent digestibility analysis. For laboratory processing, samples were dried at 65°C and finely ground through a 1-mm sieve. Dry matter (DM; method 930.15), crude protein (CP; method 2001.11), neutral detergent fiber (NDF; method 2002.04), acid detergent fiber (ADF; method 973.18), and ether extract (EE; method 920.39) were measured following AOAC protocols [15, 16]. Gross energy (GE) was quantified using a calorimeter (5E-C5508, Kaiyuan Instruments, China), and apparent total-tract digestibility was determined via the acid-insoluble ash method [17].

Rumen fluid collection and fermentation analysis

Rumen contents were collected using an oral intubation technique, inserting a tube approximately 120–150 cm into the esophagus. The first 100 mL of aspirated content was discarded to minimize saliva contamination, and 100–150 mL of rumen fluid was collected for analysis. The pH of the rumen fluid was measured immediately on-site using a portable pH meter (BPHPOCKET-E, BELL Analytical Instruments, Dalian, China). The fluid was then filtered through two layers of sterile gauze into sterile centrifuge tubes and flash-frozen in liquid nitrogen. Volatile fatty acids (VFAs) were analyzed by Gas Chromatography (7890A, Agilent, USA) as previously described [18], while ammonia nitrogen concentrations were determined using a UV-2300 Spectrophotometer (Shimadzu, Kyoto, Japan) at an absorbance of 700 nm.

DNA extraction and metagenomic sequencing

DNA was extracted from rumen fluid by Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China) using a commercial kit, following the manufacturer's instructions. DNA quality was verified with 1% agarose gel

electrophoresis, and concentration and purity were evaluated using both Qubit 2.0 and Nanodrop One instruments (Thermo Fisher Scientific, USA). Sequencing libraries were prepared using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina®, with unique index codes assigned to each sample. Library quality was assessed using Qubit 3.0 and the Agilent 4200 system. Finally, libraries were sequenced on the Illumina Hiseq X-ten platform, generating 150 bp paired-end reads.

Bioinformatic processing of rumen metagenomic data

Raw sequencing reads were first quality-filtered using Trimmomatic (v.0.36, http://www.usadellab.org/cms/index.php?page=trimmomatic) to generate clean data suitable for downstream analysis. Clean reads were then assembled with MEGAHIT (v1.0.6, https://github.com/voutcn/megahit). Scaffolds containing ambiguous "N" bases were split to produce continuous sequences, referred to as scaftigs. Scaftigs with a length ≥500 bp were retained for open reading frame (ORF) prediction using MetaGeneMark (v3.38, http://exon.gatech.edu/GeneMark/metagenome/Prediction), applying default parameters and discarding predicted ORFs shorter than 90 nt. Redundant sequences were removed with CD-HIT (v4.7, http://www.bioinformatics.org/cd-hit/), producing a non-redundant initial gene catalog. Clustering was performed at 95% sequence identity and 90% coverage, with the longest sequence in each cluster chosen as the representative. Clean reads from individual samples were mapped to this initial gene catalog using BBMAP,[1] generating mapped read counts for each gene in every sample. Gene abundance was then calculated based on read counts normalized to gene length. Functional annotation was performed using DIAMOND (v0.8.35, https://github.com/bbuchfink/diamond/) by aligning unigenes against bacterial, fungal, archaeal, and viral sequences from the NCBI NR (non-redundant protein sequence database). Alignments with e-values $\leq 1\times10^{-10}$ were selected for annotation using the lowest common ancestor (LCA) algorithm. Gene depth and abundance tables at different taxonomic levels (kingdom, phylum, class, order, family, genus, species) were constructed based on LCA results and gene abundance metrics.

Taxonomic abundance data were used to generate clustering heatmaps and principal component analyses (PCA). Differences between groups were assessed via ANOSIM analysis. Differential taxa were identified using LEfSe analysis, applying a default LDA score threshold of 2. Visualization was conducted with the R package ggplot2 (v4.3.1), while clustering heatmaps were produced using Pheatmap. KEGG pathway differences were visualized using OmicStudio tools.[2] Carbohydrate-active enzyme (CAZyme) annotation was performed with dbCAN4,[3] and the resulting CAZy classifications were visualized as circular heatmaps and bar charts via the ChiPlot online tool.[5] Finally, Spearman correlation analysis was applied to explore relationships between differential GH enzymes and apparent differential indices, with results displayed using the R packages linkET and ggplot2.

Statistical analysis

All data related to apparent nutrient digestibility and rumen fermentation were initially organized in Excel 2019 (Microsoft Corporation, United States) and subsequently subjected to statistical evaluation using SPSS 22.0 (SPSS, Inc., United States). The Shapiro–Wilk test was applied to determine whether each variable followed a normal distribution. Variables that met the normality assumption were analyzed using one-way analysis of variance (ANOVA), with Bonferroni adjustments applied for multiple pairwise comparisons. Differences were considered statistically significant at $p < 0.05$, values between 0.05 and 0.10 were interpreted as indicative of a tendency, and $p \geq 0.10$ was regarded as non-significant.

Results

Nutrient digestibility

Apparent nutrient digestibility in Sanhe cows across parities 1 to 4 is shown in **Table 1**. Among these, dry matter (DM) digestibility exhibited significant variation, with S4 displaying the lowest value ($p = 0.038$). In contrast, digestibility of crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), ether extract (EE), and gross energy (GE) remained largely consistent across the four parities ($p > 0.05$).

For Holstein cows, nutrient digestibility across parities 1 to 4 is summarized in **Table 2**. While CP digestibility was significantly greater in H4 than in H1, H2, and H3, the digestibility of DM, NDF, ADF, EE, and GE showed no significant differences among the four parities ($p > 0.05$).

Table 1. Apparent total-tract apparent digestibility of nutrients in Sanhe cows with 1–4 parities

Item[1]	Group[2]				SEM[3]	*p*-value
	S1	S2	S3	S4		
DM	86.42[a]	85.59[ab]	85.96[a]	84.03[b]	0.325	0.038
CP	63.80	59.72	62.63	59.88	0.832	0.220
NDF	56.98	52.86	58.96	50.91	1.201	0.059
ADF	47.47	44.49	48.52	40.14	1.642	0.260
EE	85.37	82.49	83.34	80.79	0.707	0.134
GE	58.72	52.41	56.74	52.10	1.061	0.061

[1]DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ethanol extract; GE, gross energy.

[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Sanhe dairy cattle, respectively.

[3]SEM was standard error of means.

[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

Table 2. Apparent total-tract apparent digestibility of nutrients in Holstein cows with 1–4 parities

Item[1]	Group[2]				SEM[3]	*p*-value
	H1	H2	H3	H4		
DM	84.01	83.31	83.07	84.96	0.385	0.279
CP	62.72[b]	62.36[b]	65.40[b]	69.58[a]	0.782	0.001
NDF	57.12	55.76	48.19	55.55	1.498	0.134
ADF	59.55	57.04	50.81	56.55	1.448	0.169
EE	85.65	84.37	83.14	83.79	0.613	0.507
GE	58.22	56.45	53.98	59.93	1.006	0.172

[1]DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ethanol extract; GE, gross energy.

[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Holstein dairy cattle, respectively.

[3]SEM was standard error of means.

[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

Rumen fermentation parameters

Rumen fermentation profiles varied across parities in Sanhe cows **(Table 3)**. Notably, S1 tended to have a lower rumen pH compared with later parities, though this difference was marginally significant ($p = 0.068$). Ammonia nitrogen concentrations were highest in S1 and dropped to their lowest in S2, showing a statistically significant difference ($p = 0.024$). Trends toward higher concentrations of total volatile fatty acids (VFAs) and specific components—including acetate, butyrate, isobutyrate, valerate, and isovalerate—were also observed in S1, indicating a tendency for increased rumen fermentation activity in early parity cows ($0.05 < p < 0.1$). In contrast, microbial protein content and the acetate-to-propionate ratio remained relatively stable across all Sanhe groups ($p > 0.05$).

In Holstein cows, the rumen fermentation patterns exhibited some parity-specific differences **(Table 4)**. H3 displayed a markedly lower acetate-to-propionate ratio compared with other parities ($p = 0.002$), while H1 had significantly higher isovalerate levels ($p = 0.002$). The remaining fermentation parameters—including total VFAs, acetate, butyrate, isobutyrate, valerate, isovalerate, and microbial protein—showed no significant variation from H1 through H4 ($p > 0.05$), suggesting that most aspects of rumen fermentation remained consistent across parities in Holstein cows.

Table 3. Rumen fermentation variables in Sanhe cows with 1–4 parities

Item[1]	Group[2]				SEM[3]	*p*-value
	S1	S2	S3	S4		
Rumen pH	6.52	6.77	6.73	6.56	0.040	0.068
Ammonia N (mg/dL)	8.59[a]	5.20[b]	6.67[ab]	7.75[a]	0.423	0.024
MCP (mg/mL)	1.42	1.45	1.37	1.21	0.064	0.547
Total VFA (mmol/L)	92.19	63.74	81.69	88.61	3.971	0.053
Acetate (mmol/L)	57.03	40.41	49.82	53.91	2.300	0.058
Propionate (mmol/L)	19.52	12.89	18.16	20.11	1.086	0.079

Butyrate (mmol/L)	11.91	7.79	10.26	11.08	0.565	0.056
Isobutyrate (mmol/L)	0.87	0.64	0.81	0.80	0.031	0.059
Valerate (mmol/L)	1.48	1.03	1.39	1.43	0.068	0.077
Isovalerate (mmol/L)	1.38	0.98	1.26	1.29	0.055	0.070
Acetate/Propionate	2.99	3.18	2.84	2.89	0.073	0.386

[1]MCP, microbial crude protein; VFA, volatile fatty acid.

[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Sanhe dairy cattle, respectively.

[3]SEM was standard error of means.

[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

Table 4. Rumen fermentation variables in Holstein cows with 1–4 parities

Item[1]	Group[2]				SEM[3]	*p*-value
	H1	H2	H3	H4		
Rumen pH	6.71	6.61	6.69	6.83	0.050	0.521
Ammonia N (mg/dL)	9.23	6.99	7.86	6.33	0.489	0.176
MCP (mg/mL)	1.44	1.38	1.28	1.43	0.073	0.864
Total VFA (mmol/L)	96.53	78.20	83.17	74.23	4.590	0.336
Acetate (mmol/L)	60.74	49.22	49.71	46.53	2.744	0.253
Propionate (mmol/L)	20.37	16.61	21.19	16.45	1.221	0.396
Butyrate (mmol/L)	11.76	9.50	9.09	8.69	0.627	0.289
Isobutyrate (mmol/L)	0.88	0.67	0.80	0.61	0.056	0.315
Valerate (mmol/L)	1.40	1.11	1.41	1.08	0.070	0.175
Isovalerate (mmol/L)	1.38^{a}	1.08^{b}	0.96^{bc}	$0.88b^{c}$	0.054	0.002
Acetate/Propionate	3.05^{a}	3.06^{a}	2.43^{b}	2.84^{a}	0.074	0.002

[1]MCP, microbial crude protein; VFA, volatile fatty acid.

[2]S1, S2, S3, and S4 represented first-, second-, third-, and fourth-parity Holstein dairy cattle, respectively.

[3]SEM was standard error of means.

[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

Rumen microbial composition

Sequencing of the 39 rumen samples from Sanhe cows generated over 2.7 billion clean reads (2,712,982,832), which were used to profile the microbial communities. At the highest taxonomic level, four kingdoms were identified: bacteria, eukaryota, archaea, and other minor groups **(Figure 1A)**. Across the four parity groups (S1–S4), bacteria consistently dominated the rumen microbiome, followed by eukaryotic microorganisms. Interestingly, S1 cows harbored slightly fewer bacterial sequences than S3 and S4, whereas eukaryotic populations exhibited the opposite pattern.

Exploration of community structure at the phylum level, encompassing 185 identified taxa, using principal coordinates analysis (PCoA) showed that the four parity groups formed loosely clustered but distinguishable patterns **(Figure 1B)**. ANOSIM confirmed that variability between parities was more pronounced than within-parity differences, with S1 displaying the richest microbial diversity **(Figure 1C)**. Hierarchical clustering of the 30 most abundant phyla revealed that S1 had relatively high proportions of Cyanobacteria, Basidiomycota, Blastocladiomycota, Microsporidia, Cryptomycota, Chytridiomycota, Zoopagomycota, Ascomycota, and Mucoromycota. In contrast, Proteobacteria and Candidatus Melainabacteria were enriched in S4 **(Figure 1D)**.

At the genus level, 3,528 distinct genera were detected. PCoA again reflected patterns similar to the phylum-level analysis, though separation between groups was less pronounced **(Figure 1E)**. ANOSIM suggested a tendency toward significant differences among parities **(Figure 1F)**. Further clustering of the top 30 genera highlighted Rhizophagus <glomeromycetes>, Neocallimastix, and Piromyces as being particularly abundant in S1 **(Figure 1G)**, indicating a potential association of these genera with early-parity rumen function.

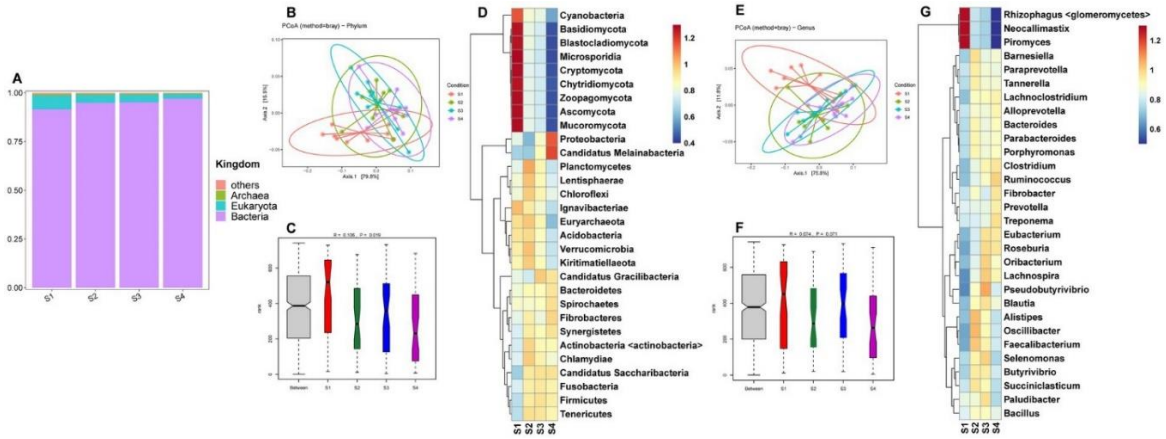


Figure 1.

Rumen microbial composition

Metagenomic sequencing of rumen samples from Sanhe cows across parities S1–S4 yielded over 2.7 billion high-quality reads, which provided a detailed view of microbial community structure. At the kingdom level, bacteria dominated the rumen microbiome, followed by eukaryotic microorganisms, with archaea and minor taxa representing smaller proportions. Interestingly, bacterial representation was slightly reduced in S1 compared with S3 and S4, while eukaryotic abundance displayed the opposite trend. Principal coordinate analysis (PCoA) at the phylum level revealed that the four parity groups formed loosely defined clusters. Statistical assessment with ANOSIM indicated that variation among parities exceeded variation within groups, with S1 exhibiting the highest microbial richness. Hierarchical clustering of the 30 most abundant phyla highlighted increased levels of Cyanobacteria, Basidiomycota, Blastocladiomycota, Microsporidia, Cryptomycota, Chytridiomycota, Zoopagomycota, Ascomycota, and Mucoromycota in S1, whereas Proteobacteria and Candidatus Melainabacteria were enriched in S4. At the genus level, 3,528 taxa were identified. Patterns observed in PCoA resembled those at the phylum level but showed less separation, while ANOSIM suggested a tendency for inter-parity differences. Hierarchical clustering of the top 30 genera revealed that Rhizophagus <glomeromycetes>, Neocallimastix, and Piromyces were more prevalent in S1, suggesting their potential role in early-parity rumen fermentation.

In Holstein cows (H1–H4), the overall kingdom-level profile was similar, with bacteria predominating but slightly lower in H1 and H2 than in H3 and H4. Conversely, eukaryotic populations increased in H1 and H2. PCoA of selected phylum- and genus-level taxa indicated that Holstein groups were less distinctly separated than in Sanhe cows. ANOSIM confirmed significant differences at the phylum level and a trend toward differences at the genus level. Hierarchical clustering further showed that H1 and H2 shared similar microbial compositions. At the phylum level, Blastocladiomycota, Microsporidia, Zoopagomycota, Basidiomycota, Ascomycota, Mucoromycota, Chytridiomycota, and Cryptomycota were enriched in these two parities. Similarly, genus-level analysis highlighted higher abundances of Rhizophagus <glomeromycetes>, Neocallimastix, and Piromyces in H1 and H2, suggesting parity-specific microbial shifts in early lactation.

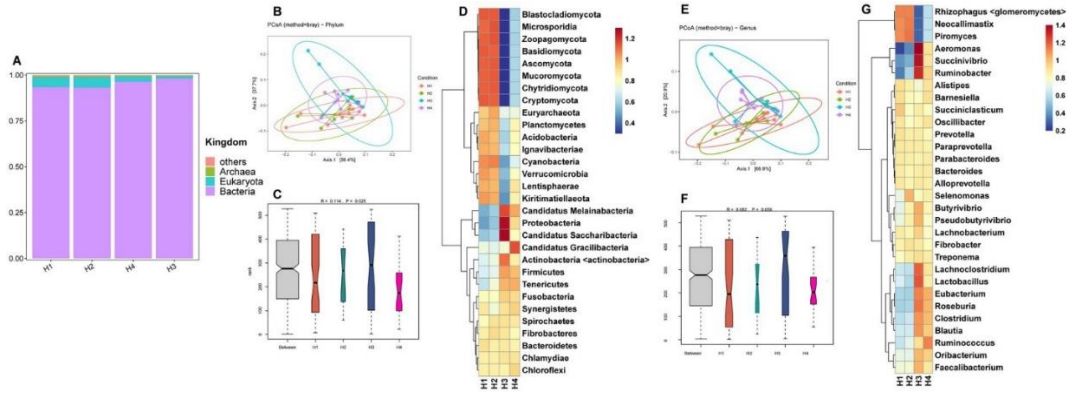


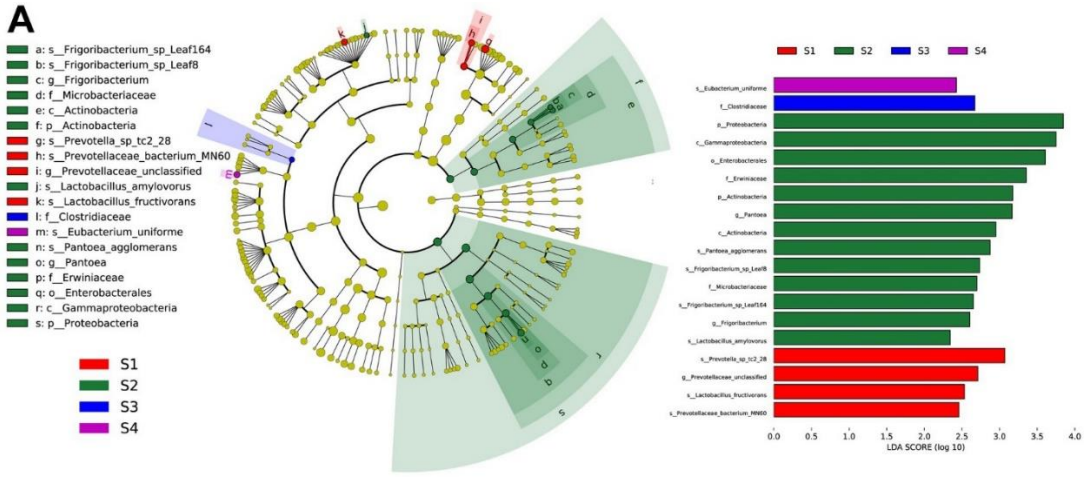
Figure 2.

Differential rumen microbes and KEGG functional pathways

To investigate parity-related differences in rumen microbial composition and identify key microbial markers, LEfSe analysis was performed for Sanhe and Holstein cows across parities 1–4. In Sanhe cows, the analysis revealed 19 significantly enriched marker taxa across the four parity groups ($p < 0.05$; **Figure 3A**). Among the four microbes overrepresented in S1, three belonged to the genus Prevotella (s_Prevotella_sp_tc2_28, g_Prevotellaceae_unclassified, s_Prevotellaceae_bacterium_MN60), while the remaining marker was s_Lactobacillus_fructivorans. S2 exhibited the highest number of differential taxa, comprising 13 markers spanning multiple taxonomic levels, including p_Proteobacteria, c_Gammaproteobacteria, o_Enterobacterales, f_Erwiniaceae, p_Actinobacteria, g_Pantoea, c_Actinobacteria, s_Pantoea_agglomerans, s_Frigoribacterium_sp_Leaf8, f_Microbacteriaceae, s_Frigoribacterium_sp_Leaf164, g_Frigoribacterium, and s_Lactobacillus_amylovorus. In contrast, S3 and S4 each exhibited a single significantly enriched taxon, namely s_Eubacterium_uniforme and f_Clostridiaceae, respectively, highlighting that the microbial shifts were most pronounced in early parities.

For Holstein cows, metagenomic analysis of rumen fluid showed similar trends in kingdom-level microbial composition, with bacteria predominating but slightly lower in H1 and H2 than in H3 and H4, while eukaryotic populations followed the opposite pattern **(Figure 2A)**. PCoA at both phylum and genus levels revealed no clear separation among Holstein groups **(Figures 2B and 2E)**. Nevertheless, ANOSIM indicated significant differences at the phylum level **(Figure 2C)** and a tendency toward differential abundance at the genus level **(Figure 2F)**. Hierarchical clustering demonstrated that H1 and H2 shared highly similar microbial profiles. At the phylum level, Blastocladiomycota, Microsporidia, Zoopagomycota, Basidiomycota, Ascomycota, Mucoromycota, Chytridiomycota, and Cryptomycota were more abundant in these two parities, while genus-level analysis highlighted elevated levels of Rhizophagus <glomeromycetes>, Neocallimastix, and Piromyces in H1 and H2 **(Figure 2D,G)**.

These findings collectively suggest that both Sanhe and Holstein cows exhibit parity-dependent shifts in rumen microbial composition, with early-parity animals showing distinctive enrichment patterns at both the phylum and genus levels.



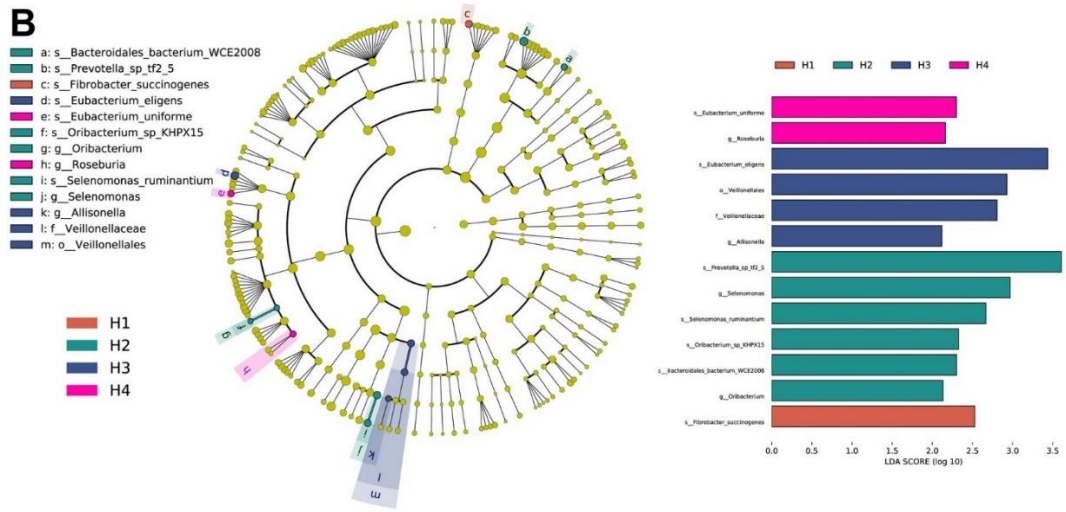
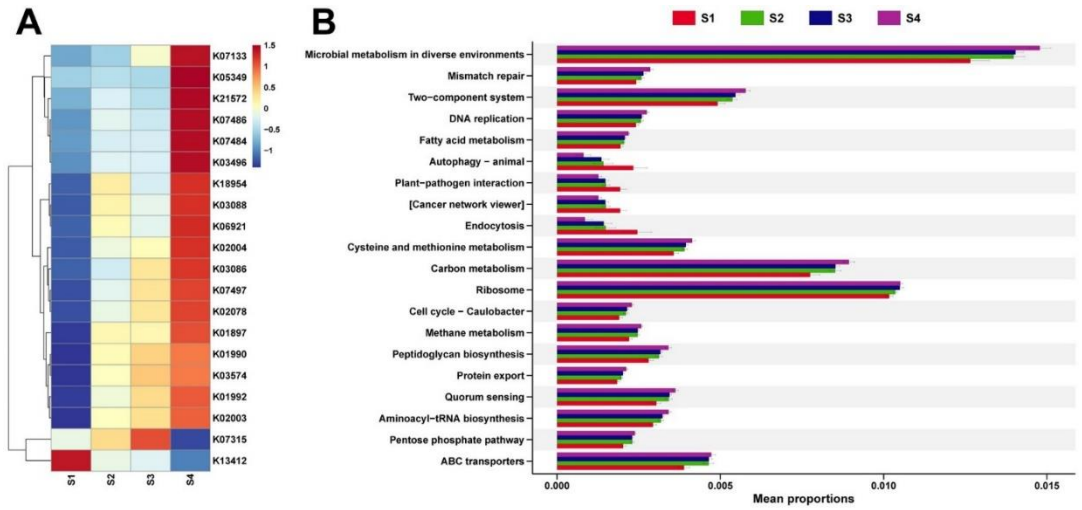


Figure 3. illustrates the LEfSe-based identification of parity-specific biomarker microbes in both Sanhe (S1–S4) and Holstein (H1–H4) cows. Panels (A) and (B) display taxonomic trees highlighting microorganisms enriched in different parities, with node colors indicating representative taxa and labels providing the specific microbial names. Microbial biomarkers were selected based on a linear discriminant analysis (LDA) score greater than 2.0 and $p < 0.05$

In Holstein cows, LEfSe analysis revealed distinct parity-related enrichments **(Figure 3B)**. H1 showed significant enrichment of s_Fibrobacter_succinogenes. H2 exhibited elevated levels of several taxa, including s_Prevotella_sp_tf2_5, g_Selenomonas, s_Selenomonas_ruminantium, s_Oribacterium_sp_KHPX15, acteroidales_bacterium_WCE2008, and g_Oribacterium. H3 was characterized by significant increases in s_Eubacterium_eligens, o_Veillonellales, f_Veillonellaceae, and g_Allisonella, whereas H4 had higher abundances of s_Eubacterium_uniforme and g_Roseburia. These results highlight clear shifts in key microbial taxa associated with parity in Holstein cows.

To explore the functional consequences of these microbial shifts, KEGG pathway analysis was performed for rumen fluid from multiparous Sanhe cows. A heatmap of the top 20 KEGG orthologies (KOs) revealed that most pathways were more abundant in S2 and S3, with the exception of K13412, which showed higher expression in S1 **(Figure 4A)**. This suggests that functional activity of the rumen microbiome varies with parity, potentially influencing nutrient metabolism and lactation-related processes.



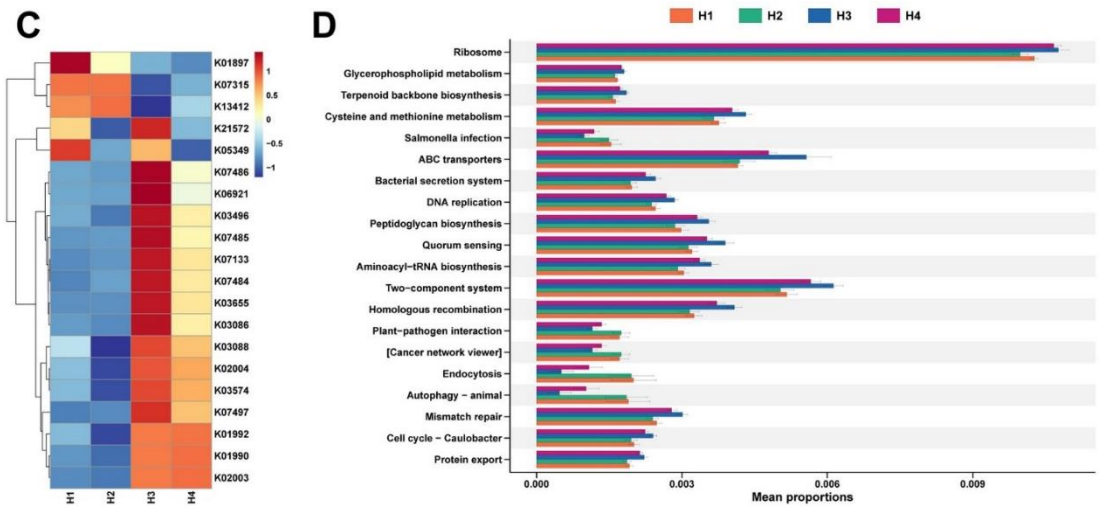


Figure 4.

Functional analysis of rumen microbiota

Functional profiling of the rumen microbiota was conducted to identify parity-related differences in metabolic potential. Using the top 20 KEGG Orthologies (KOs) and pathways ranked by abundance ($p < 0.05$, Kruskal–Wallis test), distinct patterns emerged for both Sanhe (S1–S4) and Holstein (H1–H4) cows **(Figure 4)**. In Sanhe cows, several KOs associated with ATP-binding cassette (ABC) transporters (K01990, K01992, K02003) were identified, while K13412, enriched in S1, corresponded to a calcium-dependent protein kinase. Among 425 level-3 KEGG pathways, the majority of the top 20 showed greater representation in multiparous cows (S2–S4), whereas autophagy–animal, plant–pathogen interaction, cancer network viewer, and endocytosis pathways were more active in primiparous cows (S1; **Figure 4B**).

In Holstein cows, K21572 and K05349 were notably enriched in H1 and H3, while the remaining pathways displayed similar abundances when comparing H1 with H2 and H3 with H4 **(Figure 4C)**. Early-parity Holstein cows (H1–H2) showed higher expression of pathways related to cellular signaling and metabolic regulation, potentially reflecting phosphorylation-dependent activity. Cross-species comparison revealed four KEGG pathways with higher abundance in both S2 and H1–H2, including salmonella infection, indicating conserved functional trends associated with specific parities across breeds **(Figure 4D)**.

Rumen CAZyme composition

Carbohydrate-active enzymes (CAZymes) play a critical role in the microbial breakdown of complex carbohydrates and subsequent rumen fermentation. Analysis against the CAZy database revealed six primary enzyme classes in both Sanhe and Holstein cows: auxiliary activities (AA), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), glycoside hydrolases (GH), glycosyl transferases (GT), and polysaccharide lyases (PL). GH enzymes were consistently the most prevalent across all groups.

Heatmap visualization highlighted an overall increase in CAZyme abundance in S4 compared with earlier parities **(Figure 5A)**. Among GH enzymes, 12 displayed significant parity-related variation: GH1, GH109, GH112, GH120, GH4, GH42, GH48, and GH50 were enriched in multiparous cows (S2–S4), while GH108, GH37, GH64, and GH84 predominated in primiparous cows (S1). Spearman correlation analysis linked these enzymes to key rumen and milk parameters **(Figure 5B)**. Ammonia nitrogen levels negatively correlated with GH109, GH112, and GH120, while milk protein was positively associated with GH112 and GH4. Lactose content showed positive correlations with GH64 and GH84 and negative correlations with GH109, GH42, GH48, and GH50. Fat-free dry matter correlated positively with GH64 and GH84 and negatively with GH48. These findings indicate that specific GH enzymes may directly influence fermentation efficiency and milk composition, highlighting their potential role in mediating parity-dependent changes in dairy cows.

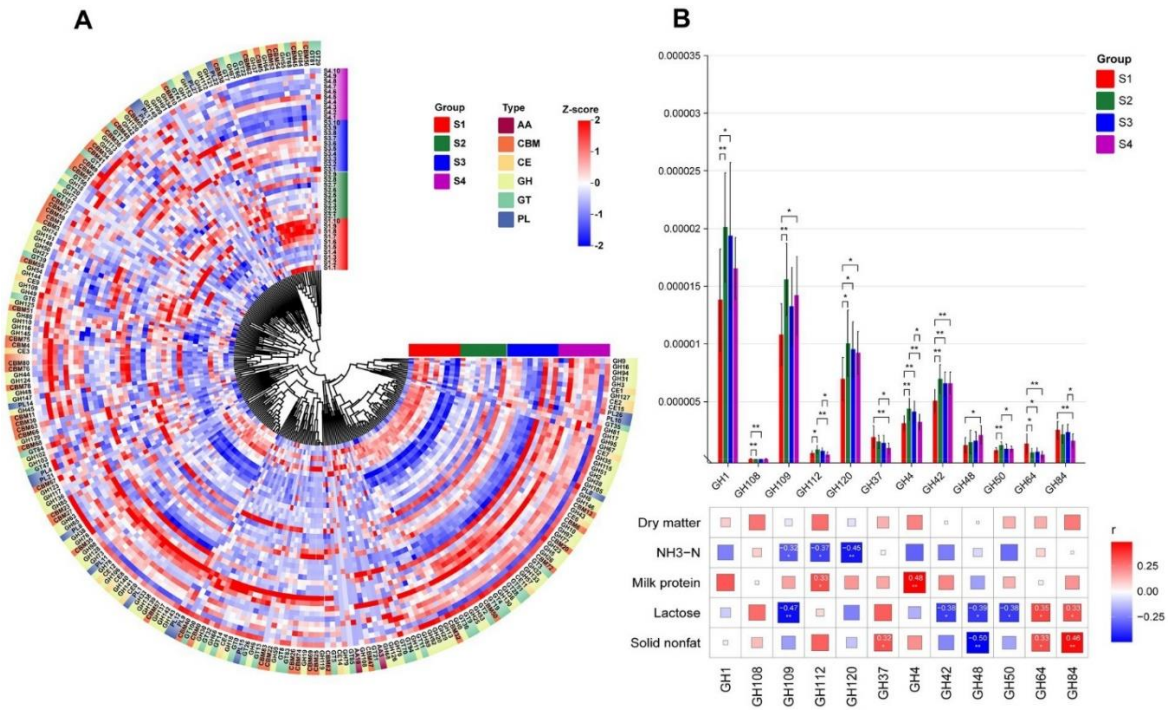


Figure 5.

CAZyme profiles in holstein Cows

Hierarchical clustering analysis of CAZymes in Holstein cows revealed that the majority of enzymes reached their highest abundance in H3 **(Figure 6A)**. Examination of glycoside hydrolases (GHs) highlighted 13 enzymes (GH119, GH124, GH18, GH19, GH27, GH37, GH6, GH64, GH65, GH73, GH84, GH85, GH87) with significant differences among parities **(Figure 6B)**. In particular, H3 displayed peak abundances for GH119, GH18, GH6, GH73, and GH85, underscoring clear variation in enzyme distribution between early (H1/H2) and later (H3/H4) lactations.

Spearman correlation analysis further revealed meaningful associations between these GH enzymes and rumen or milk parameters. The acetate-to-propionate ratio was positively associated with GH37, GH64, GH84, and GH87, while it showed negative correlations with the remaining seven enzymes. Additionally, isovalerate levels were inversely correlated with GH6 and GH65. Lactose content was positively linked to GH64, suggesting that specific GH enzymes may play influential roles in shaping rumen fermentation dynamics and impacting milk composition across different parities in Holstein cows.

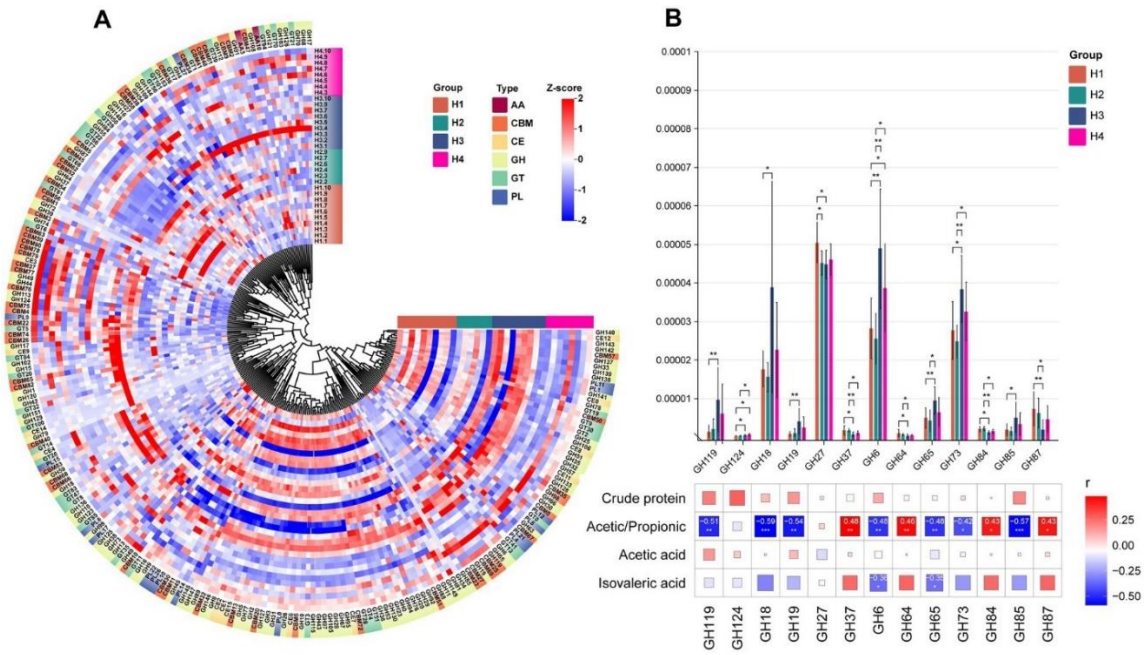


Figure 6. (A) Hierarchical clustering analysis (HCA) of the CAZy enzyme profiles in the rumen microbiota of Holstein cows across parities H1–H4. The inner ring indicates individual rumen samples from each parity group, while the outer ring displays the six CAZyme classes: auxiliary activities (AA), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), glycoside hydrolases (GH), glycosyl transferases (GT), and polysaccharide lyases (PL). (B) Spearman correlation analysis between parity-specific differential GH enzymes identified in Sanhe cows (S1–S4) and associated physiological parameters. Statistical significance is denoted as *p < 0.05, **p < 0.01, and ***p < 0.001

Discussion

To address gaps in understanding how rumen microbial communities influence production traits in Sanhe cattle, this study compared the rumen microbiota of Sanhe and Holstein cows across parities 1–4. The primary objective was to uncover parity- and breed-related differences in microbial composition and functional potential under identical feeding conditions, and to explore how these differences might relate to lactation metabolism.

In Sanhe cattle, previous work demonstrated that the milk metabolome of primiparous cows (S1) was markedly distinct from that of multiparous cows (S2–S4). Consistent with these findings, the current analysis revealed that both microbial abundance and the enrichment of functional pathways in S1 diverged significantly from those observed in cows with higher parities. Notably, several genera-level microbes that were enriched in S1, including *Neocallimastix* and *Piromyces*, are anaerobic fungi with strong cellulolytic activity [19] commonly found in the rumen and intestinal tract of ruminants [20, 21]. *Neocallimastix* exhibits exceptionally high cellulase activity, capable of degrading up to 89% of plant cell walls [22], while *Piromyces* ferments cellulose to produce acetate [23]. Previous in vitro studies have shown that introducing strains from these genera can enhance dry matter digestibility and increase VFA production [24], which aligns with the elevated VFA concentrations and digestibility trends observed in S1 in this study.

Additionally, members of the genus *Prevotella* remain among the most abundant rumen microbes worldwide [25]. These bacteria contribute to nitrogen metabolism by breaking down proteins to release ammonia [26, 27], and can also generate ammonia through amino acid deamination [28], thereby influencing NH_3-N concentrations in the rumen. While *Prevotella ruminantium* and *Prevotella bryantii* are well-known for their positive correlation with ammonia production [29], the specific Prevotella-related biomarkers identified in S1 have not been extensively characterized. Nevertheless, the findings suggest that these microbes may partly explain the elevated ruminal ammonia nitrogen levels observed in primiparous Sanhe cows.

Although no statistically significant differences were observed in total VFA concentrations across S1–S4, primiparous cows (S1) tended to exhibit higher levels of total VFAs and other short-chain fatty acids. Among the four microbial biomarkers enriched in S1, *Lactobacillus fructivorans* is known to ferment fructose, producing

acetate [30]. The remaining three biomarkers are associated with *Prevotella*, a genus previously shown to positively correlate with VFA production, thereby enhancing fermentation efficiency and promoting short-chain fatty acid generation [31–32]. Moreover, KEGG pathway analysis indicated that S1 microbiota were markedly enriched in pathways related to autophagy—animal, plant–pathogen interaction, and endocytosis, which are linked to immune response [34], cellular metabolism [35], and intracellular transport processes [36]. These findings suggest that the rumen microbial community in S1 undergoes pronounced functional shifts, potentially as an adaptive response to the physiological stress of the first lactation.

In contrast, multiparous cows (S2–S4) displayed greater enrichment of ATP-binding cassette (ABC) transporter-related KOs, including K01990, K02003, and K01992, consistent with higher representation of ABC transporter pathways in the KEGG database for these parities. Microbial ABC transporters primarily mediate nutrient uptake, particularly monosaccharides and amino acids [37], which may indirectly influence feed digestion, nutrient absorption, and metabolism in dairy cows. These observations align with our prior findings of elevated serum glucose and total protein concentrations in S2–S4 cows.

Since cows are unable to synthesize enzymes required for plant polysaccharide degradation, they rely heavily on the rumen microbiota to convert plant biomass into metabolizable carbohydrates and sugars [38]. Within CAZymes, glycoside hydrolases (GHs) represent the most diverse and abundant group, accounting for approximately 50% of all classified CAZymes [39]. These enzymes hydrolyze cellulose, hemicellulose, and starch into simple sugars that cows can absorb. The monosaccharides generated by GH-mediated hydrolysis provide critical substrates for lactose synthesis [40, 41]. For example, GH64 can cleave β-1,3-D-glucan, releasing glucose monomers that serve as carbon sources for lactose [42], while GH84 may influence glycoprotein modification, potentially affecting enzymes or intermediates involved in lactose biosynthesis [43]. However, further experimental validation is required to quantify the actual ruminal abundance of these GH enzymes, their metabolic products, and their precise impact on lactose synthesis through systemic circulation. Such studies are essential to elucidate the mechanisms linking rumen microbial activity with milk component biosynthesis.

Holstein cows are recognized as the most globally widespread high-producing dairy breed, and their rumen metagenome has been extensively investigated [44, 45]. Despite this, studies examining how rumen microbial communities evolve across successive parities or comparing Holsteins with other breeds under identical feeding conditions remain limited. Evidence from previous research suggests that, in multiparous Holstein cows, parity may act as a key driver of host–microbe interactions [46]. Earlier studies also indicated that variations in rumen microbial composition are predominantly influenced by diet, with the host exerting a comparatively smaller effect [25].

In the present study, under uniform dietary conditions, the high-abundance phyla and genera in the rumen microbiota of Japanese Black and Holstein cows exhibited similar compositional structures, although their relative abundances varied slightly across parities. Notably, the patterns of microbial shifts in Holsteins differed from those observed in Japanese Black cows, where S1 diverged from S2–S4. Specifically, H1 and H2 displayed similar microbial profiles, H3 and H4 clustered together, but H1/H2 were distinct from H3/H4. These patterns corresponded with previous observations of metabolic profiles in Holsteins, where H1 and H2 showed similar abundances that differed from H3 and H4 in heatmap analyses [6]. Collectively, these results suggest that rumen microbial composition may contribute to differences in metabolic activity and influence milk traits across breeds.

Regarding rumen fermentation, unlike S1 cows, which tended to have slightly higher levels of various VFAs than higher-parity cows, VFAs in Holsteins did not follow a consistent trend. While H3 cows exhibited somewhat lower acetate and higher propionate concentrations, the differences among groups were not statistically significant. However, the acetate-to-propionate ratio in H3 was markedly lower than in other parities, which may be linked to the elevated abundance of *Succinivibrio* and *Ruminobacter* in this group. Previous in vitro studies have shown that fermentation with a glucogenic substrate can reduce the acetate/propionate ratio and increase the abundance of these two genera [47]. Propionate is primarily generated via the decarboxylation of succinate, a pathway in which *Succinivibrio* plays a pivotal role [48].

Furthermore, several GH enzymes exhibited higher abundance in H3 and were negatively correlated with the acetate/propionate ratio. While these enzymes do not directly catalyze propionate formation, they may facilitate its production by generating monosaccharides required for microbial fermentation. Specifically, GH119 [49], GH6 [50], and GH65 [51] hydrolyze α-glucan, cellulose, and oligosaccharides into glucose, which can subsequently support propionate synthesis [52]. The bacterium *s_Fibrobacter succinogenes*, a primary lignocellulose degrader in herbivores [53], showed variable associations with isovaleric acid in prior studies; for

instance, Altay sheep with different energy diets exhibited lower isovaleric acid alongside reduced *s_Fibrobacter succinogenes* levels [54], whereas in Nellore calves, increased dietary concentrate raised isovaleric acid but decreased *s_Fibrobacter succinogenes* abundance [55]. Interestingly, in this study, H1 cows demonstrated both higher isovaleric acid and elevated *s_Fibrobacter succinogenes* abundance, which may reflect differences in feed composition or developmental stage. Additionally, *s_Eubacterium uniforme*, identified as a biomarker in both H4 and S4, is a fiber-degrading species that hydrolyzes cellobiose and xylan, previously isolated only from sheep rumen [56].

Conclusion

This study investigated the variations in rumen microbiota between multiparous Sanhe cattle and Holstein cows, revealing two key findings. First, although the overall microbial species composition in the rumen was comparable between the two breeds under identical feeding conditions, the relative abundances of these species differed. Second, patterns in the rumen microbiome were closely associated with milk metabolic profiles, yet breed remained a primary determinant of lactation performance. These findings underscore the breed-specific metabolic flexibility of dairy cows, which evolves with parity, highlighting the intricate interplay among genetics, physiological state, and metabolic regulation. Gaining insight into these complex metabolic interactions is essential for optimizing nutrition strategies, enhancing production efficiency, and promoting the health and well-being of dairy herds.

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